nutrients

Nutrition and Cancer

Edited by
Vera C. Mazurak

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Special Issue Editor

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About the Special Issue Editor

Vera C. Mazurak's research focuses on alterations in the metabolism that occur during critical illness with a focus on lipid metabolism and immunity. Following her PhD, she was employed as a research associate at the Cross Cancer Institute in Edmonton before she commenced her first faculty position at the University of Alberta in 2004, where she is currently a Professor of Human Nutrition. Her current research focuses on how changes in metabolism impact on the nutritional requirements in diseases characterized by inflammation, with a primary focus on cancer and its treatment. She is particularly interested in how providing essential dietary nutrients can improve care for patients. Her translational research program spans from experimental models of disease to human clinical trials. Dr. Mazurak developed and currently teaches a "Nutrition and Cancer" course and supervises undergraduate, graduate student (PhD and MSc) and postdoctoral fellow research programs in nutrition, as well as at the Faculty of Medicine and Dentistry as part of her research program.

Preface to "Nutrition and Cancer"

Defining better nutritional interventions for those at risk of malnutrition has been a passion of mine ever since I started research, which is why I am excited to bring forward this Special Issue focused on nutrition and cancer. The development and treatment of cancer presents a complex interaction between tumor and host. The provision of nutrients not only enables the maintenance of nutritional status, but also provides substrates and signals for immunity, tumor metabolism and the protection of the host from treatment toxicities. Unique elements in the diet can help to tip the metabolic balance in favor of the host by acting on multiple pathways concurrently, both enhancing the host's ability to fight the tumor and endure cancer treatment while simultaneously attenuating tumor growth. This Special Issue includes articles that highlight the role of certain dietary components in the prevention of cancer and host response during cancer treatment.

Fat is one dietary element that has been explored for its role in cancer development. While the bulk of these studies have been observational or experimental, the evidence assembled suggests that dietary lipids behave uniquely to prevent or promote cancers. Of particular interest are the n-3 fatty acids, consisting of linolenic acid, eicosapentaenoic acid and docosahexaenoic acid. These fatty acids are most widely recognized for their anti-inflammatory properties that attenuate cancer progression while also enhancing host immune responses. Several articles in this Special Issue focus on the n-3 fatty acids and the mechanisms involved in cancer prevention, providing a harmonious accompaniment to the reviews of the epidemiological evidence.

An additional aspect of cancer development is the role of adipose tissue, which has an expanded role beyond energy storage, contributing to the whole body metabolism. Adipose tissue is a source of and responds to inflammatory signals that may be involved in tumor development. This Special Issue also provides reviews on various aspects of adipose tissue in relation to cancer development and progression.

The contributors to this Special Issue are well recognized leaders in the field of cancer and have unique areas of focus including metabolism, immunology, biochemistry, epidemiology and nutrition. Each contribution highlights the latest research in these areas and what is known about fat and cancer.

Vera Mazurak
Special Issue Editor

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Article

Effects of Perioperative Supplementation with Omega-3 Fatty Acids on Leukotriene B_4 and Leukotriene B_5 Production by Stimulated Neutrophils in Patients with Colorectal Cancer: A Randomized, Placebo-Controlled Intervention Trial

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Abstract: Omega-3 fatty acids (*n*-3 FA) may have beneficial clinical and immune-modulating effects in surgical patients. In a randomized, double-blind, prospective, placebo-controlled trial, 148 patients referred for elective colorectal cancer surgery received an *n*-3 FA-enriched oral nutritional supplement (ONS) providing 2.0 g of eicosapentaenoic acid (EPA) and 1.0 g of docosahexaenoic acid (DHA) per day or a standard ONS for seven days before surgery. On the day of operation, there was a significant increase in the production of leukotriene B_5 (LTB_5) ($p < 0.01$) and 5-hydroxyeicosapentaenoic acid (5-HEPE) ($p < 0.01$), a significant decrease in the production of leukotriene B_4 (LTB_4) ($p < 0.01$) and a trend for a decrease in the production of 5-hydroxyeicosatetraenoic acid (5-HETE) ($p < 0.1$) from stimulated neutrophils in the active group compared with controls. There was no association between LTB_4 values and postoperative complications. In conclusion, oral *n*-3 FA exerts anti-inflammatory effects in surgical patients, without reducing the risk of postoperative complications.

Keywords: colorectal cancer; omega-3 fatty acids; immunomodulation; fish oil; leukotrienes

1. Introduction

Patients undergoing surgery are at risk of developing complications in the postoperative period [1–3]. This is believed to be partly caused by changes in the immune response following surgery [4]. Thus, initially, a hyper-inflammatory response followed by a phase of relative immune incompetence occurs in relation to major surgery [5].

The pathophysiological changes are complex, but may be driven by excessive production of various lipid mediators, including the very potent pro-inflammatory leukotriene B_4 (LTB_4) produced from the omega-6 fatty acid (*n*-6 FA) arachidonic acid (AA) present in cell membranes.

Among factors known to influence the clinical course of patients after surgery are nutritional status and specific biologically active nutrients [2,6–10] that might include the marine omega-3 fatty acids (*n*-3 FA) with the main biologically active *n*-3 FAs being eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Consumption of fish and fish oils oil increases the concentration of EPA and DHA in blood, cells and tissues [11,12] and alters the physical properties of cell membranes and the function of membrane proteins, including receptors, transporters and signalling proteins [13,14]. *n*-3 FA are incorporated into cell membranes in competition with the more abundant *n*-6 FA, AA, at the expense of the latter. AA may be liberated by phospholipases from cell membranes and induces leucocytes to produce the pro-inflammatory LTB_4 and the side product, 5-hydroxyeicosatetraenoic acid (5-HETE). In contrast, leukotriene B_5 (LTB_5) and the side product, 5-hydroxyeicosapentaenoic acid (5-HEPE), derived from EPA [15], have considerably less potent biological activities in comparison to LTB_4 [15,16]. Replacement of *n*-6 FA with *n*-3 FA in membranes of immune active cells may therefore lead to reduced formation of pro-inflammatory compounds, and by this, and other [5,12] mechanisms, *n*-3 FA may decrease infectious complications after surgery [7,17–20]. The influence of enteral feeds, including *n*-3 FA on AA-derived eicosanoids (e.g., LTB_4), has been the subject of much attention [5,21–23]. Several studies have indicated that *n*-3 FAs modulate the generation of inflammatory eicosanoids in gastrointestinal surgical patients [24–26] and may help to counteract the surgery-induced decline in antigen-presenting cell activity [20] and T-cell cytokine production [27].

The aim of the present study was to evaluate the production of LTB_4, 5-HETE, LTB_5 and 5-HEPE from stimulated neutrophils after seven days of preoperative treatment with an *n*-3 FA-enriched oral nutritional supplement (ONS) in patients undergoing colorectal cancer surgery and to study the possible impact on clinical outcome. Furthermore, the correlation between LTB_4 values and postoperative complications was investigated.

2. Materials and Methods

2.1. Study Design

This was a sub-study of a randomized, double-blind, prospective, placebo-controlled single-centre interventional trial involving 148 participants (Figure 1) awaiting colorectal cancer surgery [28]. Participants were recruited consecutively from the outpatient clinic of the Department of Surgical Gastroenterology, Aalborg University Hospital. All eligible participants were asked to participate.

Exclusion criteria were diabetes mellitus, consumption of >5 alcoholic drinks per day, emergency surgery, inability to understand the spoken and written information in Danish, untreated psychiatric conditions, pregnancy or breast-feeding, reduced kidney function (plasma creatinine > 130 μmol/L), use of *n*-3 FA supplements, anticipated poor compliance, immunosuppressive diseases and participation in another clinical trial.

After providing oral and written informed consent, participants were randomly assigned to treatment with *n*-3 FA (active treatment) or control (a standard ONS without marine *n*-3 FA), 200 mL twice per day (morning and afternoon) for 7 days before surgery. Randomization was performed using sealed non-transparent envelopes containing the randomization number and kept at the investigation site according to CONSORT (Consolidated Standards of Reporting Trials) guidelines [29]. The active and the control ONS cartons looked identical and had identical taste and scent (coffee). The main investigator and a study nurse enrolled the participants at the outpatient clinic and randomly assigned them to active treatment or control. The participants, carers, investigators and other researchers were blinded to treatment allocation throughout the study. The investigators had no access to the code until after completion of the study. Statistical analyses were completed before the code was broken.

Information collected included demographic data, tumour location and American Society of Anaesthesiologists (ASA) risk score [30]. All patients underwent standard Nutritional Risk Screening (NRS 2002) [31]. A food questionnaire focusing on consumption of seafood on a monthly basis was completed at baseline.

The study was approved by the regional ethics committee (N-VN-20050035) and conducted according to the Hong Kong amendment to the Declaration of Helsinki. The trial was registered at ClinicalTrials.gov: ID NCT00488904 [32].

2.2. Intervention

Participants in the active or control group received the ONS as a sip feed (200 mL twice a day morning and afternoon) for 7 days before surgery. The feeds (Supportan®) were isocaloric (1.5 kcal/mL) and isonitrogenous (Table 1) and were provided by Fresenius Kabi (Bad Homburg, Germany). Both feeds contained the same amounts of carbohydrate, protein, total fat and *n*-6 FA (Table 1).

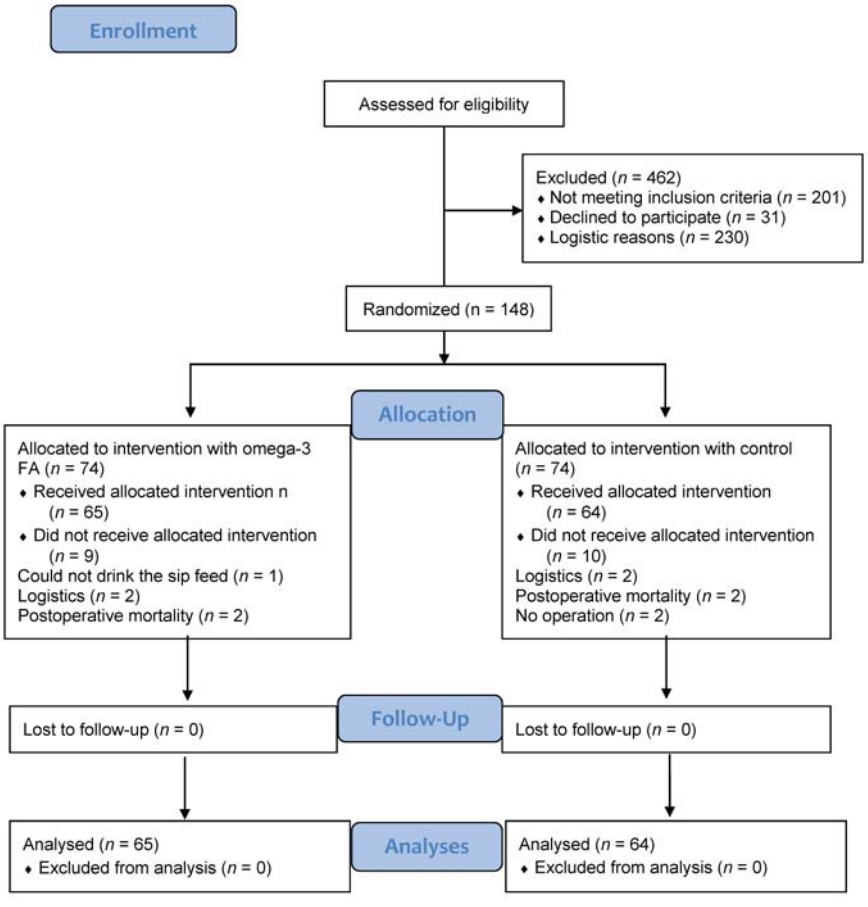

Figure 1. Patient flow through the study.

Table 1. Daily intake of energy and nutrients from the *n*-3 FA-enriched and the control oral nutritional supplement.

Daily dose	Control	*n*-3 FA (Active)
Energy (kcal)	600	600
Protein (g)	40	40
Carbohydrate (g)	49.6	49.6
Fat (g)	26.8	26.8
EPA (g)	0	2
DHA (g)	0	1
Total *n*-6 FA (g)	3.3	3.3

Fat content of the supplement was comprised of medium chain triglycerides, sunflower oil and safflower oil. The active supplement also contained additional fish oil at a level to achieve 2 g EPA and 1 g DHA per day (Table 1). Participants were provided with the sip feeds at inclusion, to consume twice a day for 7 days at home before hospitalization. A questionnaire regarding compliance preoperatively was completed, and good compliance was defined as self-reported consumption of at least 12 of the 14 ONS cartons before surgery.

2.3. Isolation of Blood Neutrophils

Blood was drawn in the fasting state on the day of the surgery. Neutrophils were separated from anticoagulated (K-EDTA 1.6 mg/mL) blood layered on top of PolymorphprepTM (AXIS-SHIELD PoC AS, Rodeloekka, Norway) and separated by a one-step centrifugation technique at 450 g for 40 min. Neutrophils were harvested and washed twice in tissue culture medium (RPMI 1640, Sigma-Aldrich, Ayrshire, UK), at ambient temperature and centrifuged for 10 min at 520× *g*. Subsequently, neutrophils were counted and red cells eliminated by the addition of ice-cold 0.2% saline for 35 s. Next, 1.6% ice-cold saline was added in order to obtain an isotonic 0.9% concentration, followed by centrifugation at 300× *g* at 5 °C for 5 min, which was repeated once. Neutrophils were then washed in a phosphate buffer containing glucose and human albumin (PBS) and resuspended in PBS adjusting the concentration to 1×10^7 neutrophils/mL PBS. Isolated neutrophils were stored at −80 °C until analysis.

2.4. Analysis of Leukotrienes, 5-HEPE and 5-HETE

The neutrophil suspension (0.9 mL of 1×10^7 granulocytes/mL PBS) was prewarmed to 37 °C, and $CaCl_2$, $MgCl_2$ and calcium ionophore (A23187) at a final concentration of 10 μM were added to initiate stimulation. After 10 min, the reaction was terminated by the addition of 100% ice-cold ethanol, and the mixtures were centrifuged at 4 °C at 700× *g*. The supernatant was stored at −80 °C for later analysis. C18 cartridges (Sep-Pak VAC RC, Waters Co., Milford, MA, USA) were used for the extraction of leukotrienes (LT), 5-HEPE and 5-HETE. The ethanol mixture was thawed and centrifuged, and international standard prostaglandin B_2 (PGB_2) and trifluoroacetic acid were added. The cartridges were conditioned and equilibrated using methyl formate, 100% ethanol and water. The acidified sample was loaded onto the cartridge, washed with 15% ethanol, water and hexane and eluted with methyl formate. The solvent was evaporated to dryness under nitrogen, and the sediment was dissolved in the mobile phase (31% H_2O, 27% methanol, 42% acetonitrile and 0.025% trifluoroacetic acid).

Analysis was performed by high pressure liquid chromatography (Dionex Ultimate LPG-3400A) on an Acclaim RSLC 2.1 mm × 100 mm C18 column (Dionex Corporation, Sunnyvale, CA, USA). Concentrations were calculated using the internal standard and response factors. The response factors were calculated by analysis of a non-stimulated neutrophil suspension after the addition of known amounts of standards of LTB_4, LTB_5, 5-HETE and 5-HEPE, as well as an internal standard (PGB_2). Samples were extracted and analysed using high performance liquid chromatography, and eventually, the recovery factors were calculated.

2.5. Granulocyte Fatty Acid Analysis

Blood was drawn in the fasting state on the day of surgery. Granulocytes were prepared as described previously [33]. FA profiles were determined by gas chromatography using a Varian 3900 gas chromatograph, CP-8400 autosampler and CP 8414 autoinjector (Varian, Middelburg, The Netherlands), as well as a flame ionization detector. In split injection mode, a CP-sil 88.60-m × 0.25-mm capillary column (Varian, Middelburg, The Netherlands), temperature programming from 90 to 205 °C, a constant flow rate of 1.0 mL/min and helium carrier gas were used. Results for individual FAs are expressed as a percentage of the total FA content.

2.6. Statistical Analysis

The basic characteristics of the trial population were analysed with Fisher's exact test for categorical variables and unpaired *t*-tests for continuous variables. Differences between treatment groups were analysed using unpaired *t*-tests. If variances differed between groups, Welch's approximation was used. The distribution of continuous data was analysed for normality. As the values for 5-HEPE, 5-HETE and LTs were right skewed distributed, log transformed observations were analysed, and these were normally distributed. Distributions of 5-HEPE, 5-HETE and LT were described with median and inter-quartile range (IQR). Relative differences between groups in medians were calculated by exponentiation of the differences between log-transformed means. Associations between LTB_4 values and postoperative complications were analysed using logistic regression. The associations between log-transformed LT values 5-HEPE and AA, EPA and AA/EPA were analysed using linear regression. Analyses were performed blinded to treatment groups. The active and control groups were not identified until after the statistical analyses had been conducted. All *p*-values were two-tailed, and differences were reported with 95% confidence intervals (CI). *p*-values below 0.05 were considered significant. All analyses were performed using Stata version 11.2 (StataCorp, 2009; Texas City, TX, USA).

3. Results

3.1. Participants Characteristics

All eligible participants (n = 610) were asked to participate, but 230 participants were not included. This was due to a change in clinical practice during the study, such that many patients were offered surgery within a five-day period, which did not allow for participants to complete the seven-day intervention. Furthermore, some participants did not meet the inclusion criteria (201), and 31 participants declined to participate. Baseline characteristics of the included *vs.* the non-included participants did not differ.

A total of 148 consecutive patients (68 females, 80 males; mean age 71 (range 41–89) years) were included in the study. The majority of participants had open surgery; laparoscopic resection was only performed in nine patients in the control group and nine in the *n*-3 FA group. Participant characteristics did not differ between treatment groups (Table 2).

Table 2. Characteristics of patients in the control and active groups.

Variable	Control (*n* = 74)	Active (*n* = 74)	*p*
Demographic data			
Age, years, mean (SD)	71 (10)	69 (11)	0.164
Sex (male/female)	36/38	44/30	0.248
Body weight, kg, mean (SD)	76 (19)	77(17)	0.570
Height, cm, mean (SD)	169 (11)	171 (9)	0.301
BMI, kg/m^2, mean (SD)	26 (5)	26 (5)	0.651
Weight loss * (*n*)	19	11	0.068
Clinical characteristics			
Smoking/non-smoking (*n*)	11/60	17/54	0.292
Unknown smoking status	3	3	
Cancer location			
Colon/rectum (*n*)	40/34	38/36	0.869
Surgical procedure			0.977
Right hemicolectomy + transverse colon (*n*)	16	17	
Left hemicolectomy + sigmoid colon (n)	10	12	
Laparoscopic resection of sigmoid colon (*n*)	9	9	
Low anterior resection of rectum or abdominoperineal resection	28	30	
Colectomy (*n*)	4	3	
Other rectum resection (n)	7	3	
Nutritional status **			0.089
No risk (NRS score <3) (*n*)	23	34	
At risk (NRS score ≥3) (*n*)	50	39	
Unknown (*n*)	1	1	

Notes: There were no significant differences between groups ($p > 0.068$); BMI, body mass index; * defined as loss of more than 5% of body weight; ** defined according to NRS 2002 [31].

3.2. Fatty Acid Composition of Neutrophils

Neutrophil EPA and DHA were significantly higher, and AA and linoleic acid was significantly lower in the group receiving *n*-3 FA than in the control group (Table 3) [28].

The food questionnaire indicated an average dietary intake of *n*-3 FA of 0.6 g per day, with no difference in preoperative intake between groups ($p = 0.770$). None of the included participants received more than 150 mg of anti-inflammatory drugs daily. Both supplements were well tolerated with no adverse effects reported. Nine participants randomized to active treatment and 10 participants in the control group did not receive the allocated intervention for reasons listed in Figure 1.

Preoperatively, 63 of 65 participants in the active group were compliant compared with 56 of 64 participants in the control group ($p = 0.266$). Two participants died in each group. In the active group, death was caused by pneumonia and a myocardial infarction, whereas the participants in the control group died from septicaemia and sudden cardiac death.

Table 3. Granulocyte fatty acids on day of operation in the control and active groups.

	Weight% of Total FA Content	
	Control	**Active**
EPA	0.54 (0.42–0.74)	2.10 (1.83–2.55) *
DPA	0.54 (0.42–0.74)	2.11 (1.83–2.55) *
DHA	1.31 (1.10–1.57)	1.61 (1.34–1.84) *
Total *n*-3 FA	2.44 (1.98–2.97)	5.95 (5.20–6.75) *
Arachidonic acid	12.51 (11.65–13.19)	11.61 (10.67–12.48) *
Linoleic acid	8.94 (8.24–9.49)	9.42 (8.72–10.19) **

Notes: Values are the median (IQR); FA, fatty acid; ONS, oral nutritional supplement; EPA, eicosapentaenoic acid; DPA, docosapentaenoic acid; DHA, docosahexaenoic acid; * $p < 0.001$; ** $p < 0.05$ *versus* the control group.

3.3. Production of Mediators from Neutrophils

Furthermore, compared to neutrophils from controls, those from participants in the *n*-3 FA group showed a significantly higher (by 176% and 306%, respectively) production of LTB_5 ($p < 0.001$) and 5-HEPE ($p < 0.001$) (Table 4).

Table 4. Formation of leukotrienes (LT) and side products (5-HEPE; 5-HETE) from activated neutrophils according to treatment group.

Eicosanoids	Control	Active	% Difference
LTB_5	5.8 (4.9–7.6)	17.5 (13.5–22.8)	176 *** (143–215)
LTB_4	186.8 (156.8–230.7)	163.5 (136.6–199.9)	−12 *** (−21–−3)
5-HETE	293.1 (246.5–357.8)	273.7 (221.8–320.7)	−7 * (−14–−0)
5-HEPE	34.2 (25.7–55.3)	154.7 (122.4–190.4)	306 *** (255–364)
LTB_4/LTB_5	31.8 (25.0–40.1)	9.6 (7.8–11.4)	−68*** (−72–−65)

Notes: Data are the median (IQR) $ng/10^7$ neutrophils and the percentage of difference between estimated medians; *** indicates $p < 0.01$; * indicates $p < 0.1$; $ng/10^7$ = nanogram/10^7 neutrophils.

Conversely, in the active group, neutrophils showed a significantly lower (by 12%) production of LTB_4 ($p < 0.001$) and a trend towards lower (by 7%) production of 5-HETE ($p = 0.059$). LTB_4/LTB_5 was significantly different between groups (by 68%) ($p < 0.001$) (Table 4). There was no statistically significant difference in clinical outcomes (total number of complications, infectious complications, non-infectious complications, intensive care unit stay, mortality, readmissions and hospital stay) between groups, as reported previously [28].

There was no statistically significant association between the values of the proinflammatory LTB_4 production and any clinical outcome, including total number of complications ($p = 0.524$), infectious complications ($p = 0.660$) and non-infectious complications ($p = 0.307$) (Table 5). The ratio LTB_4/LTB_5 did not have a statistically significant association with the total number of complications ($p = 0.707$), infectious complications ($p = 0.711$) and non-infectious complications ($p = 0.143$) (Table 4).

However, There were strong associations between the content of AA and EPA in neutrophils and production of LTB_4 and LTB_5 (Figure 2) (all $p < 0.01$).

These graphs illustrate that the higher the content of EPA in the cell membranes, the higher the production of LTB_5. Furthermore, it can be seen that the higher the content of AA in cell membranes, the lower the production of LTB_5. Furthermore, there were strong associations between AA/EPA in neutrophils and LTB_4 and LTB_5 production (both $p < 0.01$) (results not shown) and between AA, EPA, AA/EPA and 5-HEPE production (all $p < 0.001$) (results not shown).

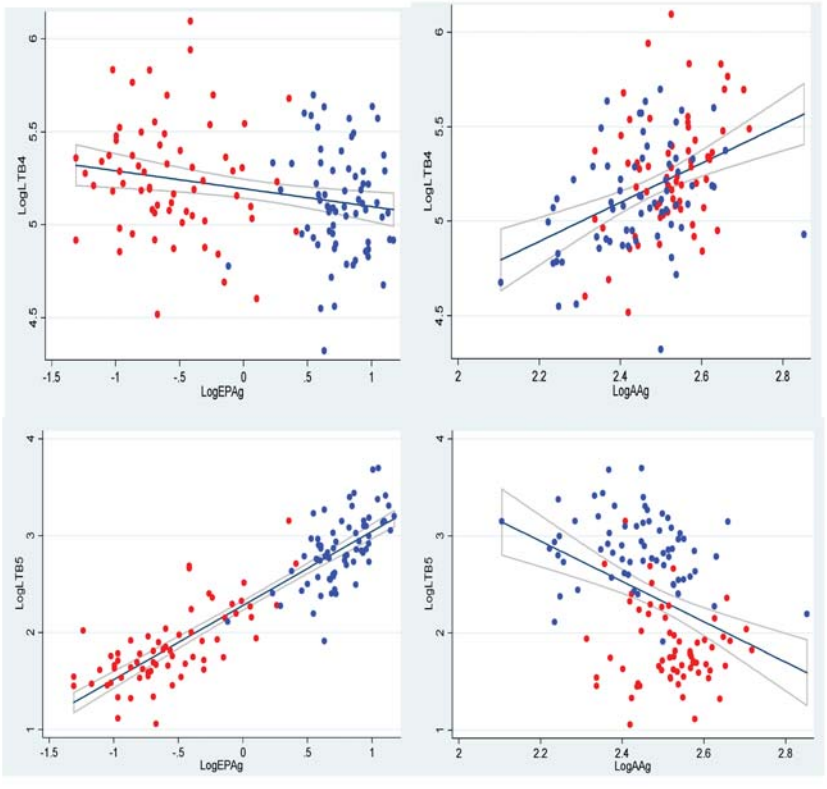

Figure 2. Associations between log-transformed AAg (AA content in the cell membranes of the granulocytes) and EPAg (EPA content in the cell membranes of the granulocytes) in the neutrophils, as well as the formation of LTs (LTB_4 and LTB_5 ($ng/10^7$)) by neutrophils, illustrated using scatter plots with regression lines and confidence bands added. Control group, red dots; active group, blue dots.

Table 5. Associations between LTs production by neutrophils and clinical outcome described by odds ratio (OR), CI and *p*-values for a unit change in LTB_4 and LTB_4/LTB_5, respectively.

	LTB_4			LTB_4/LTB_5		
	OR	**CI**	***p***	**OR**	**CI**	***p***
Infectious complications	1.00	1.00–1.01	0.660	1.00	0.97–1.03	0.711
Non-infectious complications	1.00	0.99–1.00	0.307	0.98	0.94–1.00	0.143
Total number of complications	1.00	0.99–1.00	0.524	1.00	0.98–1.03	0.707

4. Discussion

In this prospective randomized, double-blind, single-centre, placebo controlled study, it was demonstrated that seven days of enteral supplementation with 3 g of EPA + DHA daily resulted in a higher neutrophil production of LTB_5 and a lower production of LTB_4 compared to the control group. In addition, there was a higher neutrophil 5-HEPE production and a trend to lower production of 5-HETE in the active group compared to the control. While the differences in LTB_4 and LTB_5 production indicate an anti-inflammatory action of the supplement, it is unknown whether the difference in formation of 5-HETE and 5-HEPE between groups is of clinical relevance. However, 5-HETE enhances lymphocyte

proliferation, whereas 5-HEPE only has one-tenth the potency of 5-HETE regarding this [34]. Thus, the current study demonstrates that preoperative supplementation with *n*-3 FA for one week can modulate immune function, assessed as the production of lipoxygenase mediators, in participants admitted for elective colorectal cancer surgery. However, this was not associated with a decrease in postoperative complication rates [28].

The strengths of the present study are that it was a randomized, prospective, relatively large clinical study, was double-blind, with an identical appearance for the sip feed cartons, the taste of *n*-3 FA was undetectable and that compliance was acceptable. Furthermore, the study population was relatively homogenous.

One limitation of the present study is that we only had data on eicosanoid formation from the day of surgery, whereas postoperative changes would also have been of interest. The short duration of the intervention (seven days) may have limited the incorporation of *n*-3 FA and the decrease of AA in neutrophils and, thereby, limited the impact on LTB_4 production, but a longer period of supplementation was not possible, as participants were operated on soon after cancer diagnosis. However, we showed in earlier publications that 3 g of *n*-3 FA for seven days before surgery was sufficient to assure significant incorporation of *n*-3 FA into neutrophils and into colonic tissue [28,35]. The required sample size was based on a reduction in postoperative infection rates from 30% to 10% and was calculated to be 148 participants in all, but we were only able to analyse data from 129 of these. A final limitation is the discrepancy between the number of eligible (610) and analysed participants (129) due to a change in clinical practice during the study, such that many patients were offered surgery within a five-day period, which did not allow for participants to complete the seven-day intervention.

Our findings are consistent with the results from three recent studies in humans [24,36,37]. In a prospective double-blind study, Wang *et al.* [37] randomized 64 participants with a need for postoperative parenteral nutrition after surgery into two groups. The study population was a mix of surgical patients (22 gastric cancers; 29 colonic cancers; 13 with other digestive diseases). They received either fish oil containing lipid emulsion (a mixture of soybean oil, MCT and fish oil) as part of the intravenous regimen, or a mix of soybean oil and MCT for 5 days after surgery. There was a significant increase in the neutrophil LTB_5/LTB_4 ratio but no effect on clinical outcome, infectious complications and bleeding events. Grimm *et al.* [36] randomized 33 participants undergoing major abdominal surgery into two groups in a prospective double-blind study to receive parenteral nutrition providing either a fish oil containing lipid emulsion (a mixture of soybean oil, MCT, olive oil and fish oil) or soybean oil for five days after surgery. The study population was again a mix of surgical patients. The initial production of LTB_4 and LTB_5 by neutrophils was similar in both groups. The production of LTB_5 from neutrophils was significantly increased, and the release of LTB_4 was decreased, though not significantly, in the participants receiving fish oil. The length of hospital stay was significantly shorter in the intervention group. Finally, Köller *et al.* [24] conducted a prospective double-blind randomized study with 30 participants undergoing colorectal surgery. Participants received parenteral nutrition, providing either a fish oil containing lipid emulsion (a mixture of soybean oil, MCT and fish oil) or soybean oil for five days post-surgery. This study also found a significant increase in LTB_5 production by leukocytes in the fish oil group, but without a concomitant decrease in LTB_4 production. These three studies all made use of intravenous (IV) nutrition given postoperatively. The present study, which used enteral nutrition given preoperatively, agrees with these earlier findings of increased LTB_5 production and decreased LTB_4 production after fish oil provision, but with limited clinical impact.

Some earlier studies have reported beneficial effects of oral *n*-3 FA supplementation in gastrointestinal surgery patients. Wachtler *et al.* [26] analysed leukocyte function in 40 participants undergoing major upper gastrointestinal surgery in a placebo-controlled double-blind study. One group received an *n*-3 FA-enriched (0.33 g/100 mL) oral supplement, also containing arginine and ribonucleic acid, and the other group received a standard control supplement for five days preoperatively. There was a significantly higher production of LTB_5 from neutrophils in the intervention group when compared to controls. However, no changes in LTB_4 were evident in the intervention

group. The authors reported a low number of postoperative complications. In another study, Shimizu *et al.* [38] gave 12 children with ulcerative colitis 1.8 g EPA orally per day for two months. LTB_4 production by leucocytes and colonic mucosa were assessed before and after the intervention. Biopsies were taken from the rectal mucosa during sigmoidoscopy before and after initiation of EPA supplementation. After two months of supplementation, there was a decrease in LTB_4 production by leucocytes and colonic mucosa, while no information regarding LTB_5 production was given.

IV administration of *n*-3 FA ensures a quicker incorporation of the presumed active substances (*n*-3 FA) into the membranes of immune cells [39,40]. However, this format is not feasible in the pre-operative setting. ONSs are less expensive and enable the use of the gut prior to surgery. Two studies providing IV *n*-3 FA for five days post-operatively did not show any decrease in the production of LTB_4 [24,36]. Importantly many of these studies did not provide explicit information about the amount of *n*-3 FA given.

In the present study, seven days of oral supplementation with 3 g of *n*-3 FA daily ensured significant incorporation of EPA into neutrophils and a significant decrease in the formation of LTB_4. However, to achieve an anti-inflammatory response meditated by *n*-3 FA, it is probably more important that the formation of the AA-derived LTB_4 is suppressed than an increase in LTB_5. Despite this, there was no effect on clinical outcome in the current study. One explanation for this may be that *n*-3 FA incorporation was not sufficiently high. A higher *n*-3 FA dose or a longer duration of intervention could have had an impact on clinical outcome. The ratio LTB_4/LTB_5 was 68% lower in the active group, which is a considerable decrease, but still did not have any effect on clinical outcome.

One other important factor may be that the nutritional status of the patients, evaluated by NRS 2002, was generally good for most participants. A weight loss of more than 5% of body weight was only detected in 23% of participants entering the study. This could account for the lack of clinical improvement with the *n*-3 FA-enriched ONS, since it is likely that malnourished participants might benefit the most from ONS and from oral *n*-3 FA.

5. Conclusions

In summary, the current study shows that an ONS providing 3 g of *n*-3 FA daily for seven days before surgery was able to induce a significant decrease in the formation of the pro-inflammatory LTB_4 from neutrophils with a simultaneous increased production of LTB_5. A decrease in the formation of 5-HETE, though not significant, and a significant rise in 5-HEPE was also seen. However, the clinical consequences of these changes are unknown. Associations between values of LTB_4 or LTB_4/LTB_5 and postoperative complication rates were not seen. This indicates either that the changes observed were too small or that the formation of LTs from activated neutrophils is not an important determinant of surgical complications. Whether a longer period (months) of *n*-3 FA intake could be of a benefit for patients operated on for colorectal cancer regarding shorter stay in hospital or longer survival needs to be investigated in larger trials.

Abbreviations

AA	arachidonic acid
ASA	American Society of Anaesthesiologists
BMI	body mass index
CI	confidence intervals
DHA	docosahexaenoic acid
EPA	eicosapentaenoic acid
5-HEPE	5-hydroxyeicosapentaenoic acid
5-HETE	5-hydroxyeicosatetraenoic acid
IV	intravenous;
IQR	inter-quartile range
LT	leukotrienes
LTB_4	leukotriene B_4
LTB_5	leukotriene B_5

n-3 FA	*n*-3 fatty acids
n-6 FA	*n*-6 fatty acids
MCT	medium-chain triglycerides
ng	nanograms
NRS 2002	Nutritional Risk Screening
ONS	oral nutritional supplement
OR	odds ratio
PBS	phosphate-buffered saline
PGB_2	prostaglandin B_2

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References

1. Platt, J.J.; Ramanathan, M.L.; Crosbie, R.A.; Anderson, J.H.; McKee, R.F.; Horgan, P.G.; McMillan, D.C. C-reactive protein as a predictor of postoperative infective complications after curative resection in patients with colorectal cancer. *Ann. Surg. Oncol.* **2012**, *19*, 4168–4177. [CrossRef]
2. Zhu, M.W.; Tang, D.N.; Hou, J.; Wei, J.M.; Hua, B.; Sun, J.H.; Cui, H.Y. Impact of fish oil enriched total parenteral nutrition on elderly patients after colorectal cancer surgery. *Chin. Med. J. (Engl.)* **2012**, *125*, 178–181.
3. The Danish Colorectal Cancer Database, The Danish Colorectal Cancer Group. Annual review. Available online: http://www.dccg.dk/03_Publikation/02_arsraport_pdf/aarsrapport_2011.png (accessed on 3 April 2013).
4. Kehlet, H.; Dahl, J.B. Anaesthesia, surgery, and challenges in postoperative recovery. *Lancet* **2003**, *362*, 1921–1928. [CrossRef]
5. Calder, P.C. *n*-3 fatty acids, inflammation, and immunity—Relevance to postsurgical and critically ill patients. *Lipids* **2004**, *39*, 1147–1161. [CrossRef]
6. Bozzetti, F.; Braga, M.; Gianotti, L.; Gavazzi, C.; Mariani, L. Postoperative enteral *versus* parenteral nutrition in malnourished patients with gastrointestinal cancer: A randomised multicentre trial. *Lancet* **2001**, *358*, 1487–1492. [CrossRef]
7. Braga, M.; Gianotti, L.; Vignali, A.; Carlo, V.D. Preoperative oral arginine and *n*-3 fatty acid supplementation improves the immunometabolic host response and outcome after colorectal resection for cancer. *Surgery* **2002**, *132*, 805–814. [CrossRef]
8. Gustafsson, U.O.; Ljungqvist, O. Perioperative nutritional management in digestive tract surgery. *Curr. Opin. Clin. Nutr. Metab. Care* **2011**, *14*, 504–509. [CrossRef]
9. Jie, B.; Jiang, Z.M.; Nolan, M.T.; Zhu, S.N.; Yu, K.; Kondrup, J. Impact of preoperative nutritional support on clinical outcome in abdominal surgical patients at nutritional risk. *Nutrition* **2012**, *28*, 1022–1027. [CrossRef]

10. Ryan, A.M.; Reynolds, J.V.; Healy, L.; Byrne, M.; Moore, J.; Brannelly, N.; McHugh, A.; McCormack, D.; Flood, P. Enteral nutrition enriched with eicosapentaenoic acid (EPA) preserves lean body mass following esophageal cancer surgery: Results of a double-blinded randomized controlled trial. *Ann. Surg.* **2009**, *249*, 355–363. [CrossRef]
11. Senkal, M.; Haaker, R.; Linseisen, J.; Wolfram, G.; Homann, H.H.; Stehle, P. Preoperative oral supplementation with long-chain Omega-3 fatty acids beneficially alters phospholipid fatty acid patterns in liver, gut mucosa, and tumor tissue. *J. Parenter. Enter. Nutr.* **2005**, *29*, 236–240. [CrossRef]
12. Senkal, M.; Geier, B.; Hannemann, M.; Deska, T.; Linseisen, J.; Wolfram, G.; Adolph, M. Supplementation of omega-3 fatty acids in parenteral nutrition beneficially alters phospholipid fatty acid pattern. *J. Parenter. Enter. Nutr.* **2007**, *31*, 12–17. [CrossRef]
13. Calder, P.C. Mechanisms of action of (*n*-3) fatty acids. *J. Nutr.* **2012**, *142*, 592S–599S. [CrossRef]
14. Stubbs, C.D.; Smith, A.D. The modification of mammalian membrane polyunsaturated fatty acid composition in relation to membrane fluidity and function. *Biochim. Biophys. Acta* **1984**, *779*, 89–137. [CrossRef]
15. Calder, P.C. The relationship between the fatty acid composition of immune cells and their function. *Prostaglandins Leukot. Essent. Fatty Acids* **2008**, *79*, 101–108. [CrossRef]
16. Terano, T.; Salmon, J.A.; Moncada, S. Biosynthesis and biological activity of leukotriene B5. *Prostaglandins* **1984**, *27*, 217–232. [CrossRef]
17. Jiang, Z.M.; Wilmore, D.W.; Wang, X.R.; Wei, J.M.; Zhang, Z.T.; Gu, Z.Y.; Wang, S.; Han, S.M.; Jiang, H.; Yu, K. Randomized clinical trial of intravenous soybean oil alone versus soybean oil plus fish oil emulsion after gastrointestinal cancer surgery. *Br. J. Surg.* **2010**, *97*, 804–809. [CrossRef]
18. Senkal, M.; Mumme, A.; Eickhoff, U.; Geier, B.; Spath, G.; Wulfert, D.; Joosten, U.; Frei, A.; Kemen, M. Early postoperative enteral immunonutrition: Clinical outcome and cost-comparison analysis in surgical patients. *Crit. Care Med.* **1997**, *25*, 1489–1496. [CrossRef]
19. Tsekos, E.; Reuter, C.; Stehle, P.; Boeden, G. Perioperative administration of parenteral fish oil supplements in a routine clinical setting improves patient outcome after major abdominal surgery. *Clin. Nutr.* **2004**, *23*, 325–330.
20. Weiss, G.; Meyer, F.; Matthies, B.; Pross, M.; Koenig, W.; Lippert, H. Immunomodulation by perioperative administration of *n*-3 fatty acids. *Br. J. Nutr.* **2002**, *87* (Suppl. 1), S89–S94. [CrossRef]
21. Furst, P.; Kuhn, K.S. Fish oil emulsions: What benefits can they bring? *Clin. Nutr.* **2000**, *19*, 7–14.
22. Grimm, H.; Mayer, K.; Mayser, P.; Eigenbrodt, E. Regulatory potential of *n*-3 fatty acids in immunological and inflammatory processes. *Br. J. Nutr.* **2002**, *87* (Suppl. 1), S59–S67. [CrossRef]
23. Mayer, K.; Grimm, H.; Grimminger, F.; Seeger, W. Parenteral nutrition with *n*-3 lipids in sepsis. *Br. J. Nutr.* **2002**, *87* (Suppl. 1), S69–S75. [CrossRef]
24. Koller, M.; Senkal, M.; Kemen, M.; Konig, W.; Zumtobel, V.; Muhr, G. Impact of omega-3 fatty acid enriched TPN on leukotriene synthesis by leukocytes after major surgery. *Clin. Nutr.* **2003**, *22*, 59–64. [CrossRef]
25. Morlion, B.J.; Torwesten, E.; Lessire, H.; Sturm, G.; Peskar, B.M.; Furst, P.; Puchstein, C. The effect of parenteral fish oil on leukocyte membrane fatty acid composition and leukotriene-synthesizing capacity in patients with postoperative trauma. *Metabolism* **1996**, *45*, 1208–1213. [CrossRef]
26. Wachtler, P.; Axel, H.R.; Konig, W.; Bauer, K.H.; Kemen, M.; Koller, M. Influence of a pre-operative enteral supplement on functional activities of peripheral leukocytes from patients with major surgery. *Clin. Nutr.* **1995**, *14*, 275–282. [CrossRef]
27. Schauder, P.; Rohn, U.; Schafer, G.; Korff, G.; Schenk, H.D. Impact of fish oil enriched total parenteral nutrition on DNA synthesis, cytokine release and receptor expression by lymphocytes in the postoperative period. *Br. J. Nutr.* **2002**, *87* (Suppl. 1), S103–S110. [CrossRef]
28. Sorensen, L.S.; Thorlacius-Ussing, O.; Schmidt, E.B.; Rasmussen, H.H.; Lundbye-Christensen, S.; Calder, P.C.; Lindorff-Larsen, K. Randomized clinical trial of perioperative omega-3 fatty acid supplements in elective colorectal cancer surgery. *Br. J. Surg.* **2014**, *101*, 33–42. [CrossRef]
29. Moher, D.; Hopewell, S.; Schulz, K.F.; Montori, V.; Gotzsche, P.C.; Devereaux, P.J.; Elbourne, D.; Egger, M.; Altman, D.G. CONSORT 2010 Explanation and Elaboration: Updated guidelines for reporting parallel group randomised trials. *J. Clin. Epidemiol.* **2010**, *63*, e1–e37. [CrossRef]
30. Wolters, U.; Wolf, T.; Stutzer, H.; Schroder, T. ASA classification and perioperative variables as predictors of postoperative outcome. *Br. J. Anaesth.* **1996**, *77*, 217–222. [CrossRef]

31. Kondrup, J.; Rasmussen, H.H.; Hamberg, O.; Stanga, Z. Nutritional risk screening (NRS 2002): A new method based on an analysis of controlled clinical trials. *Clin. Nutr.* **2003**, *22*, 321–336. [CrossRef]
32. A Service of the U.S. National Institutes of Health. Available online: http://clinicaltrials.gov/ct2/show/NCT00488904?term=NCT00488904&rank=1 (accessed on 4 September 2010).
33. Nielsen, M.S.; Gammelmark, A.; Madsen, T.; Obel, T.; Aardestrup, I.; Schmidt, E.B. The effect of low-dose marine *n*-3 fatty acids on the biosynthesis of pro-inflammatory 5-lipoxygenase pathway metabolites in overweight subjects: A randomized controlled trial. *Prostaglandins Leukot. Essent. Fatty Acids* **2012**, *87*, 43–48. [CrossRef]
34. Powell, W.S.; Gravel, S.; Gravelle, F. Formation of a 5-oxo metabolite of 5,8,11,14,17-eicosapentaenoic acid and its effects on human neutrophils and eosinophils. *J. Lipid Res.* **1995**, *36*, 2590–2598.
35. Sorensen, L.S.; Rasmussen, H.H.; Aardestrup, I.V.; Thorlacius-Ussing, O.; Lindorff-Larsen, K.; Schmidt, E.B.; Calder, P.C. Rapid Incorporation of omega-3 Fatty Acids Into Colonic Tissue After Oral Supplementation in Patients With Colorectal Cancer: A Randomized, Placebo-Controlled Intervention Trial. *J. Parenter. Enter. Nutr.* **2013**, *38*, 617–624. [CrossRef]
36. Grimm, H.; Mertes, N.; Goeters, C.; Schlotzer, E.; Mayer, K.; Grimminger, F.; Furst, P. Improved fatty acid and leukotriene pattern with a novel lipid emulsion in surgical patients. *Eur. J. Nutr.* **2006**, *45*, 55–60.
37. Wang, J.; Yu, J.C.; Kang, W.M.; Ma, Z.Q. Superiority of a fish oil-enriched emulsion to medium-chain triacylglycerols/long-chain triacylglycerols in gastrointestinal surgery patients: A randomized clinical trial. *Nutrition* **2012**, *28*, 623–629. [CrossRef]
38. Shimizu, T.; Fujii, T.; Suzuki, R.; Igarashi, J.; Ohtsuka, Y.; Nagata, S.; Yamashiro, Y. Effects of highly purified eicosapentaenoic acid on erythrocyte fatty acid composition and leukocyte and colonic mucosa leukotriene B4 production in children with ulcerative colitis. *J. Pediatr. Gastroenterol. Nutr.* **2003**, *37*, 581–585. [CrossRef]
39. Carpentier, Y.A.; Simoens, C.; Siderova, V.; Vanweyenberg, V.; Eggerickx, D.; Deckelbaum, R.J. Recent developments in lipid emulsions: Relevance to intensive care. *Nutrition* **1997**, *13*, 73S–78S. [CrossRef]
40. Barros, K.V.; Carvalho, P.O.; Cassulino, A.P.; Andrade, I.; West, A.L.; Miles, E.A.; Calder, P.C.; Silveira, V.L. Fatty acids in plasma, white and red blood cells, and tissues after oral or intravenous administration of fish oil in rats. *Clin. Nutr.* **2013**, *32*, 993–998. [CrossRef]

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Review

The Use of Dietary Supplements to Alleviate Androgen Deprivation Therapy Side Effects during Prostate Cancer Treatment

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Abstract: Prostate cancer (PCa), the most commonly diagnosed cancer and second leading cause of male cancer death in Western societies, is typically androgen-dependent, a characteristic that underlies the rationale of androgen deprivation therapy (ADT). Approximately 90% of patients initially respond to ADT strategies, however many experience side effects including hot flashes, cardiotoxicity, metabolic and musculoskeletal alterations. This review summarizes pre-clinical and clinical studies investigating the ability of dietary supplements to alleviate adverse effects arising from ADT. In particular, we focus on herbal compounds, phytoestrogens, selenium (Se), fatty acids (FA), calcium, and Vitamins D and E. Indeed, there is some evidence that calcium and Vitamin D can prevent the development of osteoporosis during ADT. On the other hand, caution should be taken with the antioxidants Se and Vitamin E until the basis underlying their respective association with type 2 diabetes mellitus and PCa tumor development has been clarified. However, many other promising supplements have not yet been subjected large-scale clinical trials making it difficult to assess their efficacy. Given the demographic trend of increased PCa diagnoses and dependence on ADT as a major therapeutic strategy, further studies are required to objectively evaluate these supplements as adjuvant for PCa patients receiving ADT.

Keywords: prostate cancer; androgen deprivation therapy; adverse effects; dietary supplements; alternative therapies

1. Introduction

Prostate Cancer (PCa) is the most commonly diagnosed male cancer and the second leading cause of cancer death among men in Western societies [1,2]. Radical prostatectomy or primary radiation therapy are the preferred treatment modalities in men with locally confined PCa. For advanced tumors or tumor recurring after primary surgery or radiation therapy the androgen receptor (AR) and its signaling network are the prime targets of therapy. The androgen receptor orchestrates crucial oncogenic factors in PCa etiology since androgens drive proliferation, differentiation, and survival of benign and malignant prostate cells [3]. Hence, upon initial diagnosis, 80%–90% of PCa are androgen-dependent [4], an observation that underlies the rationale of androgen deprivation therapy (ADT), the current mainstay systemic treatment for advanced PCa [5]. Although highly

effective, ADT is associated with considerable side effects that negatively affect the patient's quality of life [6,7]. These adverse events include hot flashes [8], metabolic effects such as an induced metabolic syndrome (MetS) [9–12] including insulin resistance [13,14], cardiovascular (CV) diseases [15,16], musculoskeletal side effects characterized by reduced lean body mass and muscle strength, and osteoporosis [17–20] as well as depression and sexual dysfunction. Although several medical regimens have been developed [21], their impact on minimizing ADT side effects and improving quality of life is still under discussion. Recent statistics revealed an increasing use of complementary and alternative substances by PCa patients [22]. Indeed, approximately one in four patients with PCa uses at least one complementary or alternative method with the primary aim of ameliorating ADT-induced adverse effects [23,24]. In particular, herbal and dietary supplements appeal to patients because they are perceived as being "natural" with fewer side effects than prescription medicines. Despite the widespread use of alternatives to medical treatment options, little is known about their safety, efficacy and mechanism of action. The limitation of clinical studies investigating this issue leads to a lack of information concerning the use of different types of alternative interventions. This article focuses on the metabolic and musculoskeletal side effects of ADT, which are not alleviated by current treatment strategies. In particular, we discuss several adjuvant dietary options including herbs, phytoestrogens, selenium (Se), fatty acids (FA), calcium, and Vitamins D and E, whose use in the treatment of ADT side effects is supported by scientific evidence derived either from cell-based models, animal models or clinical trials.

2. ADT in the Treatment of PCa

The androgenic hormones testosterone (T) and 5α-dihydrotestosterone (DHT), which mediate their action through the AR, are essential for normal prostate development but also contribute to prostate tumor growth by regulating cell proliferation and differentiation. The concept of hormonal manipulation using ADT to restrict PCa growth was first described in 1941 by Huggins and Hodges [25] and is based on the observation that 80%–90% of PCa are androgen dependent. Since then, multiple strategies have been established to reduce serum androgen levels or to interfere with their function by inhibiting the AR. Current strategies for hormonal blockade used in the treatment of PCa have been reviewed recently [26] and include bilateral orchiectomy, luteinizing hormone-releasing hormone (LHRH) agonists or antagonists and anti-androgens (Figure 1 and Table 1).

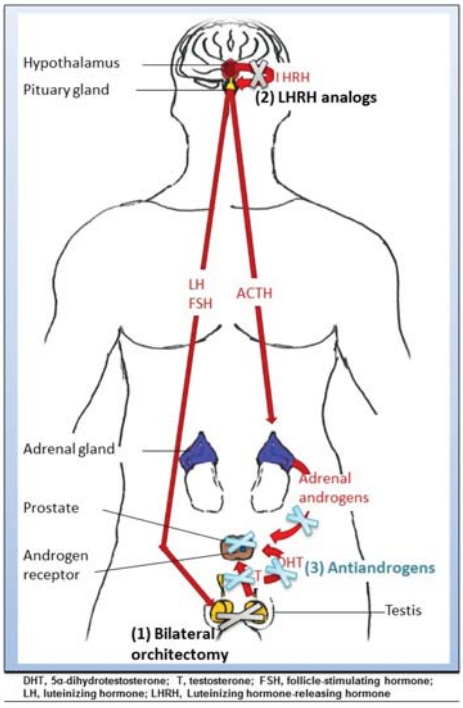

Figure 1. Mechanisms of action of androgen deprivation therapy (ADT) for blockage of the hypothalamus-pituary axis.

Current ADT strategies used in the treatment of PCa include bilateral orchiectomy (surgical castration), LHRH agonists or antagonists and anti-androgens (medical castration). (1) Bilateral orchiectomy is the surgical removal of both testicles inhibiting the production of testicular testosterone (T) and estradiol; (2) LHRH analogs reversibly decrease T production by the testis resulting in the down-regulation of LHRH receptors and thus reduced levels of luteinizing hormone (LH), follicle stimulating hormone (FSH) and T; (3) Anti-androgens (including flutamide, nilutamide, and bicalutamide) may bind competitively to the AR in cells thereby inhibiting its activation, or (abiraterone) block *de novo* androgen synthesis, both leading to apoptosis and reduced prostate tumor growth.

Table 1. Treatment options for hormone reduction.

Modality	Drug	Mechanism	Side Effects
Surgical Castration			
Bilateral Orchiectomy	-	Surgical removal of testicles, ↓T	Hot flashes, reduced muscle mass and energy, anemia and osteoporosis
Medical Castration			
LHRH Interference	*LHRH agonists* Leuproreline acetate (Trenantone®, Eligard®) Goserelin (Zoladex®) Triptorelin (Trelstar®) Histrelin (Vantas®)	LHRH receptor downregulation after initial flare, ↓LH, ↓FSH, ↓T	Hot flashes, reduced muscle mass and energy, anemia and osteoporosis, flare phenomenon, CV events, cardiotoxicity
	LHRH antagonists Degarelix (Firmagon®)	Blockade of LHRH receptor, ↓LH, ↓FSH, ↓T	Hot flashes, reduced muscle mass and energy, anemia and osteoporosis, CV events, histamine release
Antiandrogens	*Non-Steroidal* Flutamide (Eulexin®) Bicalutamide (Casodex®) Nilutamide (Nilandron®) Enzalutamide (Xtandi®)	Antagonizes AR in target tissues, ↑T	Gynecomastia, hepatotoxicity (flutamide), visual and respiratory disturbance and alcohol intolerance (nilutamide), GI problems ,fatigue and hot flushes (enzalutamide)
	Steroidal Cyproterone acetate (Androcur®, Cyprostat®)	Antagonizes AR in target tissues, suppress LHRH secretion, ↓LH, ↓T	Gynecomastia, cardiovascular events, fluid retention, GI problems
	Abiraterone acetate (Zytiga®)	Inhibition of Cyp17A1 enzyme, suppresses T, estrogen and glucocorticoid biosynthesis	Vomiting, GI problems, swellings, weakness, cough, high blood pressure

LHRH, luteinizing hormone-releasing hormone; LH, luteinizing hormone; T, testosterone; AR, androgen receptor; GI, gastrointestinal; CV, cardiovascular.

Although accompanied by fewer side effects than "medical castration", the use of surgical bilateral orchiectomy is currently limited in developed countries. Rather, medical-based approaches to achieve castration levels of circulating androgens are preferred. In contrast to surgical castration, medical castration using LHRH analogs reversibly decrease T production by the testis and are therefore the preferred treatment modality. There are two different classes of LHRH analogs: LHRH agonists and antagonists. LHRH agonists stimulate the LHRH receptors in the pituitary gland resulting in a temporary increase in LH and FSH secretion, which in turn causes an initial rise in T production (the so-called "flare phenomenon [27]"). However, constant LHRH stimulation leads to a negative feedback loop resulting in the down-regulation of LHRH receptors and thus reduced levels of LH, FSH and T. In contrast to LHRH agonists, LHRH antagonists competitively bind to their receptors in the pituitary gland thereby blocking their activation by the natural ligand, inducing a rapid decrease in LH, FSH, and T levels without any flare.

Anti-androgens differ mechanistically from the above-described castration therapies as they do not alter androgen induction by direct modification of the hypothalamic-pituitary-gonadal axis in the brain. Rather, most anti-androgens (including flutamide, nilutamide, and bicalutamide) bind

competitively to the AR in cells inhibiting its activation, leading to apoptosis and reduced prostate tumor growth. By contrast, abiraterone blocks *de novo* androgen synthesis via irreversible inhibition of CYP17-A1, a rate-limiting enzyme that catalyzes the conversion of cholesterol to androgen and estrogen precursors. The new-generation drugs abiraterone and enzalutamide have been developed during the past 5 years [28] and thus knowledge concerning their side effects is still limited. Since serum T and estrogen levels are maintained for all anti-androgen drugs that target the AR, hypogonadal side effects are generally less pronounced [29]. However, anti-androgens are often used in combination with LHRH analogs, thus this article will not discuss the side effect profile of anti-androgen monotherapy.

3. Adverse Effects of ADT

Frequent side effects of ADT that result in poor quality of life include hot flashes, metabolic effects such as gynacomastia as well as an increased body mass index, insulin resistance, metabolic syndrome (MetS), cardiovascular (CV) diseases, and musculoskeletal effects including reduced muscle mass, osteoporosis, and also sexual dysfunction (summarized Table 2) [1,7,30–35] Of these adverse events, metabolic and musculoskeletal effects are the most prevalent and distressing side effects reported by patients [21].

ADT targets gonadal function. Consequently, hypogonadism is prevalent in PCa patients undergoing ADT compared to those that undergo surgery and/or radiation therapy or compared to age-matched controls [7]. Thus, ADT induces a profound hypogonadism, which in turn is responsible for increased body mass index, increased fat mass, reduced lean body mass and muscle strength, and osteoporosis. Besides the desired physiological consequences of ADT in reducing serum androgens, hormonal castration is also associated with a decrease in circulating estrogens that are synthesized from androgens by peripheral aromatization (Figure 2). Despite having normal to elevated serum T levels, men with congenital aromatase deficiency (and thus, non-detectable serum estrogen levels) have a high prevalence of osteoporosis, insulin resistance and metabolic syndrome (MetS) [36], an observation that underscores the importance of estrogens in men. Moreover, it is decreased estrogen rather than T levels that are responsible for decreased bone density, accelerated rate of bone loss, and increased fracture incidence [37]. Thus, side effects induced by ADT leading to hot flashes, osteoporosis, MetS and higher CV events are related to androgen as well as estrogen deficiencies [35,38]. However, the relative contribution of T and estrogen to these adverse effects remains unclear.

ADT is associated with multiple adverse effects, many of which are related to androgen as well as estrogen deficiency that occur as a result of treatment [30,35].

Table 2. Summary of dietary supplements for the management of androgen deprivation therapy (ADT) side effects.

Side Effect	Postulated Management	Efficacy	Reference
Hot Flushes	**Herbs**		
	Black cohosh	Minimal, ↓sweating symptoms, may ↓hot flashes in women.	[39,40]
	Dong quai	No benefit in women or men.	[41]
	Ginseng	Minimal effects in women.	[42]
	Phyto-Estrogens		
	Soy-Isoflavons	No effect with supplements in men; possible impact with dietary source seen in women.	[43–49]
	Flaxseed	Unknown, initial studies showed impact on hormonal levels and serum lipids.	[50]
	Vitamin E	Previously recommended, but increased risk for diabetes. Might also increase the risk for PCa (SELECT trial).	[43,51–58]
Osteoporosis	**Herbs**		
	Black cohosh	Potentially effective in preclinical studies (only studied in female animals).	[40,59]
	Phyto-estrogens		
	Soy-Isoflavones	Potentially effective in preclinical studies (castrated male animals).	[60,61]
	Fatty acids		
	Omega-3 FA(CLA)	Potentially effective in preclinical studies (male and female animals).	[62–66]
	Calcium and Vitamin D	Effective in men and postmenopausal women.	[67–72]
Cardiovascular Events	**Herbs**		
	Ginseng	No benefit.	[42]
	Garlic	Showed to reduce blood pressure.	[73,74]
	Phyto-Estrogens		
	Soy-Isoflavones	No effect. Might reduce ↓LDL-cholesterol.	[46,75,76]
	Flaxseed	Potentially effective, reduced ↓LDL-cholesterol in postmenopausal women.	[77]
	Fatty acids		
	Omega-3 FA	No benefit. Postulated mechanism: ↓TG	[78–82]
	Selenium	No effect. Might have adverse effects on diabetes. Increased PCa risk (SELECT trial).	[24,56,58,84–90]
	Vitamin E	Negative association with CV health. Might increase PCa risk. No benefit in women.	[43,51,55,58,86,92]

CV, cardiovascular; CLA, conjugated linoleic acid; LDL, low density lipoprotein; TG, triglyceride.

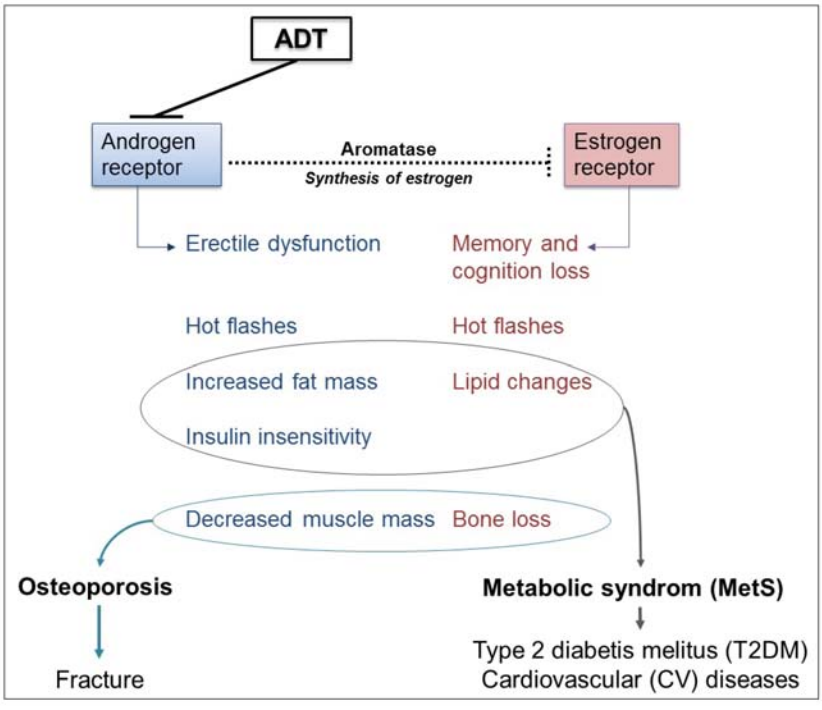

Figure 2. Side effects associated with androgen deprivation therapy (ADT).

3.1. Metabolic and Cardiovascular Side Effects

A number of prospective studies have shown that ADT increases abdominal fat and serum triglycerides (TG) and decreases insulin sensitivity [93]. Additionally, cross-sectional studies have shown that men receiving ADT are more likely to meet the diagnostic criteria of MetS [94,95]. Moreover, long term ADT therapy caused significantly higher levels of fasting insulin and glucose compared to men with PCa not on ADT as well as to age-matched controls [96]. A retrospective study involving 44 PCa patients who received ADT showed an increase in fasting blood glucose, total cholesterol, LDL cholesterol, and TG, but a decrease in HDL cholesterol in these patients [34]. In addition, longitudinal studies indicate that lower T levels in men independently predict MetS [97,98] and type 2 diabetes mellitus (T2DM) [99]. Because MetS is independently associated with CV mortality [100], it is plausible that the positive association between lower serum T levels and MetS may at least in part explain the higher CV mortality in men with PCa receiving ADT (with therapy-induced hypogonadism as the likely trigger of these events) [101]. The protective role of T on the CV system and its relationship to MetS, CV morbidity and mortality has been recently reviewed [98]. Since CV disease has become the most common cause of non-PCa-related death among this patient group [102], the potential risks of ADT with regards to CV events should be carefully weighed against the expected benefits. Moreover, since metabolic complications predominantly occur within 3–6 months after starting ADT therapy, close observation of patients especially during the first year of ADT is highly recommended [93].

3.2. Musculoskeletal Side Effects

Given that 25–40% of patients already have osteoporosis at PCa diagnosis, it must be considered that the symptoms will worsen upon administration of ADT. In general, two different forms of osteoporosis are known [103]. Whereas primary osteoporosis is caused by malnutrition and genetic

predisposition, secondary osteoporosis predominantly arises in men due to hypogonadism, including that experienced by PCa patients receiving ADT [104]. Longitudinal studies report that approximately 35% of men on LHRH agonists experience at least one skeletal fracture and approximately 20% will be diagnosed as osteoporotic or osteopenic within 7 years of starting therapy [105]. The absolute excess risk of fractures ranges from 5%–7% over an average of 6 years follow-up compared to men not receiving ADT [31,106]. In particular, treatment with either LHRH analogs or orchiectomy is associated with a significant decline in bone mineral density (BMD) and increased risk of bone fractures [31,106]. In this context, it should be noted that bone fractures in men with PCa have been associated with higher mortality rates [107]. Cross-sectional studies have shown that BMD at the hip and lumbar spine decreases with the duration of continuous ADT [108]. However, BMD appears to initially decline at a fast rate. For example, greater declines of BMD were observed among recent *versus* chronic ADT users with stronger declines in both groups in contrast to ADT naïve men [109]. BMD declined at multiple sites including the lumbar spine and hip in the first year after starting ADT, however subsequent changes in the second year were much smaller and no longer statistically significant [110]. A larger study followed 618 men on ADT for up to 7 years with annual dual-energy X-ray absorptiometry (DXA) scans [111]. Whilst steady annual declines in BMD among men with normal BMD at baseline were noted, only 38 men with normal BMD at baseline still remained in the study by 3 years [111]. Thus, due to the lack of longitudinal studies, it is currently not possible to determine whether BMD loss beyond the first year of ADT is attenuated.

4. Dietary Supplements

The use of dietary supplements to alleviate side effects of ADT is an attractive approach for the majority of PCa patients. Indeed, an array of dietary supplements proposed to be useful in intervening with side effects due to declining hormone levels in men and women are promoted typically via media that lack scientific evidence. However, it should be noted that many of these supplements have been studied in the context of hormone deprivation during menopause or in the course of breast cancer therapy in women with no clinical studies carried out in men thus far (e.g., black cohosh, ginseng, garlic). Thus, it is difficult to conclude whether these dietary supplements will exhibit a similar efficacy in PCa patients receiving ADT. Of the few dietary supplement studies that have been performed in men, most were primarily aimed at reducing the risk of PCa development or reducing the risk of disease progression after initial diagnosis or first line treatment. Thus, studies in men receiving ADT are rare. Consequently, it is inappropriate to assume that beneficial (or non-beneficial) effects on PCa development or progression will similarly translate to modifying the adverse effects associated with ADT.

4.1. Herbs

Herbal supplemental approaches to manage metabolic and musculoskeletal side effects like hot flashes and osteoporosis have been extensively evaluated in breast cancer and menopausal women in small randomized trials with varying success [112,113]. Thus, it seems evident to evaluate the potential of the most promising of these herbs for the treatment of side effects in men who are being treated for PCa with ADT.

4.1.1. Black Cohosh (*Cimicifuga racemosa*)

The herbal supplement black cohosh, which is approved for the treatment of menopausal symptoms by the German health authority (Commission E), has also shown to have MetS-preventive and bone-protective properties in recent preclinical studies [59,114]. A randomized, placebo-controlled trial found no difference in the frequency of hot flashes but a significantly lower incidence of sweating in menopausal women with a history of breast cancer in the black cohosh group compared to placebo [39]. This finding is particularly pertinent given that a significant placebo effect is consistently observed in investigations of hot flashes, with the placebo effect reportedly sufficient to reduce hot flashes by up

to 75% [115]. On the other hand, significant evidence of potentially beneficial effects on bone tissue was shown, although it should be noted that all clinical studies were conducted in females [59,116]. Of interest is one study that was carried out in orchidectomized male rats and showed to prevent osteoporosis *in vivo* [117].

The mechanism underlying the activity of black cohosh remains poorly understood. There is a lack of scientific evidence regarding its proposed estrogenic activity. However, it was recently shown to attenuate nucleoside uptake into cells and thus may have an impact on tumor treatment by nucleoside analogs [118]. One may speculate that its modulation of adenosine signaling may be beneficial for CV side effects of ADT [119], but this has yet to be investigated. Despite the lack of knowledge regarding the mechanism of action of black cohosh, its long history of use reveals that black cohosh is well tolerated. The use of black cohosh in PCa patients undergoing ADT may be warranted for hot flushes, CV and osteoporotic side effects. Importantly, further systematic studies assessing the safety and efficacy of black cohosh in alleviating ADT-induced side effects may be worth pursuing.

4.1.2. Dong Quai (*Angelica sinensis*)

Dong quai is a traditional Chinese herbal remedy most commonly used in the treatment of female reproductive problems. According to our knowledge, there are no pre-clinical studies addressing its effects in PCa. However, a small randomized clinical trial was conducted in men receiving ADT where dong quai was shown to be ineffective in reducing hot flashes [41]. Similarly, randomized trials in women also found no effect of dong quai on hot flashes beyond a placebo, irrespective of whether the herb was used alone or as part of a complex multi-ingredient intervention [120]. Taken together, the current evidence does not support the use of dong quai in patients undergoing ADT.

4.1.3. Ginseng (*Panax ginseng*)

Ginseng extract is widely used in traditional Chinese medicine and was reported to reduce fatigue, insomnia and depression in post-menopausal women, although there was no significant benefit on hot flashes [121]. However, a recent review of studies examining the efficacy of ginseng on menopausal symptoms highlighted the poor quality and bias of many randomized clinical trials conducted to date, raising doubt as to the usefulness of this herb in managing menopause symptoms [42]. Nonetheless, several pre-clinical and clinical studies have shown that ginseng may possess anti-cancer and hypoglycemic properties, the latter of potential benefit in ameliorating MetS in men receiving ADT [122,123].

4.1.4. Garlic (*Allium sativum*)

Garlic is frequently used as a dietary supplement for the treatment of hyperlipidemia, heart disease, and hypertension [124]. In addition, there is evidence that garlic is associated with blood pressure reduction in patients with elevated systolic blood pressure (10–12 mmHg systolic, 6–9 mmHg diastolic) but not in normotensive patients [73,74]. In this respect, it is conceivable that garlic may also reduce CV effects in PCa patients undergoing ADT. However, there is currently insufficient data to support this hypothesis and further studies that specifically address this question would be required [125].

4.2. Phytoestrogens

In the past, suppression of T was achieved using high doses of estrogens (estradiol) or selective estrogen receptor modulators [126–129]. However, these treatments were prone to severe and even fatal CV side effects [130,131]. Consequently, these treatments have been replaced by other therapeutics such as LHRH analogs. Phytoestrogens (plant estrogens) are non-steroidal naturally occurring phenolic compounds with known estrogenic effects and estrogen receptor (ER-β and/or ERα) binding properties [132–134]. Thus, these "mild" estrogens could possibly serve as natural alternatives with potentially fewer side effects. Indeed, phytoestrogens have been shown to improve

metabolic health, reduce CV risk, and improve BMD and brain function [60,75,135]. There are three classes of phyto-estrogens, which are categorized according to their chemical structure as isoflavones, lignans or coumestans. Isoflavones are the largest and also the most extensively studied group of phytoestrogens, which includes genistein, daidzein and glycitein. Isoflavones are found in highest amounts in soybeans, flaxseed and legumes. Soy is stably integrated into Asian diets where daily intake is at least 40 times higher than among Western populations [136]. This is considered to be one of the factors contributing to a much lower incidence of prostate and breast cancer in Asian countries [137]. In addition, increased consumption of isoflavones has been associated with decreased incidence of diabetes and heart disease in South and East Asia [44,138]. In clinical trials, isoflavones have shown an improvement in hot flash severity, glycemic control, MetS and inflammatory profile in both men and postmenopausal women [45,76,139]. However, in a recent double-blinded, randomized, placebo-controlled pilot study, phytoestrogens failed to improve metabolic or inflammatory parameters of men with PCa during ADT [47]. This is consistent with another pilot study conducted in 33 men where high dose isoflavones showed no significant improvement in cognition, vasomotor symptoms or any other aspect of quality of life measures compared to placebo in androgen deprived men [140]. Thus, there is currently no clinical evidence for the proposed improvement of ADT-induced side effects by phytoestrogens, although limitations of these studies including small cohort sizes and short treatment durations should be taken into account.

4.3. Selenium (Se)

Se is an essential trace element, which is incorporated as selenocysteine into selenoproteins, many of which are reactive oxygen species (ROS) scavenging enzymes, such as glutathione peroxidase and thioredoxin reductase [141,142]. Thus, Se may be useful in decreasing oxidative stress and low-grade inflammation. Notably, plasma biomarkers of oxidative stress and low-grade inflammation are associated with MetS, obesity and insulin resistance, conditions that are common side effects of ADT [143]. Se was shown to have strong anti-proliferative and pro-apoptotic effects on human PCa cells and to improve severe metabolic side effects in patients [83,144]. Moreover, cancer prevention studies indicated that Se decreases ROS and is associated with decreased incidence of PCa [145,146]. Taken together, these observations provided a strong mechanistic rationale to combine ADT and Se for the treatment of PCa [5] and led to a number of epidemiological studies and clinical trials [51,83]. However, epidemiological studies have suggested that supranutritional Se intake and high plasma Se levels are not necessarily preventive against cancer, and may even be a possible risk factor for developing T2DM [83]. For example, supplementation with Se and/or Vitamin E in the large-scale Selenium and Vitamin E Cancer Prevention Trial (SELECT) did not prevent the development of PCa, rather, the incidence of newly diagnosed T2DM increased among the Se-supplemented participants [51,83]. Whilst the Nutritional Prevention of Cancer (NPC) study reported a decreased risk of PCa among Se supplemented men, an increased risk of T2DM was also observed in participants with baseline plasma Se levels in the top tertile [147]. Since then, several longitudinal studies have failed to support a causal role of Se in T2DM, although cross-sectional studies continued to find significant associations between circulating Se levels and T2DM [148]. For example, serum Se was observed to be associated with adipocytokines, such as TNF-α, VCAM-1, leptin, FABP-4, and MCP-1 [149,150] and adiponectin [151]. Although on the other hand, an analysis across randomized groups showed that Se supplementation had no effect on adiponectin levels after six months of treatment [84]. Thus, it appears likely that the reported link between Se and T2DM is due to indirect effects of Se-containing ROS scavenging enzymes, affecting the hydrogen peroxide level that in turn modulates both glucose-induced insulin secretion and insulin-induced signaling [152,153]. Moreover, Se homeostasis is modulated by factors related to carbohydrate metabolism, suggesting that low serum Se levels may themselves be a consequence of dysregulated energy metabolism in T2DM [153]. In addition, there appears to be a significant interaction between dietary intake of phytoestrogens and Se with important implications for heart disease, cancer, diabetes, and other conditions related to body weight [154].

From the current prospective—especially since to date there has been no study specifically evaluating the supplementation of Se in PCa patients receiving ADT—a combination of ADT with Se cannot be recommended given that many patients develop pre-diabetes.

4.4. Fatty Acids (FA)

The relative amounts and different types of dietary FA are thought to play critical roles in PCa associated MetS. Total FA intake and the ratio of omega-3 (ω-3) to omega-6 (ω-6) polyunsaturated FA (PUFAs) in the Western diet have increased significantly since the Industrial Revolution [155]. The effects of ω-3 PUFAs on CV disease, cancer as well as MetS have been investigated extensively [78, 156–161]. Consequently, the United States Food and Drug Administration (FDA) has approved ω-3 PUFAs for the prevention of CV adverse outcomes by the postulated mechanism of lowering serum triglyceride levels [162,163]. Additionally, three common dietary ω-3 FA—alpha-linolenic acid (ALA), eicosa-pentaenoic acid (EPA), and docosahexaenoic acid (DHA)—were proposed to exhibit anti-inflammatory properties [50,79,164]. Since inflammation is a major risk factor for the development of CV disease, these ω-3 FA may reduce the risk of CV disease [50,79,164]. Various pre-clinical, epidemiological and clinical studies have investigated the influence of ω-3 and ω-6 FA on the development and progression of PCa [80,155,158,161,165–173]. However, these studies have yielded contradictory results. In particular, dietary intake of long-chain ω-3 PUFA or its individual components (EPA, DHA, docosapentaenoic acid (DPA) and ALA) have been associated with PCa risk and progression [80,155,158,161,165–173]. On the other hand, encouraging results were obtained from clinical trials showing potential anti-inflammatory effects of ω-3 FA [77,174–177]. Other trials investigating higher doses of ω-3 FA (>1 g/day EPA and/or DHA) in populations at high risk for CV disease reported improvements (*i.e.*, reduced concentrations) of selected inflammatory markers [81, 178,179]. However, it should be noted that in these studies the same dosage of ω-3 FA showed mixed response rates in healthy adults, and no beneficial effect in patients that received lower doses (<1 g/day) [81,174,175,178–185]. A single study has reported that very high intakes (6.6 g/day), which are well beyond the current recommendations (500 mg/day–1 g/day), may raise blood concentrations of some inflammation markers such as soluble tumor necrosis factor receptors 1 and 2 (sTNF-Rs 1 and 2) [79,185]. It is likely that differences in dosage, study population characteristics, the source of ω-3 FA, study duration, and background diet may explain the inconsistencies in these findings. In this respect, it may be noted that the majority of clinical trials have used marine sources of ω-3 FA (EPA and DHA from fish), whereas few have examined FA from plants (ALA from flax oil) and only two trials compared both sources [82,175]. The heterogeneous results from these studies might also be due to variations in measuring FA consumption of individuals and different techniques assessing their diet [186–190]. The main mechanisms underlying the purported anticancer effects of modulating dietary fat appear to be through reduced insulin-like growth factor signaling and alterations in membrane ω-6 to ω-3 FA ratios leading to suppressed COX-2-dependent PGE-2 production and reduction of inflammation via modification of the eicosanoid pathway [191–195]. Moreover, decreased PGE-2 levels are expected to decrease estrogen production and further also modify androgen production [190].

In summary, although ω-3 FA exhibits some anti-inflammatory potential, there is still a lack of consensus regarding their optimal use and dosage. Moreover, results from the aforementioned studies have to be interpreted with caution, since the metabolic conditions during ADT are different. Further research is warranted to better elucidate the mechanism of action and ideal consumption of ω-3 FA for potential CV health benefits during ADT.

4.5. Calcium and Vitamin D

Osteoporosis is a common and one of the most debilitating side effects of ADT. The most important nutritional factors contributing to osteoporosis include deficiencies in Vitamin D and calcium. Measurement of Vitamin D levels in osteoporotic males by a large multi-center study in the US (MrOs) revealed a deficiency in 26% and an insufficiency in 72% of subjects [54]. Because

deterioration of BMD occurs soon after initiation of ADT therapy [67], the European Association of Urology does not specify recommendations but states that calcium supplementation is protective [196]. The National Comprehensive Cancer Network cite the National Osteoporosis foundation guidelines that recommend calcium (1000 mg daily) and Vitamin D (800–1000 IU daily) supplementation for all men aged 50 or above [197]. Notably however, data supporting this recommendation are lacking as shown in a recent systematic review [196]. One cross-sectional study suggested an association between low calcium intake and a greater likelihood of osteoporosis in men with PCa of whom 71 % were undergoing ADT. Unfortunately, Vitamin D use was not examined in this study [198]. Another trial reported a positive association of calcium and Vitamin D use (examined together) on hip and lumbar spine BMD in men on ADT [199]. Importantly, results from 12 different clinical trials revealed that the commonly recommended doses, of 500–1000 mg calcium and 200–500 IU Vitamin D per day still result in BMD loss in men receiving ADT [196]. Alibhai *et al.* examined long-term effects of calcium and Vitamin D in a prospective 3-year matched cohort study comparing PCa patients with and without ADT. This study found that Vitamin D but not calcium may be protective particularly in the first year of ADT [32]. In a multivariate analysis, it was further shown that the mean daily calcium intake in men receiving ADT was significantly lower in men who suffered from osteoporosis compared to those without osteoporosis [198].

In summary, calcium and Vitamin D supplementation is a recommended complementary therapy not only in elderly men with osteoporosis but also in men undergoing ADT even though the long-term impact of ADT on BMD and the value of calcium and Vitamin D in ameliorating negative effects remains to be elucidated more precisely.

4.6. Vitamin E

Vitamin E is a potent antioxidant, which is of interest to ameliorate hot flushes and CV associated side effects of ADT. Vitamin E has a long history in the treatment of pre-eclampsia (characterized by high blood pressure in pregnant women), premenstrual syndrome, painful periods, menopausal syndrome, hot flashes associated with breast cancer, and breast cysts even though randomized controlled clinical studies did not reveal any benefit [52,53,86,200]. Moreover, the Physicians Health Study II concluded that Vitamin E does not reduce the risk of major CV events (non-fatal myocardial infarction, non-fatal stroke, or CV disease death) [57]. Similarly, the Women's Health Study, which comprised approximately 40,000 healthy women, found that Vitamin E did not reduce the risk of death or major CV events. Interestingly however, there was a significant reduction in the secondary endpoint of CV deaths and in major CV events among a subgroup of women aged 65 or over [55]. The Women's Antioxidant Cardiovascular Study found that there were no overall effects of Vitamin E on CV events among women at high risk of CV disease [91]. Moreover, the intake of Vitamin E has been shown to increase all-cause mortality and may even have negative effects on CV health [92].

There have been a number of studies in men, which have purported a positive effect of Vitamin E on hot flushes and high blood pressure [201]. However, most of these studies have yielded inconclusive or conflicting findings or a lack of benefit for its use, illustrating the need for studies of higher quality in this area. Thus, clinical trials have failed to recapitulate the promising findings of *in vitro* and many observational studies. Possible reasons for this discrepancy may be that clinical trials are too short in duration to reverse the results of decades of oxidative stress contributing to atherosclerosis or that the antioxidants selected for study were chosen for their ease of availability rather than proven efficacy (Vitamin E) [202]. Recent evidence from the SELECT trial revealed an increased risk for PCa in the Vitamin E supplemented group. Taken together, current evidence does not support a beneficial effect for Vitamin E, and its use as a supplementary treatment is therefore not recommended [56,88].

5. Discussion

The administration of ADT is associated with a diverse set of known side effects that have a significant impact on patient quality of life, overall health, and mortality. Some of the dietary

supplements discussed in this review may be beneficial for patients undergoing ADT. The value of calcium and Vitamin D supplementation remains to be elucidated more precisely; however, because of their long and safe history of usage they may be recommended in the prevention of osteoporosis during ADT. Phytoestrogens were shown to prevent hot flashes and other climacteric complaints and exert anti-osteoporotic effects in women. However, positive effects on CV health are still questionable and require further elucidation, especially with respect to their effects in PCa patients receiving ADT. In addition, further clinical trials evaluating the efficacy of isoflavones must be conducted before their use for relieving ADT-induced side effects can be advocated. We can conclude that dietary interventions with herbal substances may in fact be helpful in the treatment of adverse effects arising from ADT [43]; however, more clinical randomized studies in PCa patients on ADT are highly warranted to support these findings. The long history of use and lack of adverse effects of black cohosh in the treatment of climacteric complaints in women is particularly encouraging. Further evaluation of its proposed osteo-protective and anti-metabolic effects in conjunction with ADT in randomized and controlled clinical studies is also warranted. Recent data obtained in the large SELECT trial suggest that combined supplementation of Vitamin E and supranutritional Se may increase the risk of PCa, making this a non-recommended treatment for men receiving ADT. From the current perspective, a combination of ADT with Se, which has been associated with an increased risk for the development of T2DM, cannot be recommended given that many patients develop pre-diabetes.

6. Conclusions

In summary, dietary supplements are active compounds with as yet mostly poorly defined effects. As such, the unregulated self-prescription of active compounds such as soy, Se or Vitamin E may in some cases even prove to be harmful or negatively interfere with cancer treatment. Given the prevalent use of alternative dietary supplements in PCa patients, there is an urgent need to (1) perform rigorous research to determine the precise physiological effects of these different supplements with respect to relieving side effects of ADT; (2) conduct clinical trials of these supplements in men undergoing ADT and (3) establish more open lines of communication between patients and physicians regarding the use of dietary supplements and their integration into conventional treatment strategies.

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References and Notes

1. Siegel, R.; Naishadham, D.; Jemal, A. Cancer statistics, 2012. *Cancer J. Clin.* **2012**, *62*, 10–29. [CrossRef]
2. Siegel, R.; Ma, J.; Zou, Z.; Jemal, A. Cancer statistics, 2014. *Cancer J. Clin.* **2014**, *64*, 9–29. [CrossRef]
3. Heinlein, C.A.; Chang, C. Androgen receptor in prostate cancer. *Endocr. Rev.* **2004**, *25*, 276–308. [CrossRef] [PubMed]
4. Denis, L.J. The role of active treatment in early prostate cancer. *Radiother. Oncol.* **2000**, *57*, 251–258. [CrossRef] [PubMed]
5. Lieberman, R. Androgen deprivation therapy for prostate cancer chemoprevention: Current status and future directions for agent development. *Urology* **2001**, *58*, 83–90. [CrossRef] [PubMed]
6. Molina, A.; Belldegrun, A. Novel therapeutic strategies for castration resistant prostate cancer: Inhibition of persistent androgen production and androgen receptor mediated signaling. *J. Urol.* **2011**, *185*, 787–794. [CrossRef] [PubMed]

7. Basaria, S.; Davda, M.N.; Travison, T.G.; Ulloor, J.; Singh, R.; Bhasin, S. Risk factors associated with cardiovascular events during testosterone administration in older men with mobility limitation. *J. Gerontol. Series A Biol. Sci. Med. Sci.* **2013**, *68*, 153–160. [CrossRef]
8. Hot flashes are a sudden wave of mild or intense body heat caused by rushes of hormonal changes resulting from decreased levels of estrogen. They can occur at any time and may last from a few seconds to half an hour. They are due to blood vessel dilation and constriction. Their frequency is increased in menopausal women and men receiving ADT.
9. The "metabolic" or "insulin-resistance" syndrome (MetS) is a cluster of cardio-vascular (CV) risks related to insulin resistance [10,11]. Diagnosis of the syndrome is defined by the adult treatment panel III (ATP III) and the World health organization (WHO) as a combination of low HDL, increased waist circumference, increased triglycerides, increased fasting glucose and hypertension [11]. The syndrome itself is further associated with various metabolic abnormalities, including insulin resistance as well as other factors such as male hypogonadism [12].
10. Ferrannini, E. Metabolic syndrome: A solution in search of a problem. *J. Clin. Endocrinol. Metab.* **2007**, *92*, 396–398. [CrossRef] [PubMed]
11. Kahn, R.; Buse, J.; Ferrannini, E.; Stern, M. The metabolic syndrome: Time for a critical appraisal: Joint statement from the American Diabetes Association and the European Association for the Study of Diabetes. *Diabetes Care* **2005**, *28*, 2289–2304. [CrossRef] [PubMed]
12. Cho, L.W. Metabolic syndrome. *Singap. Med. J.* **2011**, *52*, 779–785.
13. Insulin resistance is defined clinically as the inability of a known quantity of exogenous or endogenous insulin to increase glucose uptake and utilization in an individual as much as it does in a normal population [14].
14. Lebovitz, H.E. Insulin resistance: Definition and consequences. *Exp. Clin. Endocrinol. Diabetes* **2001**, *109*, 135–148. [CrossRef] [PubMed]
15. Cardiovascular events refer to any incidents that may cause damage to the heart muscle involving the heart and/or blood vessels which include: arterial embolic or thrombotic events, hemorrhagic or ischemic cerebrovascular conditions, myocardial infarction, and other ischemic heart disease. Severe CV events include conditions such as myocardial infarction or congestive heart failure which may be fatal [16].
16. WHO Fact Sheet—Cardiovascular diseases (CVDs). Available online: Http://www.who.int/mediacentre/factsheets/fs317/en/ (accessed on 4 September 2014).
17. Osteoporosis is a skeletal disorder characterized by compromised bone strength predisposing to an increased risk for bone fractures. Bone strength reflects the integration of two main features: bone mineral density (BMD) and bone quality [18]. Osteoporosis is defined by the WHO as a BMD of 2.5 standard deviations or more below the mean peak bone mass (average of young, healthy adults) as measured by dual-energy X-ray absorptiometry (DXA). A BMD value allows fracture risk to be calculated using FRAX or CAROC assessment algorithms, which incorporate a group of clinical risk factors in addition to femoral neck BMD [19,20].
18. Kilbanski, A.; Adams-Campbell, L.; Bassford, T.; Blair, S.N.; Boden, S.D.; Dickersin, K.; Gifford, D.R.; Glasse, L.; Goldring, S.R.; Hruska, K.; *et al.* Osteoporosis prevention, diagnosis, and therapy. *JAMA J. American Medical Association* **2001**, *285*, 785–795. [CrossRef]
19. Leslie, W.D.; Lix, L.M.; Johansson, H.; Oden, A.; McCloskey, E.; Kanis, J.A. Independent clinical validation of a Canadian FRAX tool: Fracture prediction and model calibration. *J. Bone Miner. Res.* **2010**, *25*, 2350–2358. [CrossRef] [PubMed]
20. Leslie, W.D.; Tsang, J.F.; Lix, L.M. Simplified system for absolute fracture risk assessment: Clinical validation in Canadian women. *J. Bone Miner. Res.* **2009**, *24*, 353–360. [CrossRef] [PubMed]
21. Grossmann, M.; Zajac, J.D. Androgen deprivation therapy in men with prostate cancer: How should the side effects be monitored and treated? *Clin. Endocrinol.* **2011**, *74*, 289–293.
22. Truant, T.L.; Porcino, A.J.; Ross, B.C.; Wong, M.E.; Hilario, C.T. Complementary and alternative medicine (CAM) use in advanced cancer: A systematic review. *J. Support. Oncol.* **2013**, *11*, 105–113. [CrossRef] [PubMed]
23. Philippou, Y.; Hadjipavlou, M.; Khan, S.; Rane, A. Complementary and alternative medicine (CAM) in prostate and bladder cancer. *BJU Int.* **2013**, *112*, 1073–1079. [CrossRef] [PubMed]
24. Klempner, S.J.; Bubley, G. Complementary and alternative medicines in prostate cancer: From bench to bedside? *The oncologist* **2012**, *17*, 830–837. [CrossRef]

25. Huggins, C.; Hodges, C.V. Studies on prostatic cancer: I. The effect of castration, of estrogen and of androgen injection on serum phosphatases in metastatic carcinoma of the prostate. *J. Urol.* **2002**, *168*, 9–12. [CrossRef] [PubMed]
26. Schroder, F.; Crawford, E.D.; Axcrona, K.; Payne, H.; Keane, T.E. Androgen deprivation therapy: Past, present and future. *BJU Int.* **2012**, *109*, 1–12. [CrossRef] [PubMed]
27. The initial administration of an LHRH agonist first causes an increase of LH with a corresponding rise in serum T over 1–2 weeks. This increase in T may initially stimulate and worsen the disease and related symptoms. To prevent this phenomenon, administration of an anti-androgen or estrogen for one week before and during the first few weeks of LHRH agonist therapy is often employed.
28. Rodrigues, D.N.; Butler, L.M.; Estelles, D.L.; de Bono, J.S. Molecular pathology and prostate cancer therapeutics: From biology to bedside. *J. Pathol.* **2014**, *232*, 178–184. [CrossRef] [PubMed]
29. Kolvenbag, G.J.; Iversen, P.; Newling, D.W. Antiandrogen monotherapy: A new form of treatment for patients with prostate cancer. *Urology* **2001**, *58*, 16–23. [CrossRef] [PubMed]
30. Grossmann, M.; Zajac, J.D. Management of side effects of androgen deprivation therapy. *Endocrinol. Metab. Clin. N. Am.* **2011**, *40*, 655–671. [CrossRef]
31. Alibhai, S.M.; Duong-Hua, M.; Sutradhar, R.; Fleshner, N.E.; Warde, P.; Cheung, A.M.; Paszat, L.F. Impact of androgen deprivation therapy on cardiovascular disease and diabetes. *J. Clin. Oncol.* **2009**, *27*, 3452–3458. [CrossRef] [PubMed]
32. Alibhai, S.M.; Mohamedali, H.Z.; Gulamhusein, H.; Panju, A.H.; Breunis, H.; Timilshina, N.; Fleshner, N.; Krahn, M.D.; Naglie, G.; Tannock, I.F.; *et al.* Changes in bone mineral density in men starting androgen deprivation therapy and the protective role of vitamin D. *Osteoporos. Int.* **2013**, *24*, 2571–2579. [CrossRef] [PubMed]
33. Yuan, J.Q.; Xu, T.; Zhang, X.W.; Yu, L.P.; Li, Q.; Liu, S.J.; Huang, X.B.; Wang, X.F. Metabolic syndrome and androgen deprivation therapy in metabolic complications of prostate cancer patients. *Chin. Med. J.* **2012**, *125*, 3725–3729. [PubMed]
34. Saglam, H.S.; Kose, O.; Kumsar, S.; Budak, S.; Adsan, O. Fasting blood glucose and lipid profile alterations following twelve-month androgen deprivation therapy in men with prostate cancer. *Sci. World J.* **2012**, *2012*. [CrossRef]
35. Freedland, S.J.; Eastham, J.; Shore, N. Androgen deprivation therapy and estrogen deficiency induced adverse effects in the treatment of prostate cancer. *Prostate Cancer Prostatic Dis.* **2009**, *12*, 333–338. [CrossRef] [PubMed]
36. Carani, C.; Qin, K.; Simoni, M.; Faustini-Fustini, M.; Serpente, S.; Boyd, J.; Korach, K.S.; Simpson, E.R. Effect of testosterone and estradiol in a man with aromatase deficiency. *N Engl. J. Med.* **1997**, *337*, 91–95. [CrossRef] [PubMed]
37. Khosla, S. Update in male osteoporosis. *J. Clin. Endocrinol. Metab.* **2010**, *95*, 3–10. [CrossRef] [PubMed]
38. Lee, R.J.; Saylor, P.J.; Smith, M.R. Contemporary therapeutic approaches targeting bone complications in prostate cancer. *Clin. Genitourin. Cancer* **2010**, *8*, 29–36. [CrossRef] [PubMed]
39. Jacobson, J.S.; Troxel, A.B.; Evans, J.; Klaus, L.; Vahdat, L.; Kinne, D.; Lo, K.M.; Moore, A.; Rosenman, P.J.; Kaufman, E.L.; *et al.* Randomized trial of black cohosh for the treatment of hot flashes among women with a history of breast cancer. *J. Clin. Oncol.* **2001**, *19*, 2739–2745. [PubMed]
40. Fritz, H.; Seely, D.; McGowan, J.; Skidmore, B.; Fernandes, R.; Kennedy, D.A.; Cooley, K.; Wong, R.; Sagar, S.; Balneaves, L.G.; *et al.* Black cohosh and breast cancer: A systematic review. *Integr. Cancer Ther.* **2014**, *13*, 12–29. [CrossRef] [PubMed]
41. Al-Bareeq, R.J.; Ray, A.A.; Nott, L.; Pautler, S.E.; Razvi, H. Dong Quai (*Angelica sinensis*) in the treatment of hot flashes for men on androgen deprivation therapy: Results of a randomized double-blind placebo controlled trial. *Can. Urol. Assoc. J.* **2010**, *4*, 49–53. [PubMed]
42. Kim, M.S.; Lim, H.J.; Yang, H.J.; Lee, M.S.; Shin, B.C.; Ernst, E. Ginseng for managing menopause symptoms: A systematic review of randomized clinical trials. *J. Ginseng Res.* **2013**, *37*, 30–36. [CrossRef] [PubMed]
43. Moyad, M.A. Complementary/alternative therapies for reducing hot flashes in prostate cancer patients: Reevaluating the existing indirect data from studies of breast cancer and postmenopausal women. *Urology* **2002**, *59*, 20–33. [CrossRef]
44. Zhang, X.; Shu, X.O.; Gao, Y.T.; Yang, G.; Li, Q.; Li, H.; Jin, F.; Zheng, W. Soy food consumption is associated with lower risk of coronary heart disease in Chinese women. *J. Nutr.* **2003**, *133*, 2874–2878. [PubMed]

45. Azadbakht, L.; Kimiagar, M.; Mehrabi, Y.; Esmaillzadeh, A.; Padyab, M.; Hu, F.B.; Willett, W.C. Soy inclusion in the diet improves features of the metabolic syndrome: A randomized crossover study in postmenopausal women. *Am. J. Clin. Nutr.* **2007**, *85*, 735–741. [PubMed]
46. Thelen, P.; Burfeind, P.; Schweyer, S.; Scharf, J.G.; Wuttke, W.; Ringert, R.H. Molecular principles of alternative treatment approaches for hormone-refractory prostate cancer. *Der. Urol. Ausg. A* **2007**, *46*, 1271–1274. [CrossRef]
47. Napora, J.K.; Short, R.G.; Muller, D.C.; Carlson, O.D.; Odetunde, J.O.; Xu, X.; Carducci, M.; Travison, T.G.; Maggio, M.; Egan, J.M.; *et al.* High-dose isoflavones do not improve metabolic and inflammatory parameters in androgen-deprived men with prostate cancer. *J. Androl.* **2011**, *32*, 40–48. [CrossRef] [PubMed]
48. Albertazzi, P.; Pansini, F.; Bonaccorsi, G.; Zanotti, L.; Forini, E.; de Aloysio, D. The effect of dietary soy supplementation on hot flushes. *Obstetrics Gynecol.* **1998**, *91*, 6–11. [CrossRef]
49. Charles, C.; Yuskavage, J.; Carlson, O.; John, M.; Tagalicud, A.S.; Maggio, M.; Muller, D.C.; Egan, J.; Basaria, S. Effects of high-dose isoflavones on metabolic and inflammatory markers in healthy postmenopausal women. *Menopause* **2009**, *16*, 395–400. [CrossRef] [PubMed]
50. James, M.J.; Gibson, R.A.; Cleland, L.G. Dietary polyunsaturated fatty acids and inflammatory mediator production. *Am. J. Clin. Nutr.* **2000**, *71*, 343–348.
51. Lippman, S.M.; Klein, E.A.; Goodman, P.J.; Lucia, M.S.; Thompson, I.M.; Ford, L.G.; Parnes, H.L.; Minasian, L.M.; Gaziano, J.M.; Hartline, J.A.; *et al.* Effect of selenium and vitamin E on risk of prostate cancer and other cancers: The Selenium and Vitamin E Cancer Prevention Trial (SELECT). *Jama* **2009**, *301*, 39–51. [CrossRef] [PubMed]
52. Rubenstein, B.B. Vitamin E diminishes the vasomotor symptoms of menopause. *Fed. Proc.* **1948**, *7*, 106. [PubMed]
53. Raffy, A. Menopause and vitamin E. *Ther. Hung.* **1955**, *4*, 12–14. [PubMed]
54. Orwoll, E.; Nielson, C.M.; Marshall, L.M.; Lambert, L.; Holton, K.F.; Hoffman, A.R.; Barrett-Connor, E.; Shikany, J.M.; Dam, T.; Cauley, J.A. Vitamin D deficiency in older men. *J. Clin. Endocrinol. Metab.* **2009**, *94*, 1214–1222. [CrossRef] [PubMed]
55. Liu, S.; Lee, I.M.; Song, Y.; Van Denburgh, M.; Cook, N.R.; Manson, J.E.; Buring, J.E. Vitamin E and risk of type 2 diabetes in the women's health study randomized controlled trial. *Diabetes* **2006**, *55*, 2856–2862. [CrossRef] [PubMed]
56. Klein, E.A.; Thompson, I.M., Jr.; Tangen, C.M.; Crowley, J.J.; Lucia, M.S.; Goodman, P.J.; Minasian, L.M.; Ford, L.G.; Parnes, H.L.; Gaziano, J.M.; *et al.* Vitamin E and the risk of prostate cancer: The Selenium and Vitamin E Cancer Prevention Trial (SELECT). *JAMA* **2011**, *306*, 1549–1556. [CrossRef] [PubMed]
57. Sesso, H.D.; Buring, J.E.; Christen, W.G.; Kurth, T.; Belanger, C.; MacFadyen, J.; Bubes, V.; Manson, J.E.; Glynn, R.J.; Gaziano, J.M. Vitamins E and C in the prevention of cardiovascular disease in men: The Physicians' Health Study II randomized controlled trial. *JAMA* **2008**, *300*, 2123–2133. [CrossRef] [PubMed]
58. Peters, U.; Littman, A.J.; Kristal, A.R.; Patterson, R.E.; Potter, J.D.; White, E. Vitamin E and selenium supplementation and risk of prostate cancer in the Vitamins and lifestyle (VITAL) study cohort. *Cancer Causes Control* **2008**, *19*, 75–87. [CrossRef] [PubMed]
59. Seidlova-Wuttke, D.; Stecher, G.; Kammann, M.; Haunschild, J.; Eder, N.; Stahnke, V.; Wessels, J.; Wuttke, W. Osteoprotective effects of Cimicifuga racemosa and its triterpene-saponins are responsible for reduction of bone marrow fat. *Phytomedicine* **2012**, *19*, 855–860. [CrossRef] [PubMed]
60. Kuhnle, G.G.; Ward, H.A.; Vogiatzoglou, A.; Luben, R.N.; Mulligan, A.; Wareham, N.J.; Forouhi, N.G.; Khaw, K.T. Association between dietary phyto-oestrogens and bone density in men and postmenopausal women. *Br. J. Nutr.* **2011**, *106*, 1063–1069. [CrossRef] [PubMed]
61. Soung, D.Y.; Devareddy, L.; Khalil, D.A.; Hooshmand, S.; Patade, A.; Lucas, E.A.; Arjmandi, B.H. Soy affects trabecular microarchitecture and favorably alters select bone-specific gene expressions in a male rat model of osteoporosis. *Calcif. Tissue Int.* **2006**, *78*, 385–391. [CrossRef] [PubMed]
62. Hogstrom, M.; Nordstrom, P.; Nordstrom, A. n-3 Fatty acids are positively associated with peak bone mineral density and bone accrual in healthy men: The NO2 Study. *Am. J. Clin. Nutr.* **2007**, *85*, 803–807. [PubMed]
63. Watkins, B.A.; Li, Y.; Lippman, H.E.; Seifert, M.F. Omega-3 polyunsaturated fatty acids and skeletal health. *Exp. Biol Med. (Maywood)* **2001**, *226*, 485–497.

64. Fernandes, G.; Bhattacharya, A.; Rahman, M.; Zaman, K.; Banu, J. Effects of n-3 fatty acids on autoimmunity and osteoporosis. *Front. Biosci.* **2008**, *13*, 4015–4020. [CrossRef] [PubMed]
65. Shen, C.L.; Yeh, J.K.; Rasty, J.; Chyu, M.C.; Dunn, D.M.; Li, Y.; Watkins, B.A. Improvement of bone quality in gonad-intact middle-aged male rats by long-chain n-3 polyunsaturated fatty acid. *Calcif. Tissue Int.* **2007**, *80*, 286–293. [CrossRef] [PubMed]
66. Rahman, M.M.; Bhattacharya, A.; Banu, J.; Fernandes, G. Conjugated linoleic acid protects against age-associated bone loss in C57BL/6 female mice. *J. Nutr. Biochem.* **2007**, *18*, 467–474. [CrossRef] [PubMed]
67. Tang, B.M.; Eslick, G.D.; Nowson, C.; Smith, C.; Bensoussan, A. Use of calcium or calcium in combination with vitamin D supplementation to prevent fractures and bone loss in people aged 50 years and older: A meta-analysis. *Lancet* **2007**, *370*, 657–666. [CrossRef] [PubMed]
68. Gates, B.J.; Das, S. Management of osteoporosis in elderly men. *Maturitas* **2011**, *69*, 113–119. [CrossRef] [PubMed]
69. Gielen, E.; Boonen, S.; Vanderschueren, D.; Sinnesael, M.; Verstuyf, A.; Claessens, F.; Milisen, K.; Verschueren, S. Calcium and vitamin D supplementation in men. *J. Osteoporos.* **2011**, *2011*. [CrossRef]
70. Verbrugge, F.H.; Gielen, E.; Milisen, K.; Boonen, S. Who should receive calcium and vitamin D supplementation? *Age Ageing* **2012**, *41*, 576–580. [CrossRef]
71. Cranney, A.; Horsley, T.; O'Donnell, S.; Weiler, H.; Puil, L.; Ooi, D.; Atkinson, S.; Ward, L.; Moher, D.; Hanley, D.; *et al.* Effectiveness and safety of vitamin D in relation to bone health. *Evid. Rep. Technol. Assess.* **2007**, 1–235.
72. Moyad, M.A. Complementary therapies for reducing the risk of osteoporosis in patients receiving luteinizing hormone-releasing hormone treatment/orchiectomy for prostate cancer: A review and assessment of the need for more research. *Urology* **2002**, *59*, 34–40. [CrossRef] [PubMed]
73. Stabler, S.N.; Tejani, A.M.; Huynh, F.; Fowkes, C. Garlic for the prevention of cardiovascular morbidity and mortality in hypertensive patients. *Cochrane Database Syst. Rev.* **2012**, *8*. [CrossRef]
74. Shouk, R.; Abdou, A.; Shetty, K.; Sarkar, D.; Eid, A.H. Mechanisms underlying the antihypertensive effects of garlic bioactives. *Nutr Res.* **2014**, *34*, 106–115. [CrossRef] [PubMed]
75. Clarkson, T.B. Soy, soy phytoestrogens and cardiovascular disease. *J. Nutr.* **2002**, *132*, 566–569.
76. Atteritano, M.; Marini, H.; Minutoli, L.; Polito, F.; Bitto, A.; Altavilla, D.; Mazzaferro, S.; D'Anna, R.; Cannata, M.L.; Gaudio, A.; *et al.* Effects of the phytoestrogen genistein on some predictors of cardiovascular risk in osteopenic, postmenopausal women: A two-year randomized, double-blind, placebo-controlled study. *J. Clin. Endocrinol. Metab.* **2007**, *92*, 3068–3075. [CrossRef] [PubMed]
77. Bloedon, L.T.; Balikai, S.; Chittams, J.; Cunnane, S.C.; Berlin, J.A.; Rader, D.J.; Szapary, P.O. Flaxseed and cardiovascular risk factors: Results from a double blind, randomized, controlled clinical trial. *J. Am. Coll. Nutr.* **2008**, *27*, 65–74. [CrossRef] [PubMed]
78. Jacobson, T.A. Beyond lipids: The role of omega-3 fatty acids from fish oil in the prevention of coronary heart disease. *Curr. Atheroscler. Rep.* **2007**, *9*, 145–153. [CrossRef] [PubMed]
79. Kris-Etherton, P.M.; Harris, W.S.; Appel, L.J. Fish consumption, fish oil, omega-3 fatty acids, and cardiovascular disease. *Circulation* **2002**, *106*, 2747–2757. [CrossRef] [PubMed]
80. Simopoulos, A.P. The importance of the ratio of omega-6/omega-3 essential fatty acids. *Biomed. Pharmacother.* **2002**, *56*, 365–379. [CrossRef] [PubMed]
81. Hjerkinn, E.M.; Seljeflot, I.; Ellingsen, I.; Berstad, P.; Hjermann, I.; Sandvik, L.; Arnesen, H. Influence of long-term intervention with dietary counseling, long-chain n-3 fatty acid supplements, or both on circulating markers of endothelial activation in men with long-standing hyperlipidemia. *Am. J. Clin. Nutr.* **2005**, *81*, 583–589. [PubMed]
82. Dewell, A.; Marvasti, F.F.; Harris, W.S.; Tsao, P.; Gardner, C.D. Low- and high-dose plant and marine (n-3) fatty acids do not affect plasma inflammatory markers in adults with metabolic syndrome. *J. Nutr.* **2011**, *141*, 2166–2171. [CrossRef] [PubMed]
83. Koyama, H.; Mutakin; Abdulah, R.; Yamazaki, C.; Kameo, S. Selenium supplementation trials for cancer prevention and the subsequent risk of type 2 diabetes mellitus. *Nihon Eiseigaku Zasshi* **2013**, *68*, 1–10. [CrossRef] [PubMed]
84. Rayman, M.P.; Blundell-Pound, G.; Pastor-Barriuso, R.; Guallar, E.; Steinbrenner, H.; Stranges, S. A randomized trial of selenium supplementation and risk of type-2 diabetes, as assessed by plasma adiponectin. *PLoS One* **2012**, *7*, 45269. [CrossRef]

85. Faghihi, T.; Radfar, M.; Barmal, M.; Amini, P.; Qorbani, M.; Abdollahi, M.; Larijani, B. A randomized, placebo-controlled trial of selenium supplementation in patients with type 2 diabetes: Effects on glucose homeostasis, oxidative stress, and lipid profile. *Am. J. Ther.* **2013**. [CrossRef]
86. Dennehy, C.; Tsourounis, C. A review of select vitamins and minerals used by postmenopausal women. *Maturitas* **2010**, *66*, 370–380. [CrossRef] [PubMed]
87. Algotar, A.M.; Stratton, M.S.; Stratton, S.P.; Hsu, C.H.; Ahmann, F.R. No effect of selenium supplementation on serum glucose levels in men with prostate cancer. *Am. J. Med.* **2010**, *123*, 765–768. [CrossRef] [PubMed]
88. Kristal, A.R.; Darke, A.K.; Morris, J.S.; Tangen, C.M.; Goodman, P.J.; Thompson, I.M.; Meyskens, F.L., Jr.; Goodman, G.E.; Minasian, L.M.; Parnes, H.L.; *et al.* Baseline selenium status and effects of selenium and vitamin E supplementation on prostate cancer risk. *J. Nat. Cancer Inst.* **2014**, *106*. [CrossRef] [PubMed]
89. Algotar, A.M.; Stratton, M.S.; Ahmann, F.R.; Ranger-Moore, J.; Nagle, R.B.; Thompson, P.A.; Slate, E.; Hsu, C.H.; Dalkin, B.L.; Sindhwani, P.; *et al.* Phase 3 clinical trial investigating the effect of selenium supplementation in men at high-risk for prostate cancer. *Prostate* **2013**, *73*, 328–335. [CrossRef] [PubMed]
90. b>Algotar, A.M.; Hsu, C.H.; Singh, P.; Stratton, S.P. Selenium supplementation has no effect on serum glucose levels in men at high risk for Prostate Cancer. *J. Diabetes* **2013**, *5*, 465–470. [CrossRef] [PubMed]
91. Cook, N.R.; Albert, C.M.; Gaziano, J.M.; Zaharris, E.; MacFadyen, J.; Danielson, E.; Buring, J.E.; Manson, J.E. A randomized factorial trial of vitamins C and E and beta carotene in the secondary prevention of cardiovascular events in women: Results from the Women's Antioxidant Cardiovascular Study. *Arch. Internal Med.* **2007**, *167*, 1610–1618. [CrossRef]
92. Saremi, A.; Arora, R. Vitamin E and cardiovascular disease. *Am. J. Ther.* **2010**, *17*, 56–65. [CrossRef]
93. Shahani, S.; Braga-Basaria, M.; Basaria, S. Androgen deprivation therapy in prostate cancer and metabolic risk for atherosclerosis. *J. Clin. Endocrinol. Metab.* **2008**, *93*, 2042–2049. [CrossRef] [PubMed]
94. Smith, M.R.; Lee, H.; McGovern, F.; Fallon, M.A.; Goode, M.; Zietman, A.L.; Finkelstein, J.S. Metabolic changes during gonadotropin-releasing hormone agonist therapy for prostate cancer: Differences from the classic metabolic syndrome. *Cancer* **2008**, *112*, 2188–2194. [CrossRef] [PubMed]
95. Braga-Basaria, M.; Dobs, A.S.; Muller, D.C.; Carducci, M.A.; John, M.; Egan, J.; Basaria, S. Metabolic syndrome in men with prostate cancer undergoing long-term androgen-deprivation therapy. *J. Clin. Oncol.* **2006**, *24*, 3979–3983. [CrossRef] [PubMed]
96. Basaria, S.; Muller, D.C.; Carducci, M.A.; Egan, J.; Dobs, A.S. Hyperglycemia and insulin resistance in men with prostate carcinoma who receive androgen-deprivation therapy. *Cancer* **2006**, *106*, 581–588. [CrossRef] [PubMed]
97. Laaksonen, D.E.; Niskanen, L.; Punnonen, K.; Nyyssonen, K.; Tuomainen, T.P.; Valkonen, V.P.; Salonen, R.; Salonen, J.T. Testosterone and sex hormone-binding globulin predict the metabolic syndrome and diabetes in middle-aged men. *Diabetes Care* **2004**, *27*, 1036–1041. [CrossRef] [PubMed]
98. Tirabassi, G.; Gioia, A.; Giovannini, L.; Boscaro, M.; Corona, G.; Carpi, A.; Maggi, M.; Balercia, G. Testosterone and cardiovascular risk. *Intern. Emerg. Med.* **2013**, *8*, 65–69. [CrossRef] [PubMed]
99. Stellato, R.K.; Feldman, H.A.; Hamdy, O.; Horton, E.S.; McKinlay, J.B. Testosterone, sex hormone-binding globulin, and the development of type 2 diabetes in middle-aged men: Prospective results from the Massachusetts male aging study. *Diabetes Care* **2000**, *23*, 490–494. [CrossRef] [PubMed]
100. Eckel, R.H.; Alberti, K.G.; Grundy, S.M.; Zimmet, P.Z. The metabolic syndrome. *Lancet* **2010**, *375*, 181–183. [CrossRef] [PubMed]
101. Fink, R.I.; Kolterman, O.G.; Griffin, J.; Olefsky, J.M. Mechanisms of insulin resistance in aging. *J. Clin. Investig.* **1983**, *71*, 1523–1535. [CrossRef] [PubMed]
102. Lu-Yao, G.; Stukel, T.A.; Yao, S.L. Changing patterns in competing causes of death in men with prostate cancer: A population based study. *J. Urol.* **2004**, *171*, 2285–2290. [CrossRef] [PubMed]
103. WHO Assessment of fracture risk and its application to screening for postmenopausal osteoporosis. Report of a WHO Study Group. *World Health Organ. Technical Rep. Ser.* **1994**, *843*, 1–129.
104. Lee, R.J.; Saylor, P.J.; Smith, M.R. Treatment and prevention of bone complications from prostate cancer. *Bone* **2011**, *48*, 88–95. [CrossRef] [PubMed]
105. Krupski, T.L.; Smith, M.R.; Lee, W.C.; Pashos, C.L.; Brandman, J.; Wang, Q.; Botteman, M.; Litwin, M.S. Natural history of bone complications in men with prostate carcinoma initiating androgen deprivation therapy. *Cancer* **2004**, *101*, 541–549. [CrossRef] [PubMed]

106. Shahinian, V.B.; Kuo, Y.F.; Freeman, J.L.; Goodwin, J.S. Risk of fracture after androgen deprivation for prostate cancer. *N. Engl. J. Med.* **2005**, *352*, 154–164. [CrossRef] [PubMed]
107. Oefelein, M.G.; Ricchiuti, V.; Conrad, W.; Resnick, M.I. Skeletal fractures negatively correlate with overall survival in men with prostate cancer. *J. Urol.* **2002**, *168*, 1005–1007. [CrossRef] [PubMed]
108. Kiratli, B.J.; Srinivas, S.; Perkash, I.; Terris, M.K. Progressive decrease in bone density over 10 years of androgen deprivation therapy in patients with prostate cancer. *Urology* **2001**, *57*, 127–132. [CrossRef] [PubMed]
109. Greenspan, S.L.; Coates, P.; Sereika, S.M.; Nelson, J.B.; Trump, D.L.; Resnick, N.M. Bone loss after initiation of androgen deprivation therapy in patients with prostate cancer. *J. Clin. Endocrinol. Metab.* **2005**, *90*, 6410–6417. [CrossRef] [PubMed]
110. Morote, J.; Orsola, A.; Abascal, J.M.; Planas, J.; Trilla, E.; Raventos, C.X.; Cecchini, L.; Encabo, G.; Reventos, J. Bone mineral density changes in patients with prostate cancer during the first 2 years of androgen suppression. *J. Urol.* **2006**, *175*, 1679–1683. [CrossRef] [PubMed]
111. Wadhwa, V.K.; Weston, R.; Mistry, R.; Parr, N.J. Long-term changes in bone mineral density and predicted fracture risk in patients receiving androgen-deprivation therapy for prostate cancer, with stratification of treatment based on presenting values. *BJU Int.* **2009**, *104*, 800–805. [CrossRef] [PubMed]
112. Lloyd, K.B.; Hornsby, L.B. Complementary and alternative medications for women's health issues. *Nutr. Clin. Prac.* **2009**, *24*, 589–608. [CrossRef]
113. Dennehy, C.E. The use of herbs and dietary supplements in gynecology: An evidence-based review. *J. Midwifery Women's Health* **2006**, *51*, 402–409. [CrossRef]
114. Seidlova-Wuttke, D.; Eder, N.; Stahnke, V.; Kammann, M.; Stecher, G.; Haunschild, J.; Wessels, J.T.; Wuttke, W. Cimicifuga racemosa and its triterpene-saponins prevent the Metabolic Syndrome and deterioration of cartilage in the knee joint of ovariectomized rats by similar mechanisms. *Phytomedicine* **2012**, *19*, 846–853. [CrossRef]
115. Mahon, S.M.; Kaplan, M. Placebo effect in hot flush research. *Lancet Oncol.* **2012**, *13*, 188. [CrossRef]
116. Wuttke, W.; Gorkow, C.; Seidlova-Wuttke, D. Effects of black cohosh (*Cimicifuga racemosa*) on bone turnover, vaginal mucosa, and various blood parameters in postmenopausal women: A double-blind, placebo-controlled, and conjugated estrogens-controlled study. *Menopause* **2006**, *13*, 185–196. [CrossRef]
117. Seidlova-Wuttke, D.; Jarry, H.; Pitzel, L.; Wuttke, W. Effects of estradiol-17beta, testosterone and a black cohosh preparation on bone and prostate in orchidectomized rats. *Maturitas* **2005**, *51*, 177–186. [CrossRef]
118. Dueregger, A.; Guggenberger, F.; Barthelmes, J.; Stecher, G.; Schuh, M.; Intelmann, D.; Abel, G.; Haunschild, J.; Klocker, H.; Ramoner, R.; *et al.* Attenuation of nucleoside and anti-cancer nucleoside analog drug uptake in prostate cancer cells by Cimicifuga racemosa extract BNO-1055. *Phytomedicine* **2013**, *20*, 1306–1314. [CrossRef]
119. Molina-Arcas, M.; Casado, F.J.; Pastor-Anglada, M. Nucleoside transporter proteins. *Curr. Vasc. Pharmacol.* **2009**, *7*, 426–434. [CrossRef]
120. Cheema, D.; Coomarasamy, A.; El-Toukhy, T. Non-hormonal therapy of post-menopausal vasomotor symptoms: A structured evidence-based review. *Arch. Gynecol. Obstet.* **2007**, *276*, 463–469. [CrossRef]
121. Wiklund, I.K.; Mattsson, L.A.; Lindgren, R.; Limoni, C. Effects of a standardized ginseng extract on quality of life and physiological parameters in symptomatic postmenopausal women: A double-blind, placebo-controlled trial. Swedish Alternative Medicine Group. *Int. J. Clin. Pharmacol. Res.* **1999**, *19*, 89–99.
122. Nag, S.A.; Qin, J.J.; Wang, W.; Wang, M.H.; Wang, H.; Zhang, R. Ginsenosides as Anticancer Agents: *In vitro* and *in vivo* Activities, Structure-Activity Relationships, and Molecular Mechanisms of Action. *Front. Pharmacol.* **2012**, *3*, 25. [CrossRef]
123. Wang, Z.; Wang, J.; Chan, P. Treating type 2 diabetes mellitus with traditional chinese and Indian medicinal herbs. *Evid. Based Complement. Altern. Med.* **2013**, *2013*. [CrossRef]
124. Rahman, K.; Lowe, G.M. Garlic and cardiovascular disease: A critical review. *J. Nutr.* **2006**, *136*, 736–740.
125. Rabito, M.J.; Kaye, A.D. Complementary and alternative medicine and cardiovascular disease: An evidence-based review. *Evid. Based Complement. Altern. Med.* **2013**, *2013*. [CrossRef]
126. Smith, M.R.; Malkowicz, S.B.; Chu, F.; Forrest, J.; Sieber, P.; Barnette, K.G.; Rodriquez, D.; Steiner, M.S. Toremifene improves lipid profiles in men receiving androgen-deprivation therapy for prostate cancer: Interim analysis of a multicenter phase III study. *J. Clin. Oncol.* **2008**, *26*, 1824–1829. [CrossRef]

127. Smith, M.R.; Morton, R.A.; Barnette, K.G.; Sieber, P.R.; Malkowicz, S.B.; Rodriguez, D.; Hancock, M.L.; Steiner, M.S. Toremifene to reduce fracture risk in men receiving androgen deprivation therapy for prostate cancer. *J. Urol.* **2013**, *189*, 45–50. [CrossRef]
128. Kearns, A.E.; Northfelt, D.W.; Dueck, A.C.; Atherton, P.J.; Dakhil, S.R.; Rowland, K.M., Jr.; Fuloria, J.; Flynn, P.J.; Dentchev, T.; Loprinzi, C.L. Osteoporosis prevention in prostate cancer patients receiving androgen ablation therapy: Placebo-controlled double-blind study of estradiol and risedronate: N01C8. *Support. Care Cancer* **2010**, *18*, 321–328. [CrossRef]
129. Wibowo, E.; Schellhammer, P.; Wassersug, R.J. Role of estrogen in normal male function: Clinical implications for patients with prostate cancer on androgen deprivation therapy. *J. Urol.* **2011**, *185*, 17–23. [CrossRef]
130. Treatment and survival of patients with cancer of the prostate. The Veterans Administration Co-operative Urological Research Group. *Surg. Gynecol. Obstet.* **1967**, *124*, 1011–1017.
131. Byar, D.P.; Corle, D.K. Hormone therapy for prostate cancer: Results of the Veterans Administration Cooperative Urological Research Group studies. *NCI Monographs* **1987**, *7*, 165–170.
132. Murkies, A.L.; Wilcox, G.; Davis, S.R. Clinical review 92: Phytoestrogens. *J. Clin. Endocrinol. Metab.* **1998**, *83*, 297–303.
133. Turner, J.V.; Agatonovic-Kustrin, S.; Glass, B.D. Molecular aspects of phytoestrogen selective binding at estrogen receptors. *J. Pharm. Sci.* **2007**, *96*, 1879–1885. [CrossRef]
134. Dahlman-Wright, K.; Cavailles, V.; Fuqua, S.A.; Jordan, V.C.; Katzenellenbogen, J.A.; Korach, K.S.; Maggi, A.; Muramatsu, M.; Parker, M.G.; Gustafsson, J.A. International Union of Pharmacology. LXIV. Estrogen receptors. *Pharmacol. Rev.* **2006**, *58*, 773–781. [CrossRef]
135. Khosla, S.; Melton, L.J.; Riggs, B.L. The unitary model for estrogen deficiency and the pathogenesis of osteoporosis: Is a revision needed? *J. Bone Miner. Res.* **2011**, *26*, 441–451. [CrossRef]
136. De Kleijn, M.J.; van der Schouw, Y.T.; Wilson, P.W.; Adlercreutz, H.; Mazur, W.; Grobbee, D.E.; Jacques, P.F. Intake of dietary phytoestrogens is low in postmenopausal women in the United States: The Framingham study(1–4). *J. Nutr.* **2001**, *131*, 1826–1832.
137. Jemal, A.; Siegel, R.; Ward, E.; Hao, Y.; Xu, J.; Murray, T.; Thun, M.J. Cancer statistics, 2008. *Cancer J. Clin.* **2008**, *58*, 71–96. [CrossRef]
138. Villegas, R.; Gao, Y.T.; Yang, G.; Li, H.L.; Elasy, T.A.; Zheng, W.; Shu, X.O. Legume and soy food intake and the incidence of type 2 diabetes in the Shanghai Women's Health Study. *Am. J. Clin. Nutr.* **2008**, *87*, 162–167.
139. Huang, Y.; Cao, S.; Nagamani, M.; Anderson, K.E.; Grady, J.J.; Lu, L.J. Decreased circulating levels of tumor necrosis factor-alpha in postmenopausal women during consumption of soy-containing isoflavones. *J. Clin. Endocrinol. Metab.* **2005**, *90*, 3956–3962. [CrossRef]
140. Sharma, P.; Wisniewski, A.; Braga-Basaria, M.; Xu, X.; Yep, M.; Denmeade, S.; Dobs, A.S.; DeWeese, T.; Carducci, M.; Basaria, S. Lack of an effect of high dose isoflavones in men with prostate cancer undergoing androgen deprivation therapy. *J. Urol.* **2009**, *182*, 2265–2272. [CrossRef]
141. Fairweather-Tait, S.J.; Bao, Y.; Broadley, M.R.; Collings, R.; Ford, D.; Hesketh, J.E.; Hurst, R. Selenium in human health and disease. *Antioxid. Redox signal.* **2011**, *14*, 1337–1383. [CrossRef]
142. Steinbrenner, H.; Sies, H. Protection against reactive oxygen species by selenoproteins. *Biochim. Biophys. Acta* **2009**, *1790*, 1478–1485. [CrossRef]
143. Holvoet, P. Relations between metabolic syndrome, oxidative stress and inflammation and cardiovascular disease. *Verhandelingen* **2008**, *70*, 193–219.
144. Yang, M.; Sytkowski, A.J. Differential expression and androgen regulation of the human selenium-binding protein gene hSP56 in prostate cancer cells. *Cancer Res.* **1998**, *58*, 3150–3153.
145. Clark, L.C.; Dalkin, B.; Krongrad, A.; Combs G.F., Jr.; Turnbull, B.W.; Slate, E.H.; Witherington, R.; Herlong, J.H.; Janosko, E.; Carpenter, D.; *et al.* Decreased incidence of prostate cancer with selenium supplementation: Results of a double-blind cancer prevention trial. *Br. J. Urol.* **1998**, *81*, 730–734. [CrossRef]
146. Yoshizawa, K.; Willett, W.C.; Morris, S.J.; Stampfer, M.J.; Spiegelman, D.; Rimm, E.B.; Giovannucci, E. Study of prediagnostic selenium level in toenails and the risk of advanced prostate cancer. *J. Nat. Cancer Inst.* **1998**, *90*, 1219–1224. [CrossRef]
147. Duffield-Lillico, A.J.; Dalkin, B.L.; Reid, M.E.; Turnbull, B.W.; Slate, E.H.; Jacobs, E.T.; Marshall, J.R.; Clark, L.C. Selenium supplementation, baseline plasma selenium status and incidence of prostate cancer: An analysis of the complete treatment period of the Nutritional Prevention of Cancer Trial. *BJU Int.* **2003**, *91*, 608–612. [CrossRef]

148. Rayman, M.P.; Stranges, S. Epidemiology of selenium and type 2 diabetes: Can we make sense of it? *Free Radic. Biol. Med.* **2013**, *65*, 1557–1564.
149. Savini, I.; Catani, M.V.; Evangelista, D.; Gasperi, V.; Avigliano, L. Obesity-associated oxidative stress: Strategies finalized to improve redox state. *Int J. Mol. Sci.* **2013**, *14*, 10497–10538. [CrossRef]
150. Hassanzadeh, M.; Faridhosseini, R.; Mahini, M.; Faridhosseini, F.; Ranjbar, A. Serum Levels of TNF-, IL-6, and Selenium in Patients with Acute and Chronic Coronary Artery Disease. *Iran. J. Immunol.* **2006**, *3*, 142–145.
151. Zhang, Y.; Chen, X. Reducing selenoprotein P expression suppresses adipocyte differentiation as a result of increased preadipocyte inflammation. *Am. J. Physiol. Endocrinol. Metab.* **2011**, *300*, 77–85. [CrossRef]
152. Steinbrenner, H.; Speckmann, B.; Pinto, A.; Sies, H. High selenium intake and increased diabetes risk: Experimental evidence for interplay between selenium and carbohydrate metabolism. *J. Clin. Biochem. Nutr.* **2011**, *48*, 40–45. [CrossRef]
153. Steinbrenner, H. Interference of selenium and selenoproteins with the insulin-regulated carbohydrate and lipid metabolism. *Free Radic. Biol. Med.* **2013**, *65*, 1538–1547. [CrossRef]
154. Quiner, T.E.; Nakken, H.L.; Mason, B.A.; Lephart, E.D.; Hancock, C.R.; Christensen, M.J. Soy content of basal diets determines the effects of supplemental selenium in male mice. *J. Nutr.* **2011**, *141*, 2159–2165. [CrossRef]
155. Simopoulos, A.P. Essential fatty acids in health and chronic disease. *Am. J. Clin. Nutr.* **1999**, *70*, 560–569.
156. Nettleton, J.A. *Omega-3 Fatty Acids and Health*; Springer US: New York, NY, USA, 1995; pp. 64–76.
157. Heinze, V.M.; Actis, A.B. Dietary conjugated linoleic acid and long-chain *n*-3 fatty acids in mammary and prostate cancer protection: A review. *Int. J. Food Sci. Nutr.* **2012**, *63*, 66–78. [CrossRef]
158. Tziomalos, K.; Athyros, V.G.; Mikhailidis, D.P. Fish oils and vascular disease prevention: An update. *Curr. Med. Chem.* **2007**, *14*, 2622–2628. [CrossRef]
159. Carpentier, Y.A.; Portois, L.; Malaisse, W.J. n-3 fatty acids and the metabolic syndrome. *Am. J. Clin. Nutr.* **2006**, *83*, 1499–1504.
160. Barre, D.E. The role of consumption of alpha-linolenic, eicosapentaenoic and docosahexaenoic acids in human metabolic syndrome and type 2 diabetes–a mini-review. *J. Oleo Sci.* **2007**, *56*, 319–325. [CrossRef]
161. Chen, Y.Q.; Edwards, I.J.; Kridel, S.J.; Thornburg, T.; Berquin, I.M. Dietary fat-gene interactions in cancer. *Cancer Metastasis Rev.* **2007**, *26*, 535–551. [CrossRef]
162. Ballantyne, C.M.; Bays, H.E.; Kastelein, J.J.; Stein, E.; Isaacsohn, J.L.; Braeckman, R.A.; Soni, P.N. Efficacy and safety of eicosapentaenoic acid ethyl ester (AMR101) therapy in statin-treated patients with persistent high triglycerides (from the ANCHOR study). *Am. J. Cardiol.* **2012**, *110*, 984–992. [CrossRef]
163. Bays, H.E.; Ballantyne, C.M.; Kastelein, J.J.; Isaacsohn, J.L.; Braeckman, R.A.; Soni, P.N. Eicosapentaenoic acid ethyl ester (AMR101) therapy in patients with very high triglyceride levels (from the Multi-center, plAcebo-controlled, Randomized, double-blINd, 12-week study with an open-label Extension [MARINE] trial). *Am. J. Cardiol.* **2011**, *108*, 682–690. [CrossRef]
164. Connor, W.E. Importance of *n*-3 fatty acids in health and disease. *Am. J. Clin. Nutr.* **2000**, *71*, 171–175.
165. Wang, S.; Wu, J.; Suburu, J.; Gu, Z.; Cai, J.; Axanova, L.S.; Cramer, S.D.; Thomas, M.J.; Perry, D.L.; Edwards, I.J.; *et al.* Effect of dietary polyunsaturated fatty acids on castration-resistant Pten-null prostate cancer. *Carcinogenesis* **2012**, *33*, 404–412. [CrossRef]
166. Friedrichs, W.; Ruparel, S.B.; Marciniak, R.A.; deGraffenried, L. Omega-3 fatty acid inhibition of prostate cancer progression to hormone independence is associated with suppression of mTOR signaling and androgen receptor expression. *Nutr. Cancer* **2011**, *63*, 771–777. [CrossRef]
167. Cavazos, D.A.; Price, R.S.; Apte, S.S.; deGraffenried, L.A. Docosahexaenoic acid selectively induces human prostate cancer cell sensitivity to oxidative stress through modulation of NF-kappaB. *Prostate* **2011**, *71*, 1420–1428. [CrossRef]
168. McEntee, M.F.; Ziegler, C.; Reel, D.; Tomer, K.; Shoieb, A.; Ray, M.; Li, X.; Neilsen, N.; Lih, F.B.; O'Rourke, D.; Whelan, J. Dietary n-3 polyunsaturated fatty acids enhance hormone ablation therapy in androgen-dependent prostate cancer. *Am. J. Pathol.* **2008**, *173*, 229–241. [CrossRef]
169. Epstein, M.M.; Kasperzyk, J.L.; Mucci, L.A.; Giovannucci, E.; Price, A.; Wolk, A.; Hakansson, N.; Fall, K.; Andersson, S.O.; Andren, O. Dietary fatty acid intake and prostate cancer survival in Orebro County, Sweden. *Am. J. Epidemiol.* **2012**, *176*, 240–252. [CrossRef]

170. Aronson, W.J.; Kobayashi, N.; Barnard, R.J.; Henning, S.; Huang, M.; Jardack, P.M.; Liu, B.; Gray, A.; Wan, J.; Konijeti, R.; *et al.* Phase II prospective randomized trial of a low-fat diet with fish oil supplementation in men undergoing radical prostatectomy. *Cancer Prev Res. (Phila)* **2011**, *4*, 2062–2071. [CrossRef]
171. Fradet, V.; Cheng, I.; Casey, G.; Witte, J.S. Dietary omega-3 fatty acids, cyclooxygenase-2 genetic variation, and aggressive prostate cancer risk. *Clin. Cancer Res.* **2009**, *15*, 2559–2566. [CrossRef]
172. Berquin, I.M.; Edwards, I.J.; Chen, Y.Q. Multi-targeted therapy of cancer by omega-3 fatty acids. *Cancer Lett.* **2008**, *269*, 363–377. [CrossRef]
173. Szymanski, K.M.; Wheeler, D.C.; Mucci, L.A. Fish consumption and prostate cancer risk: A review and meta-analysis. *Am. J. Clin. Nutr.* **2010**, *92*, 1223–1233. [CrossRef]
174. Nelson, T.L.; Stevens, J.R.; Hickey, M.S. Inflammatory markers are not altered by an eight week dietary alpha-linolenic acid intervention in healthy abdominally obese adult males and females. *Cytokine* **2007**, *38*, 101–106. [CrossRef]
175. Thies, F.; Miles, E.A.; Nebe-von-Caron, G.; Powell, J.R.; Hurst, T.L.; Newsholme, E.A.; Calder, P.C. Influence of dietary supplementation with long-chain *n*-3 or *n*-6 polyunsaturated fatty acids on blood inflammatory cell populations and functions and on plasma soluble adhesion molecules in healthy adults. *Lipids* **2001**, *36*, 1183–1193. [CrossRef]
176. Zhao, G.; Etherton, T.D.; Martin, K.R.; West, S.G.; Gillies, P.J.; Kris-Etherton, P.M. Dietary alpha-linolenic acid reduces inflammatory and lipid cardiovascular risk factors in hypercholesterolemic men and women. *J. Nutr.* **2004**, *134*, 2991–2997.
177. Pot, G.K.; Geelen, A.; Majsak-Newman, G.; Harvey, L.J.; Nagengast, F.M.; Witteman, B.J.; van de Meeberg, P.C.; Hart, A.R.; Schaafsma, G.; Lund, E.K.; *et al.* Increased consumption of fatty and lean fish reduces serum C-reactive protein concentrations but not inflammation markers in feces and in colonic biopsies. *J. Nutr.* **2010**, *140*, 371–376. [CrossRef]
178. Micallef, M.A.; Garg, M.L. Anti-inflammatory and cardioprotective effects of n-3 polyunsaturated fatty acids and plant sterols in hyperlipidemic individuals. *Atherosclerosis* **2009**, *204*, 476–482. [CrossRef]
179. Kelley, D.S.; Siegel, D.; Fedor, D.M.; Adkins, Y.; Mackey, B.E. DHA supplementation decreases serum C-reactive protein and other markers of inflammation in hypertriglyceridemic men. *J. Nutr.* **2009**, *139*, 495–501. [CrossRef]
180. Geelen, A.; Brouwer, I.A.; Schouten, E.G.; Kluft, C.; Katan, M.B.; Zock, P.L. Intake of n-3 fatty acids from fish does not lower serum concentrations of C-reactive protein in healthy subjects. *Eur. J. Clin. Nutr.* **2004**, *58*, 1440–1442. [CrossRef]
181. Vega-Lopez, S.; Kaul, N.; Devaraj, S.; Cai, R.Y.; German, B.; Jialal, I. Supplementation with omega3 polyunsaturated fatty acids and all-rac alpha-tocopherol alone and in combination failed to exert an anti-inflammatory effect in human volunteers. *Metabolism* **2004**, *53*, 236–240. [CrossRef]
182. Damsgaard, C.T.; Frokiaer, H.; Andersen, A.D.; Lauritzen, L. Fish oil in combination with high or low intakes of linoleic acid lowers plasma triacylglycerols but does not affect other cardiovascular risk markers in healthy men. *J. Nutr.* **2008**, *138*, 1061–1066.
183. Yusof, H.M.; Miles, E.A.; Calder, P. Influence of very long-chain *n*-3 fatty acids on plasma markers of inflammation in middle-aged men. *Prostaglandins Leukot Essent Fatty Acids* **2008**, *78*, 219–228. [CrossRef]
184. Pot, G.K.; Brouwer, I.A.; Enneman, A.; Rijkers, G.T.; Kampman, E.; Geelen, A. No effect of fish oil supplementation on serum inflammatory markers and their interrelationships: A randomized controlled trial in healthy, middle-aged individuals. *Eur. J. Clin. Nutr.* **2009**, *63*, 1353–1359. [CrossRef]
185. Fujioka, S.; Hamazaki, K.; Itomura, M.; Huan, M.; Nishizawa, H.; Sawazaki, S.; Kitajima, I.; Hamazaki, T. The effects of eicosapentaenoic acid-fortified food on inflammatory markers in healthy subjects—A randomized, placebo-controlled, double-blind study. *J. Nutr. Sci. Vitaminol.* **2006**, *52*, 261–265. [CrossRef]
186. Carayol, M.; Grosclaude, P.; Delpierre, C. Prospective studies of dietary alpha-linolenic acid intake and prostate cancer risk: A meta-analysis. *Cancer Causes Control* **2010**, *21*, 347–355. [CrossRef]
187. Ma, R.W.; Chapman, K. A systematic review of the effect of diet in prostate cancer prevention and treatment. *J. Hum. Nutr Diet.* **2009**, *22*, 187–199. [CrossRef]
188. Chua, M.E.; Sio, M.C.; Sorongon, M.C.; Dy, J.S. Relationship of dietary intake of omega-3 and omega-6 Fatty acids with risk of prostate cancer development: A meta-analysis of prospective studies and review of literature. *Prostate Cancer* **2012**, *2012*. [CrossRef]

189. Chua, M.E.; Sio, M.C.; Sorongon, M.C.; Morales, M.L., Jr. The relevance of serum levels of long chain omega-3 polyunsaturated fatty acids and prostate cancer risk: A meta-analysis. *Can.Urol. Assoc. J.* **2013**, *7*, 333–343.
190. Larsson, S.C.; Kumlin, M.; Ingelman-Sundberg, M.; Wolk, A. Dietary long-chain *n*-3 fatty acids for the prevention of cancer: A review of potential mechanisms. *Am. J. Clin. Nutr.* **2004**, *79*, 935–945.
191. Ngo, T.H.; Barnard, R.J.; Cohen, P.; Freedland, S.; Tran, C.; deGregorio, F.; Elshimali, Y.I.; Heber, D.; Aronson, W.J. Effect of isocaloric low-fat diet on human LAPC-4 prostate cancer xenografts in severe combined immunodeficient mice and the insulin-like growth factor axis. *Clin. Cancer Res.* **2003**, *9*, 2734–2743.
192. Kobayashi, N.; Barnard, R.J.; Said, J.; Hong-Gonzalez, J.; Corman, D.M.; Ku, M.; Doan, N.B.; Gui, D.; Elashoff, D.; Cohen, P.; *et al.* Effect of low-fat diet on development of prostate cancer and Akt phosphorylation in the Hi-Myc transgenic mouse model. *Cancer Res.* **2008**, *68*, 3066–3073. [CrossRef]
193. Berquin, I.M.; Min, Y.; Wu, R.; Wu, J.; Perry, D.; Cline, J.M.; Thomas, M.J.; Thornburg, T.; Kulik, G.; Smith, A.; *et al.* Modulation of prostate cancer genetic risk by omega-3 and omega-6 fatty acids. *J. Clin. Investig.* **2007**, *117*, 1866–1875. [CrossRef]
194. De Marzo, A.M.; Platz, E.A.; Sutcliffe, S.; Xu, J.; Gronberg, H.; Drake, C.G.; Nakai, Y.; Isaacs, W.B.; Nelson, W.G. Inflammation in prostate carcinogenesis. *Nat. Rev. Cancer* **2007**, *7*, 256–269. [CrossRef]
195. Chan, J.M.; Gann, P.H.; Giovannucci, E.L. Role of diet in prostate cancer development and progression. *J. Clin. Oncol.* **2005**, *23*, 8152–8160. [CrossRef]
196. Datta, M.; Schwartz, G.G. Calcium and vitamin D supplementation during androgen deprivation therapy for prostate cancer: A critical review. *Oncologist* **2012**, *17*, 1171–1179. [CrossRef]
197. Cosman, F.; de Beur, S.J.; LeBoff, M.S.; Lewiecki, E.M.; Tanner, B.; Randall, S.; Lindsay, R. Clinician's Guide to Prevention and Treatment of Osteoporosis. *Osteoporos. Int.* **2014**, *25*, 2359–2381. [CrossRef]
198. Planas, J.; Morote, J.; Orsola, A.; Salvador, C.; Trilla, E.; Cecchini, L.; Raventos, C.X. The relationship between daily calcium intake and bone mineral density in men with prostate cancer. *BJU Int.* **2007**, *99*, 812–816. [CrossRef]
199. Ryan, C.W.; Huo, D.; Stallings, J.W.; Davis, R.L.; Beer, T.M.; McWhorter, L.T. Lifestyle factors and duration of androgen deprivation affect bone mineral density of patients with prostate cancer during first year of therapy. *Urology* **2007**, *70*, 122–126. [CrossRef]
200. Mata-Granados, J.M.; Cuenca-Acebedo, R.; Luque de Castro, M.D.; Quesada Gomez, J.M. Lower vitamin E serum levels are associated with osteoporosis in early postmenopausal women: A cross-sectional study. *J. Bone Miner. Metab.* **2013**, *31*, 455–460. [CrossRef]
201. Nieman, L.K. Management of surgically hypogonadal patients unable to take sex hormone replacement therapy. *Endocrinol. Metab. Clin. N. Am.* **2003**, *32*, 325–336. [CrossRef]
202. Steinhubl, S.R. Why have antioxidants failed in clinical trials? *Am. J. Cardiol.* **2008**, *101*, 14–19. [CrossRef]

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Review

n-3 Polyunsaturated Fatty Acids and Mechanisms to Mitigate Inflammatory Paracrine Signaling in Obesity-Associated Breast Cancer

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Abstract: Globally, the prevalence of obesity is increasing which subsequently increases the risk of the development of obesity-related chronic diseases. Low-grade chronic inflammation and dysregulated adipose tissue inflammatory mediator/adipokine secretion are well-established in obesity, and these factors increase the risk of developing inflammation-associated cancer. Breast cancer is of particular interest given that increased inflammation within the subcutaneous mammary adipose tissue depot can alter the local tissue inflammatory microenvironment such that it resembles that of obese visceral adipose tissue. Therefore, in obese women with breast cancer, increased inflammatory mediators both locally and systemically can perpetuate inflammation-associated pro-carcinogenic signaling pathways, thereby increasing disease severity. Herein, we discuss some of these inflammation-associated pro-carcinogenic mechanisms of the combined obese breast cancer phenotype and offer evidence that dietary long chain *n*-3 polyunsaturated fatty acids (PUFA) may have utility in mitigating the severity of obesity-associated inflammation and breast cancer.

Keywords: breast cancer; inflammation; obesity; adipokines; *n*-3 polyunsaturated fatty acids; leptin; adiponectin; aromatase; lipid rafts; eicosanoids

1. Introduction

Based on body mass index (BMI), globally 1.5 billion people are overweight (BMI $\geq$ 25.0 kg/m^2), and 500 million of these individuals are classified as obese (BMI $\geq$ 30.0 kg/m^2) [1]. The clinical consequence of obesity is that it acts as an independent risk factor for several other pathologies, including cancer [1,2]. In this context, obesity is associated with increased mortality in several types of cancer, including breast cancer (BC) [3]. It is estimated that obesity contributes to 50% of all BC cases in older women [4]. Furthermore, considering the prevalence of obesity in younger populations and the projected expansion of the obese population [1], the impact on BC incidence is likely to be exacerbated in the future. Obesity increases the risk of developing the most common BC subtype, estrogen receptor (ER)-positive and progesterone receptor (PR)-positive BC (*i.e.*, hormone-sensitive form of the disease) [4–7]. Paradoxically the incidence of hormone sensitive BC increases with age, which coincides with increasing adiposity and decreasing circulating estrogen levels [8]. In fact, in postmenopausal women the majority of BC cases associated with obesity are ER-positive with a phenotype exhibiting

larger and faster growing tumors that metastasize to axillary lymph nodes [4,5,9–11]. The positive association between BC development and obesity in postmenopausal women is well-established using multiple anthropometric indices of obesity including BMI, adiposity and waist:hip circumference ratio [4,5,9–12]. Interestingly, postmenopausal BC risk is increased by the degree of weight gain during adult life prior to menopause [13,14], thereby indicating that the effects of obesity during the premenopausal phase impact BC risk later in life. Conversely, in premenopausal women the link between BC risk and BMI as a measure of obesity status is more controversial [15–17]. Studies finding no association commonly normalize data to BMI alone, which is a poor discriminator of body fat and lean mass, and fails to account for visceral adiposity which is believed to be a more deleterious adipose depot compared to subcutaneous [18,19]. Premenopausal women with high BMI have been shown to develop significantly larger tumors and worse histopathological features including increased tumor vascularization and metastasis to axillary lymph nodes compared to healthy BMI BC patients [9]. Additionally, premenopausal obesity has been shown to increase the risk of developing triple negative BC (ER, PR and HER negative) [9,20] and hormone receptor-negative BC (ER and PR-negative) [21,22]. Collectively, these data indicate that independent of hormonal status, obesity increases overall BC risk, an effect that is, at least in part, attributed to inflammatory mechanisms and paracrine interactions (*i.e.*, cross-talk) between cell types within the mammary tissue that promote tumorigenesis [4,23,24].

The connection between obesity and ER-positive BC in post-menopausal women is likely attributable to two main interrelated factors that will be a key focus of this review, including: (i) increased adipose tissue (AT) mass and the associated increase in inflammatory mediator production (both locally and systemically); and (ii) elevated AT aromatase activation, which is up-regulated by inflammatory mediators and drives aberrant estrogen production within the AT, thereby promoting BC tumorigenesis. The obesity-associated inflammatory mammary tumor microenvironment is complex and the resultant phenotype is underscored by autocrine and paracrine interactions between adipocytes, tumor infiltrating macrophages (TAM) and epithelial cells, which produce AT-derived inflammatory mediators, collectively referred to as adipokines, which will be discussed in more detail herein. The inflammatory mammary tumor microenvironment should not be confused with "inflammatory breast cancer" (IBC), a rare (1%–6% of all breast malignancies) aggressive BC subtype with higher grade metastatic hormone receptor negative tumors that has been reviewed elsewhere [25,26]. Moreover, we provide evidence that dietary long-chain (LC) *n*-3 polyunsaturated fatty acids (PUFA), particularly fish oil (marine)-derived eicosapentaenoic acid (EPA, 20:5*n*-3) and docosahexaenoic acid (DHA, 22:6*n*3), which have well established anti-inflammatory effects in obesity [27–41] and anti-carcinogenic effects in BC [42–56], may represent an effective complementary approach in the prevention and/or treatment of obesity-associated BC by attenuating inflammatory adipokine-mediated paracrine interactions within the mammary tumor microenvironment.

2. Obese Inflammatory Phenotype

AT is an endocrine organ that secretes greater than 50 recognized proteins including several cytokines and chemokines, both of which are included in the term adipokine (*i.e.*, of AT origin) [57]. In the classic obese phenotype (reviewed elsewhere [2,58,59]), the tissue stress and remodeling that occurs in expanding visceral AT is associated with dysregulated adipokine secretion and a subsequent state of chronic, sub-clinical, low-grade, systemic inflammation [2,60]. The cellular source of these inflammatory mediators includes adipocytes and cells of the stromal vascular fraction (SVF) including endothelial cells, fibroblasts, macrophages and T cells [60]. These adipokines can influence whole-body metabolism, insulin sensitivity and inflammation through autocrine, paracrine and endocrine signaling. Most notably, in obesity both the local AT and circulating levels of inflammatory mediators, such as TNFα, IL-6, IL-1β, MCP-1, leptin and many others (reviewed by [58,59]), are elevated, while levels of adiponectin, an anti-inflammatory adipokine, are decreased [61]. Many of these same adipokines are up-regulated in obese BC and activate signaling pathways that drive inflammation-associated

malignant transformation, and therefore, when present in the mammary tissue result in a more severe BC phenotype, as discussed in detail below.

3. *n*-3 Polyunsaturated Fatty Acids and Obesity

In obesity, marine source LC *n*-3 PUFA have been shown to modulate and improve several critical aspects of the obese phenotype, collectively reducing AT inflammation. Specifically, *n*-3 PUFA modulate the production of AT-derived adipokines by increasing anti-inflammatory adiponectin levels [27–35], while decreasing production of inflammatory mediators such as leptin [36–39] and cytokines including TNFα, IL-6 and MCP-1 [29,35,40,41]. Moreover, dietary *n*-3 PUFA have been found to reverse and/or improved obesity-associated hepatic steatosis and impairments in glucose metabolism and insulin sensitivity [27–29,35,62–64]. Collectively, these anti-inflammatory effects of *n*-3 PUFA alter the obesity-associated inflammatory microenvironment and improve the overall obese phenotype. One well-documented effect of *n*-3 PUFA is the suppression of inflammation by interfering with pro-inflammatory signaling cascades via peroxisome proliferator-activated receptor (PPAR)γ-dependent and independent mechanisms that involve up-regulation of adiponectin, in murine [65] and human adipocytes [66]. Additionally, PPARγ is involved in trans-repression of nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) transcriptional activity leading to decreased expression of NFκB responsive genes including several inflammatory cytokines (TNFα, IL-1β, IL-6 and MCP-1) [67]. In this connection, *n*-3 PUFA functioning as PPAR-receptor ligands also interfere with other transcription factors involved in inflammatory signal transduction pathways including AP-1, STAT-1 and NFAT [68].

n-3 PUFA can also perturb inflammatory signaling in obesity through PPARγ independent signaling mechanisms, most notably by acting as ligands for the G-protein coupled receptor 120 (GPR120) [69]. GPR120 has been shown to be partly responsible for the anti-inflammatory effects of DHA by using the adaptor β-arrestin2 to interfere with inflammatory mediator-stimulated NFκB activation in macrophages [69]. Additionally, EPA and DHA exert anti-inflammatory effects following their selective incorporation into the phospholipid fraction of cell membranes where they can act to decrease the signaling efficiency of protein complexes in lipid rafts [70], or serve as substrates for the synthesis of anti-inflammatory bioactive lipid mediators (*i.e.*, eicosanoids) [71,72]. Taken together, *n*-3 PUFA may beneficially modulate obesity-associated pro-inflammatory paracrine interactions between the different cell types within AT. Overall, *n*-3 PUFA utilize multiple mechanisms to suppress inflammatory signaling, thereby modulating the obesity-associated inflammatory phenotype.

4. *n*-3 Polyunsaturated Fatty Acids and Breast Cancer

Marine-derived *n*-3 PUFA have well-established anti-tumorigenic effects in chemically induced, transgenic and xenograft rodent models of BC [73]. As a point of reference, amongst high LC *n*-3 PUFA consuming populations, the typical Japanese diet contains 1%–2% of daily energy as LC *n*-3 PUFA [74,75], whereas intake levels are higher amongst the Greenland Inuit who typically consume 2.4%–6.3% of daily energy as LC *n*-3 PUFA [76,77]. Although higher levels of *n*-3 PUFA intake can be achieved through supplementation, these physiologically relevant intake levels have been recapitulated in BC rodent dietary intervention studies which demonstrate a beneficial effect of *n*-3 PUFA on the BC phenotype [42–44,46,48,49,55,78]. In this connection, *n*-3 PUFA are recognized for their potential application in reducing obesity-associated inflammation and consequent tumorigenic risk [45]. In brief, LC *n*-3 PUFA are incorporated into mammary AT and tumor tissue [46,47], thereby increasing the levels of *n*-3 PUFA-derived lipid mediators at the expense of those derived from *n*-6 PUFA (*i.e.*, arachidonic acid (AA, C20:4*n*-6)-derived eicosanoids) [42,56,78], altering adipokine secretion [54] and interrupting tumorigenic signaling pathways [79]. These chemoprotective effects of *n*-3 PUFA result in decreased cell proliferation and increased apoptosis, ultimately resulting in reduced BC tumor incidence, growth, multiplicity, and metastasis in rodent models of BC [43,44,46,48–53,55,79]. Further, in a model of obese postmenopausal BC, *n*-3 PUFA supplementation reduced mammary AT

inflammation and markers of inflammatory M1 macrophage infiltration [80] which was associated with reduced tumor burden, indicating that the inflammatory microenvironment promotes tumorigenesis and that *n*-3 PUFA directly antagonize this process. Similar *n*-3 PUFA-mediated anti-tumorigenic effects have been reported in overweight humans wherein *n*-3 PUFA supplementation up-regulated the expression of several genes involved in cell cycle regulation [81]. These studies clearly demonstrate that *n*-3 PUFA can independently modulate responsiveness to cell proliferative and/or apoptotic signaling. This is further highlighted in Figure 1, which outlines the effects of *n*-3 PUFA on critical adipokine/inflammatory mediator levels that underlie the paracrine interactions within the obese mammary tumor microenvironment that ultimately impact proliferative and apoptotic signaling and will be discussed in this review.

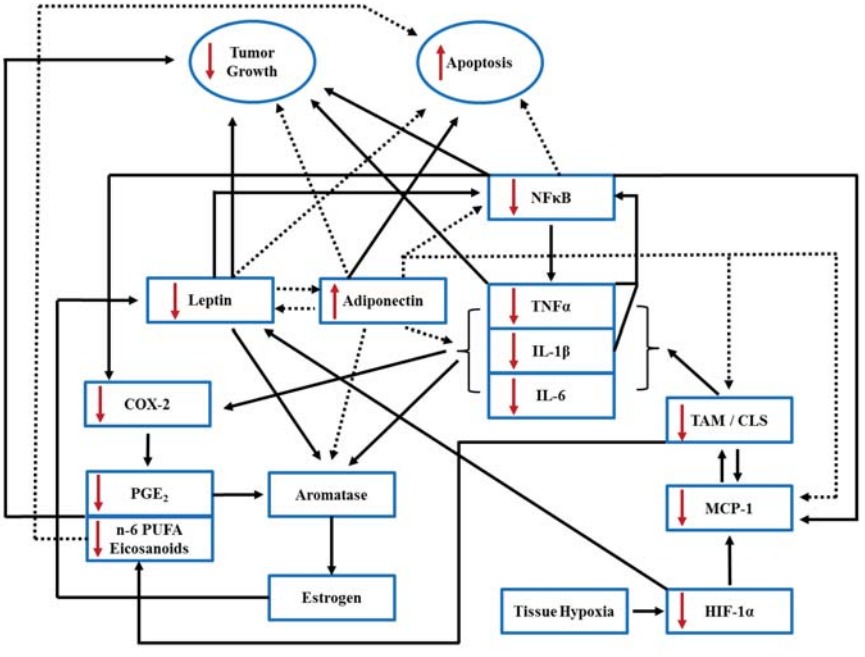

Figure 1. Summary of inflammatory mediator paracrine interactions produced within the obese mammary tissue tumor microenvironment highlighting the complex interactions mediated by adipocytes, macrophages and epithelial cells (main cellular sources of inflammatory mediators). Solid arrows denote stimulatory effects and dotted arrows denote inhibitory effects between inflammatory mediators. Red arrows indicate the effects of *n*-3 PUFA to increase or decrease inflammatory mediator levels, thereby subsequently up-regulating (adiponectin, *n*-3 PUFA-derived eicosanoids) or down-regulating (leptin, *n*-6 PUFA-derived eicosanoids, cytokines (TNFα, IL-1β, IL-6 and MCP-1) and macrophage tissue infiltration). TAM, tumor associated-macrophage; CLS, crown-like structure; HIF-1α, hypoxia induced factor-1α; COX-2, cyclooxygenase-2; PGE_2, prostaglandin E_2; TNFα, tumor necrosis factor-α; IL, interleukin; MCP-1, monocyte chemoattractant protein-1.

A recent meta-analysis that included 21 independent prospective cohort studies determined that marine source *n*-3 PUFA intake was associated with a 14% reduction in BC risk (RR = 0.86 for highest *versus* lowest category of intake (95% confidence interval 0.78–0.94)) [82]. The results were also sustained if the data was analyzed based on either reported dietary intake levels or tissue biomarker levels of *n*-3 PUFA, thereby removing concerns regarding intake compliance or accuracy in dietary recall data. In obese women, a decreased risk of BC was found to be significantly associated with both

increased intake of *n*-3 PUFA and altered dietary fatty acid composition by increasing the ratio of *n*-3:*n*-6 PUFA intake, although this association was not significant in overweight or normal weight women [83]. These data suggest that obesity status may affect the association between *n*-3 PUFA intake and BC risk. To the best of our knowledge, this is the only case-control study that has specifically investigated the relationship between obesity, *n*-3 PUFA intake and BC risk. Interestingly, the chemoprotective effect of an increased ratio of *n*-3:*n*-6 PUFA intake has been reported elsewhere [84], and several case-control studies have reported an inverse association between BC risk and increased *n*-3 PUFA intake and/or increasing the *n*-3:*n*-6 PUFA ratio in breast AT [85–88]. Although obesity status was not independently assessed, some of these studies report a trend with more normal weight women in the control group and more overweight and obese women with BC [85,88]. In contrast, others have reported no association between AT *n*-3 PUFA levels and the development of BC; however, BMI was lower among these BC cases [89]. Taken together, while higher *n*-3 PUFA intake and tissue content is more often than not associated with a decreased risk of developing BC, controversy exists surrounding the associations between BC incidence, obesity status and *n*-3 PUFA intake, warranting case-control studies that investigate a relationship between all endpoints.

5. *n*-3 PUFA, Lipid Rafts and Breast Cancer

Extensive research has indicated that *n*-3 PUFA have a unique ability to broadly affect cell signaling. A ubiquitous mechanism by which *n*-3 PUFA can alter signal transduction is by modifying lipid rafts, which are heterogeneous, highly ordered membrane microdomains that facilitate several signaling events [90,91]. Lipid rafts laterally isolate their components from the bulk membrane and then are able to coalesce in response to stimuli to form signaling platforms [92,93], thereby playing an integral role in the propagation of multiple signaling events that are involved in tumor-promoting activities, including cell proliferation, survival, migration, and invasion [94]. The physical properties that facilitate the segregation of lipid raft domains from the bulk membrane is imparted by the enrichment of cholesterol, sphingolipids, and other phospholipids with saturated, long hydrocarbon chains within lipid rafts [95,96]. *n*-3 PUFA display low affinity for cholesterol due to their high degree of unsaturation [97,98]; therefore, enrichment of the membrane with *n*-3 PUFA can alter the composition and organization of raft domains. Altering the properties of lipid rafts can then have major effects on signaling events that are initiated or propagated by these integral domains.

An important property of lipid rafts is their size and number. Changes to the size of rafts can impose substantial changes on their function. It has been shown that rafts must be small and mobile for optimal activity [99]. Studies in BC cells, in addition to many other cell types, have demonstrated the ability of *n*-3 PUFA to alter the size of lipid rafts. Specifically, DHA was found to alter the size of lipid rafts in BC cells, resulting in lipid rafts of varying height [100]. The same study illustrated that DHA decreased the total amount of lipid rafts on the order of 20%–30%. This is particularly interesting because the levels of lipid rafts are elevated in some forms of cancer, including BC [101], and perturbing these domains can sensitize cells to apoptosis [102]. In addition to altering the size and number of lipid rafts, DHA was found to reduce cell surface levels of lipid rafts by enhancing their internalization [102]. These data implicate *n*-3 PUFA-induced changes in the physical properties of lipid rafts as a mechanism by which these fatty acids exert a chemoprotective effect.

The composition of raft domains is also central to their function as signaling platforms, and *n*-3 PUFA can substantially alter the contents of lipid rafts. The lipid composition of rafts endows the properties necessary for ordering and segregation. BC cells treated with a combination of EPA and DHA were demonstrated to have significantly reduced cholesterol, sphingomyelin, and diacylglycerol lipid raft content [103]. Another study demonstrated differential effects of EPA and DHA on the lipid composition of rafts [100]. EPA was shown to displace AA from raft domains, whereas DHA reduced cholesterol and sphingomyelin content. In addition to lipids, many proteins reside in lipid rafts and require localization to lipid rafts for signal transduction. Importantly, many of these proteins are established mediators of oncogenesis, and displacing these proteins can markedly reduce their

signaling capacity. In MDA-MB-231 BC cells, several raft-associated proteins, including EGFR, Hsp90, Akt, and Src, are redistributed out of raft domains in response to DHA treatment [102], which induced increased BC cell apoptosis. Additionally, DHA was demonstrated to disrupt lipid rafts and reduce HER-2 signaling in mammary epithelial cells overexpressing HER-2 [104]. All of these proteins are involved in the regulation of cell survival and proliferation, and many are targets for cancer therapy. Another well-known therapeutic target for BC metastasis is the chemokine receptor, CXCR4. Treatment of BC cells with DHA or EPA caused redistribution of CXCR4 from lipid rafts to the cell surface [105], resulting in an overall reduction in cell migration. In addition to shifting proteins out of lipid rafts, *n*-3 PUFA can prompt the localization of some proteins into these domains. For example, CD95 (APO-1/FAS) is the transmembrane death receptor which activates the extrinsic apoptosis pathway and activation results in CD95 aggregation in the plasma membrane, followed closely by recruitment of Fas-associated death domain-containing protein (FADD) and caspase-8 to the CD95 receptor, forming the death-inducing signaling complex (DISC) [106]. EPA and DHA have been shown to induce translocation of CD95 into lipid rafts in MDA-MB-231 BC cells and this effect was accompanied by reduced cell growth [107]. Moreover, when DHA was used as a co-treatment it enhanced the chemotherapeutic effects of doxorubicin [107], an anti-neoplastic drug therapy, which has been shown to induce apoptosis and the movement of DISC to membrane rafts [108,109]. All of these data support the regulation of lipid rafts by *n*-3 PUFA as a mechanism by which they exert protective effects in BC and may also have utility as a complementary therapy in combination with pharmaceuticals, although further study is required.

6. Paracrine Interactions, Inflammatory Mediator Signaling and Breast Cancer

The majority of breast tissue is comprised of adipocytes, whereas epithelial cells account for only 10% of total breast cellular volume [110]. Mammary epithelial cells are embedded within the AT, which facilitates direct contact between epithelial cells and adjacent adipocytes and allows for direct functional interactions between AT and mammary tumor cells in a paracrine manner. Within the BC tumor microenvironment, these cellular interactions are further influenced by exposure to circulating AT adipokines [111,112]. Inflammation plays a role in the carcinogenic process and approximately 20% of all cancers originate in association with inflammation [113]. Given the chronic low grade-inflammatory state perpetuated in obesity [114,115], it is likely that obese AT-derived inflammatory mediator production could exacerbate inflammation-associated tumorigenic effects. Specifically, these mediators include *n*-6 PUFA-derived eicosanoids, and adipokines such as leptin and inflammatory cytokines (TNFα, IL-1β and IL-6) with a concomitant reduction in the anti-inflammatory adipokine, adiponectin (discussed below), which are produced in both visceral AT depots and surprisingly also within mammary AT depots, and collectively contribute to the development of a more severe BC phenotype via stimulating BC growth, invasion and metastasis. Typically in obesity-associated BC, inflammatory processes precede tumorigenesis. However, once developed, mammary tumor cells may also serve as a cellular source of inflammatory mediators and support the on-going inflammatory milieu within the mammary tumor microenvironment, thereby potentiating a feed-forward pro-tumorigenic mechanism facilitated by local inflammatory autocrine and paracrine interactions. In summary, autocrine and paracrine signaling between cell types within the mammary AT and tumor tissue are thought to play a central role in breast tumorigenesis [110,116], indicating that dysregulation of adipokines may underlie the association between obesity and BC.

One of the main cellular sources of these inflammatory mediators, apart from adipocytes, is macrophages that infiltrate obese AT and form crown-like structures (CLS) [117,118]. CLS are inflammatory lesions defined as adipocytes surrounded by an aggregation of macrophages that undergo necrosis and fuse to form a syncytium of lipid-containing giant multinucleated cells [119]. Obese mice have increased CLS formation in both visceral AT and mammary AT, which is associated with increased local inflammatory cytokine production (TNFα, IL-1β and IL-6), COX-2 induction and eicosanoid (PGE_2) production, as well as increased aromatase gene expression,

enzyme activity and subsequent estrogen synthesis [119–122]. Therefore, CLS formation represents a tissue localization wherein largely adipocyte-macrophage-mediated paracrine interactions promote both the development and persistence of an inflammatory AT microenvironment, which through further paracrine interactions signal to the mammary epithelial cells to promote BC growth and invasion [120,121]. Transformed mammary epithelial cells and/or the BC tumor itself can further serve as a cellular source for inflammatory mediator production and amplify the on-going local production of inflammatory mediators, and spread these signals to the surrounding non-involved mammary tissue. Moreover, in obesity, mammary AT, which is a subcutaneous AT depot, is transformed to mimic the inflammatory milieu that characterizes the obese visceral AT phenotype [120,121]. The obesity-associated mammary AT phenotypic switch is significant because evidence suggests that subcutaneous AT tends to be less inflammatory compared to visceral sources [118,123] and therefore, in obese mammary AT, a typically less inflammatory depot exhibits a more pronounced inflammatory phenotype that can drive tumorigenesis. In obesity, dietary *n*-3 PUFA supplementation has been shown to reduce AT CLS formation, reduce macrophage AT infiltration by reducing MCP-1 tissue expression and improve the inflammatory secretory profile, in part, by increasing adiponectin [27,28].

The mammary AT tumor microenvironment is complex. Increased local production of inflammatory adipokines underpin the paracrine signaling that regulates the cellular interactions between adipocytes, stromal epithelial cells and infiltrating macrophages, which ultimately drive and define mammary tumor development and the end-stage phenotype (tumor size, type and inflammatory status). Up-regulated paracrine interactions (*i.e.*, cross-talk) in obesity-associated BC perpetuate the carcinogenic process by stimulating multiple, overlapping signaling pathways. These pathways converge to stimulate aromatase expression/activation that aberrantly produces local estrogen, promote cell proliferation and/or inhibit apoptosis and stimulate the production of additional inflammatory mediators within mammary tissue, all of which ultimately support tumorigenesis. The critical inflammatory mediators that are up-regulated in obesity-associated BC perpetuate the carcinogenic process and exhibit redundant effects by stimulating multiple and overlapping signaling pathways that converge to stimulate aromatase expression/activation, resulting in aberrant local estrogen production, which promotes cell proliferation and/or inhibits apoptosis, and stimulate the production of additional inflammatory mediators within mammary tissue, ultimately resulting in tumorigenesis. Since a large and diverse list of hormones, adipokines and lipid mediators are implicated in promoting obesity-associated mammary tumorigenesis, our review will focus on a critical subset that work in concert to promote estrogen production (via aromatase activation) and tumorigenesis, specifically eicosanoids, inflammatory cytokines, leptin and adiponectin; however, we recognize that other mediators play a role in this process such as insulin, insulin-like growth factors, resistin nampt/visfatin and cholesterol as reviewed elsewhere [124–126]. The specific mechanisms/pathways through which obesity-associated inflammatory adipokines exert pro-tumorigenic effects are discussed in detail below, and the complexity of these paracrine interactions are shown in Figure 1.

7. The Role of Estrogen and Aromatase Activation in BC

Circulating estrogen levels are higher in obese women compared to lean women, and increased circulating estrogen is associated with approximately a two-fold increased risk of postmenopausal BC [5,6,127–129]. Additionally, obesity, particularly abdominal adiposity, increases estradiol production and bioavailability due to a reduction in hepatic synthesis of sex hormone-binding globulin (SHBG) in postmenopausal women [6,12,127,129,130]. These hormonal changes are widely believed to play an underlying role in the increased risk of BC in obese postmenopausal women [6,12,127,131]. After menopause, the primary source of estrogen are extra-ovarian sites, primarily in the AT, and aberrant AT estrogen production is attributable to increased aromatase activity, which is present at higher levels in mammary tumors compared to normal mammary tissue [132,133]. Aromatase is the rate-limiting enzyme in the estrogen biosynthesis pathway [131] which catalyzes the peripheral conversion of

androstenedione and testosterone to estrone and estradiol, respectively [110]. Downstream conversion of estrone to the biologically potent estradiol is catalyzed by 17β-hydroxysteroid dehydrogenase, which is also expressed within AT [131]. In obese individuals, aromatase expression is reported to be increased by two-fold compared to normal weight individuals [125]. Additionally, aromatase expression is four to five-fold higher within breast tumor tissue compared to non-involved tissues within the same breast [125]. Consequently, mammary tissue estrogen levels are reported to be 10–50 times higher compared to blood levels in postmenopausal healthy women, which has been shown to play a critical role in BC cell growth [134–138].

Typically, mammary tumors are located in regions of the breast with the highest aromatase expression and activity [139,140]. Furthermore, breast tissue aromatase expression is highest in the quadrant of the breast that contains the greatest proportion of adipose stromal cells, as there is little aromatase activity in mature adipocytes [141], and accordingly, aromatase expression is typically highest in the adipose stromal cells adjacent to the tumor mass [139,140,142]. Therefore, the ratio of stromal cells to adipocytes within mammary tissue may have a predictive value in potential tumor development. Moreover, mammary tumors are typically surrounded by a layer of proliferating cancer-associated fibroblasts (CAF) which have also been shown to express aromatase, thereby indicating that factors produced by the tumor may also stimulate aromatase expression in the surrounding CAF [143].

Aromatase expression and activity is strongly influenced by local inflammatory paracrine signaling within mammary tissue. For instance, malignant epithelial cells along with AT macrophages produce pro-inflammatory mediators, including the eicosanoid PGE_2, which induce aromatase activity and stimulate estrogen production in pre-adipocytes [131,143,144]. The resultant inflammatory mammary tissue microenvironment is further propagated in the obese state, thereby creating a favorable tissue microenvironment to promote the progression of BC growth [120,121]. Further, in obese human BC tissue, aromatase expression is associated with increased tissue levels of COX-2 and PGE_2 [121]. In BC tissue, COX-2 expression is induced by pro-inflammatory cytokines, notably TNFα, and the resultant increased PGE_2 levels are associated with large tumor size and high proliferation rates [131,145], due to, in part, the induction of aromatase expression via activation of cAMP-PKA and PKC-mediated signaling cascades [141,146,147]. Conversely, PGE_3, an *n*-3 PUFA-derived eicosanoid does not induce aromatase expression [148]. Aromatase expression is negatively regulated, in part, by AMP-activated protein kinase (AMPK), which also functions as a negative regulator of the Akt/mTOR signaling pathway that is frequently activated in BC [125]. Additionally, liver kinase B1 (LKB1) can function as a tumor suppressor and can regulate aromatase expression via directly phosphorylating and activating AMPK [149]. Therefore, LKB1 and AMPK both function as negative regulators of aromatase expression in BC. As many inflammatory and metabolic factors alter aromatase expression via effects on LKB1 and/or AMPK, this may provide a critical link between obesity, inflammation and aromatase expression in BC [125]. Leptin increases aromatase expression by decreasing LKB1 protein expression and phosphorylation, whereas adiponectin exerts the opposite effect by stimulating LKB1 and its activity, leading to decreased aromatase expression [150]. Additionally, PGE_2 down-regulates the phosphorylation of AMPK and LKB1, thereby promoting aromatase expression [150]. Inflammatory cytokines such as IL-6, IL-1β and TNFα have also been shown to stimulate aromatase activity [151–154]. TNFα induces aromatase expression through two mechanisms, (i) stimulating the binding of c-fos and c-jun transcription factors to activating protein-1 (AP-1) binding site; and (ii) activation of NFκB and MAPK signaling pathways [152,153]. Obese ovariectomized rodents exhibit increased NFκB activation and inflammatory mediator production (TNF-α, IL-1β, COX-2), which is accompanied by elevated levels of aromatase expression and activity in both the mammary gland and visceral fat [120]. Collectively, these data demonstrate that inflammatory mediator signaling in the mammary tissue microenvironment is driven by autocrine/paracrine interactions that regulate critical aspects of the mammary tissue tumor phenotype including aromatase activation and local estrogen production.

These inflammatory mediators can establish a positive feedback mechanism that stimulates cell proliferation and mammary tumor development.

8. Inflammatory and Chemopromotive Fatty Acid Derived Lipid Mediators: Differential Effects of *n*-6 *versus n*-3 PUFA

LC *n*-3 and *n*-6 PUFA are metabolized by the cyclooxygenase (COX), lipoxygenase (LOX) and cytochrome P450 pathways to produce eicosanoids [72]. Specifically, *n*-6 PUFA (such as AA) serves as a substrate for COX enzymes (producing two-series prostanoids such as prostaglandins (PG) and thromboxanes (TX)), LOX enzymes (producing four-series leukotrienes (LT) and hydroxyeicosatetraenoic acids (HETE), principally 5-, 12- and 15-HETE), or cytochrome P450 enzymes (producing primarily 20-HETE) [72,148,155]. The same enzymes metabolize *n*-3 PUFA to structurally different three-series prostanoids, five-series LT, and primarily 19- and 20-hydroxyeicosapentaenoic acids (HEPE) and 21- and 22-hydroxydocosahexaenoic acids (HDHE), as well as the unique lipid mediators E- and D-series resolvins and protectins [72,148,156]. Although both resolvins and protectins exert anti-inflammatory effects, resolvins can stimulate the resolution phase of inflammation to begin at an earlier point, thereby limiting the tissue exposure to inflammatory signaling [157], whereas protectins reduce inflammatory cytokine production [158]. Generally, *n*-6 PUFA-derived eicosanoids are pro-inflammatory and pro-carcinogenic, whereas *n*-3 PUFA-derived lipid mediators are less biologically active and functionally oppose the synthesis and activity *n*-6 PUFA-derived eicosanoids [72,148,159]. Excessive dietary intake of *n*-6 PUFA *versus n*-3 PUFA (five- to 20-fold greater amounts) results in a significantly greater proportion of eicosanoids generated from *n*-6 PUFA [72,160]. Importantly, the fatty acid profile of adipocyte, immune and tumor cell membrane phospholipids can be modified by increased intake of *n*-3 PUFA, thereby suppressing the biosynthesis of AA-derived eicosanoids in favor of EPA and DHA-derived lipid mediators [72,148,159,161].

AA has been shown to be preferentially taken up by MDA-MB-231 BC cells in comparison to EPA, especially in a pro-inflammatory microenvironment [162]. Also, COX-2 and 12-LOX enzymes are overexpressed in BC tumor tissue [131], thereby increasing production of AA-derived inflammatory eicosanoids, which have established pro-tumorigenic effects, and dominate the BC phenotype [131,163–166]. Specifically, PGE_2, LTB_4 and 5-, 12- and 15-HETE, have been shown to increase cell proliferation, down-regulate apoptotic pathways, and induce rapid growth and tumor metastasis, and these effects have been shown to be counteracted by *n*-3 PUFA [50,51,159,165–169]. Furthermore, *n*-6 PUFA-derived eicosanoid levels are elevated during obesity and have been shown to stimulate breast tumor growth, invasion, and metastasis [131], indicating that obesity perpetuates local inflammatory eicosanoid production.

EPA is the preferential substrate for LOX enzymes, and therefore, when present in comparable proportions, *n*-3 PUFA-derived eicosanoids will be produced at the expense of *n*-6 PUFA-derived eicosanoids [148]. Moreover, *n*-3 PUFA suppress COX-2 expression, which is associated with decreased mammary epithelial cell proliferation in MMTV-HER-2/neu transgenic mice [78]. Mammary tumor PGE_2 and 12- and 15-HETE concentrations are dose-dependently reduced by increased dietary *n*-3 PUFA intake in female nude mice injected with MDA-MB-231 cells [42,170], which is associated with increased apoptotic activity and decreased breast tumor cell proliferation, growth and lung metastasis. Similar *n*-3 PUFA-mediated anti-tumorigenic effects have been associated with suppressed cell proliferation and decreased expression of Bcl-2 and other carcinogenic proteins including Ki-67, Her-2/neu and c-Myc [50,51,159,167,168,171]. Taken together, *n*-6 PUFA-derived eicosanoids provide a link between obesity-associated chronic inflammation and the development of BC, and are a potential target for dietary LC *n*-3 PUFA intervention to mitigate the pro-carcinogenic effects of inflammatory *n*-6 PUFA-derived eicosanoids within the mammary tumor microenvironment.

9. Role of Inflammatory Cytokines

Inflammatory cytokines (TNFα, IL-1β, IL-6 and MCP-1) also contribute to the local mammary tissue milieu through paracrine signaling and through an autocrine positive-feedback mechanism to further their own on-going production, in part, by activating the transcription factor NFκB. Interestingly, NFκB activation underlies many aspects of BC cell proliferation, invasion and metastasis [126,172,173]. Moreover, aberrant NFκB signaling is proposed to be one of the mechanisms through which chronic inflammation leads to cancer, as NFκB activation promotes tumorigenesis by inhibiting apoptosis (via activation of Bcl2, Bcl-xL, cFLIP and other genes) and increasing cell proliferation by regulating expression of cyclinD1, cyclinE, CDK2, and c-Myc [174]. Several inflammatory mediators are up-regulated by NFκB activation; specifically, inflammatory *n*-6 PUFA-derived eicosanoid production is stimulated by NFκB activation of COX-2 [175]. Additionally, within chronically inflamed rodent mammary tissue, NFκB increases production of TNFα and IL-1β [120] and similar findings are reported in the mammary tissue of both pre- and postmenopausal obese women [121]. IL-1β levels are increased in patients with invasive ductal carcinoma and ductal carcinomas *in situ* compared to benign mammary tissue levels [176,177], and are overexpressed in breast carcinomas, but undetectable in normal breast tissue [178]. IL-1β levels are positively correlated with the expression of angiogenic factor expression, tumor grade and the expression of AP-1 [176–178].

IL-1β and IL-6 have been shown to stimulate BC cell proliferation in an additive manner with estrogen [154], indicative of synergy between inflammatory mediators and hormones within the mammary tumor microenvironment. TNFα, another potent inflammatory cytokine, also promotes mammary tumor development [179] and has been shown to contribute to BC cell epithelial-mesenchymal transition (EMT) by increasing matrix metalloproteinase (MMP)-9 expression, thereby enhancing migration and invasive capacity [180–182]. Additionally, the high levels of IL-6 and TNFα found in obese rodent adipocyte-conditioned media and serum have been shown to promote cancer cell EMT [183]. Interestingly, the two main cellular sources of TNFα are tumor-associated macrophages (TAM) and the BC cells themselves [131], highlighting the role of inflammatory macrophages in BC.

Macrophage infiltration into mammary tumor sites (and subsequent development of CLS) is driven, in part, by chemotactic signaling. MCP-1, also referred to as CCL2, signals to increase macrophage infiltration into the inflamed mammary tissue, thereby increasing the number of deleterious TAM that accumulate in mammary tumor tissue [184,185]. The cellular sources of MCP-1 in primary breast tumor sites are tumor cells and the TAM themselves, which indicates a feed-forward mechanism wherein macrophage accumulation/tumor recruitment is perpetuated throughout the stages of tumor growth [184,185]. Overall, TAM form CLS within the mammary AT and act as the cellular source of several inflammatory mediators/cytokines that perpetuate the local inflammatory tissue microenvironment, which, through autocrine and paracrine interactions, further promotes BC development [119–122,131]. Collectively, this highlights the critical role that macrophages play in driving tumor-associated inflammatory paracrine interactions. MCP-1 tumor expression is associated with a more advanced course of tumor progression, wherein MCP-1 promotes angiogenesis by stimulating the production of angiogenic factors (such as IL-8 and VEGF) [184,185]. In obesity, circulating and AT levels of MCP-1 are increased. This chemokine provides the main chemoattractant signal that drives visceral AT macrophage infiltration and CLS formation, up-regulating local AT inflammatory mediator production and subsequently impairing glucose metabolism [58,59]. Macrophage recruitment is also stimulated by AT hypoxia. In obesity, adipocyte hypertrophy results in decreased oxygen diffusion, leading to localized tissue AT hypoxia, as evidenced by upregulation of the hypoxia master regulator, hypoxia induced factor (HIF-1α), which stimulates MCP-1 and subsequent macrophage chemotaxis [143,186,187].

Countering these effects in obesity, *n*-3 PUFA have been shown to improve the hypoxic AT microenvironment by reducing adipocyte size [27] and to decrease obesity-associated expression of HIF-1α [81,188]. Furthermore, *n*-3 PUFA reduce visceral AT MCP-1 levels [27,28,35,189,190], thereby

reducing obesity-associated AT macrophage accumulation, CLS formation and AT inflammation [27,28]. We and others have shown that *n*-3 PUFA reduce inflammatory paracrine signaling between adipocytes and macrophages by decreasing NFκB activation and subsequent secretion of TNFα, MCP-1 and IL-6 [191,192]. Moreover, independent of cellular source, *n*-3 PUFA have been shown to inhibit NFκB activation by decreasing IκB phosphorylation and activation, thereby reducing production TNFα, IL-1β and IL-6 [29,35,40,41,67,191,193,194].

10. Role of Leptin

Leptin exerts pleiotropic effects apart from regulation of energy expenditure and food intake, including effects on immunity, inflammation, cell differentiation and proliferation [124,195], all of which have direct relevance to cancer. Classically, circulating leptin levels are proportional to the amount of body fat [196] and increased leptin levels are associated with increased risk of BC development and progression [197,198]. Leptin gene expression is detected in normal healthy breast epithelial tissue [110], consistent with its endogenous role in normal mammary gland development and lactation [199]; however, it is also capable of contributing to mammary tumorigenesis [200] and is expressed in the healthy tissue that surrounds malignant ductal lesions [201]. In primary tumors and various BC cell lines, both leptin and various isoforms of the leptin receptor (ObR) including the long signaling form ObR1 are overexpressed [197,202–207]. Recently, three single nucleotide polymorphisms in the leptin receptor gene (K109R, K656N and Q223R) were identified to be associated with increased BC risk, suggesting that tumor leptin receptor signaling can directly influence tumor growth and progression [208]. Interestingly, in BC patients high intra-tumor ObR gene expression was strongly correlated with decreased relapse-free survival [209], indicating that susceptibility to leptin signaling is strongly associated with BC disease prognosis.

Leptin signaling has been shown to exert autocrine and paracrine effects, ultimately promoting cell growth and proliferation via the activation of critical signaling pathways including those mediated by PKC, c-Jun *N*-terminal kinase (JNK), p38 MAPK, Janus Kinase2/Signal Transducer and Activator of Transcription 3 (JAK2/STAT3), PI3K/Akt/mTOR, Akt/GSK3 and MAPK/extracellular signal-related kinase 1/2 (ERK1/2) pathways [116,181,195,203,210–213]. Further, leptin increases the levels of cell cycle regulators in human MCF-7 BC cells by up-regulating the expression of cyclin dependent kinase 2 (cdk2) and cyclin D1, which advances cells from the G1 to S phase of the cell cycle [214], and induces cell proliferation in ZR-75-1 BC cells via up-regulation of cyclin D1 and c-Myc [215]. Therefore, overexpression and activation of leptin within the mammary tissue microenvironment can ultimately promote tumorigenesis. Similar to obese women, rodents with high leptin levels are more likely to develop mammary tumors [216,217]. Furthermore, in animal BC models leptin antagonism has produced successful outcomes. Specifically MMTV-TGF-α mice crossed with leptin deficient mice fail to develop tumors [218,219] and treatment with a leptin antagonist decreases the growth of murine triple-negative breast tumors [220] and 4T1 mammary cancer cell growth via reducing levels of VEGF, pSTAT3 and cyclin D1 [221].

An additional role of leptin in breast carcinogenesis is to potentiate estrogen signaling, as leptin has been shown to induce aromatase expression/activity and subsequent estrogen synthesis, thereby enhancing ERα activity in BC [210,222,223]. Leptin levels positively correlate with ER expression and BC tumor size [203,205]. Further, leptin can transactivate ERα via ERK1/2 signaling [210] and enhance ERα-dependent transcription by reducing ERα ubiquitination and degradation, even in the presence of an estrogen inhibitor [223], thereby potentiating the effects of estrogen on cell proliferation. Bi-directional influences of estrogen on leptin also exist, wherein estradiol induces leptin and ObR expression, in both AT and BC cell lines [203,224–226].

Another obesity-associated factor that can contribute to the increased local production of leptin and subsequent pro-tumorigenic effects is AT hypoxia. Leptin receptor expression can also be stimulated by tissue hypoxia [226] and, therefore, local tissue leptin expression is up-regulated by HIF-1α [227,228]. Conversely, *n*-3 PUFA have been shown to improve the hypoxic AT

microenvironment by reducing adipocyte size [27] and to decrease obesity-associated expression of HIF-1α [81,188], thereby providing a mechanism through which local leptin signaling responsiveness could be attenuated.

Dietary *n*-3 PUFA have been shown to reduce leptin AT gene expression and/or circulating levels in obese rodents [36,37] and humans [38,39], an effect that was most prominent when combined with weight loss [39]. Decreased leptin signaling represents an additional mechanism through which *n*-3 PUFA attenuate the effects of leptin. In a rodent obesity model, *n*-3 PUFA supplementation was found to decrease leptin receptor gene expression [229], thereby decreasing leptin signaling. In this connection, leptin receptors have been shown to localize to lipid rafts and downstream proliferative effects of leptin mediated through p38 MAPK signaling is lipid raft dependent [230]. Thus, *n*-3 PUFA antagonism of lipid raft size and composition in BC [100,103], as already discussed herein, may also antagonize leptin-mediated proliferative signaling within the mammary tumor microenvironment. Therefore, these findings add to the complex interplay of autocrine and paracrine interactions that underlie the obesity-associated BC inflammatory phenotype, and suggest dietary *n*-3 PUFA as an intervention that may have utility in mitigating local mammary tissue leptin production and signaling to inhibit its pro-tumorigenic effects.

11. Role of Adiponectin

Adipocytes are the primary cellular source of adiponectin, which is secreted as a monomeric protein that can be oligomerized to form both low-molecular weight and high molecular weight complexes [231]. Additionally, cleavage reactions via the action of elastase, can generate globular oligomeric complexes [232] that bind with greater affinity to the adiponectin receptor 1 (AdipoR1), whereas the AdipoR2 preferentially binds full-length and multimeric adiponectin [233].

Within the context of the tumor microenvironment, adiponectin and leptin counter-regulate each other and exert opposing effects [126]. Decreased levels of adiponectin may explain, in part, the increased risk of BC in obesity. Circulating adiponectin levels are generally inversely correlated with BMI, adiposity and visceral fat mass [234,235] and the decreased adiponectin levels in obesity [236] correlate with increased BC risk [111,237,238]. Moreover, three recent independent meta-analyses of BC observational studies confirmed the correlation of higher circulating adiponectin levels with lower BC risk in postmenopausal women [239–241]. More specifically, an increase of 3 μg/mL of circulating adiponectin corresponded to a 5% reduction in BC risk [239]. In obese postmenopausal women, hypoadiponectinemia is associated with increased BC risk, and the disease has been shown to manifest with an aggressive metastatic phenotype [235,242]. In premenopausal women, however, adiponectin levels are not associated with BC risk (95% CI −0.164 to 0.204, $p = 0.829$) [239–241]. The local breast tumor tissue mRNA and protein expression of adiponectin is low, although its receptors are still expressed, indicating that adiponectin-mediated anti-tumorigenic signaling is possible in BC [111,243,244]. In animal studies, reduced production of adiponectin is associated with earlier tumor onset and accelerated tumor growth [245], and overexpression of adiponectin results in mice with reduced mammary tumor size and weight [246]. Studies using various BC cell lines demonstrate that the anti-proliferative effect of adiponectin is mediated through AdipoR1 and AdipoR2 signaling [246–248]. There is a negative correlation between AdipoR1 expression and tumor size, which suggests that the loss of AdipoR1 signaling favours tumor growth [249]. These data are indicative of a weak autocrine/paracrine activity of this hormone within the tumor microenvironment and a loss of the beneficial anti-tumor effects of adiponectin. Despite reduced tissue levels and blunted adiponectin signaling in obesity-associated BC, treatment strategies designed to stimulate adiponectin signaling might represent a novel therapeutic approach.

Adiponectin exerts an anti-proliferative effect in BC [246,247,250–253] by impacting several signaling pathways. Specifically, adiponectin has been shown to impact the glycogen synthase kinase-3β (GSK-3β)/β-catenin signaling pathway via inhibition of phosphorylation of Akt and GSK-3β and subsequent suppression of intracellular accumulation of β-catenin and its transcriptional activities,

resulting in reduced cyclin-D1 expression [246,247,251]. Additionally, adiponectin has been shown to reduce BC cell proliferation by regulating the PTEN/PI3K/mTOR and MAPK pathways [212], specifically inactivating ERK1/2, stimulating AMPK activity and decreasing Akt phosphorylation, leading to reduced mTOR activity [4,251–253]. In MCF-7 BC cells, microarray analysis demonstrated that adiponectin represses expression of multiple important genes that regulate cell cycle (MAPK3 and ATM) and apoptosis (BAG1, BAG3, and TP53), as well as potential diagnostic/prognostic markers (ACADS, CYP19A1, DEGS1, and EVL) [250]. Adiponectin has also been shown to induce BC cell apoptosis [251,253] by down-regulating Bcl2 and up-regulating p53, Bax and p21 expression [4,248, 251]; however, this outcome is dependent on the BC cell line utilized and duration of adiponectin incubation (reviewed [212]). Adiponectin also exerts anti-inflammatory effects by inhibiting the effects of leptin [126] and inhibiting TNFα production by macrophages and adipocytes [254]. Generally, independent of cell type, adiponectin signaling down-regulates the activation of NFκB and production of inflammatory cytokines (TNFα, IL-1β, IL-6 and MCP-1) [255]. Moreover, adiponectin reduces macrophage-mediated inflammation within the tumor microenvironment by suppressing IL-6 gene expression and antagonizing NFκB, JNK and p38 MAPK mediated signaling [255,256].

Increasing the production of adiponectin in obesity may be a beneficial strategy to mitigate inflammation. *n*-3 PUFA have been shown to up-regulate adiponectin secretion in both murine [65] and human adipocytes [66]. Furthermore, dietary *n*-3 PUFA improve the obesity-associated inflammatory secretory profile, in part, by increasing adiponectin levels [27–31,35,63]. In human clinical trials, dietary *n*-3 PUFA have been shown to increase adiponectin levels [32–34] in obese and overweight subjects, thereby demonstrating the potential utility of *n*-3 PUFA to stimulate the effects of this anti-inflammatory adipokine in obesity. Considering the anti-tumorigenic effects of adiponectin and the ability of *n*-3 PUFA to restore adiponectin function in obesity [27–35,63], further research initiatives should be undertaken to determine the utility of *n*-3 PUFA in mitigating obesity-associated BC inflammation and tumor production.

12. Conclusions

In obese women with BC, increased inflammatory adipokine production, both locally in the mammary AT depot and systemically, perpetuates inflammation-associated pro-tumorigenic signaling pathways, thereby increasing disease severity. A spectrum of inflammatory mediators/adipokines are produced by adipocytes, TAM, mammary epithelial cells and tumor cells, which collectively stimulate diverse and overlapping signaling pathways that converge to stimulate aromatase activity that aberrantly increases local estrogen production, up-regulates cell proliferation and down-regulates apoptosis. The complex nature of the obesity-associated BC inflammatory pathophysiology is not likely to be attenuated or prevented by targeting any individual inflammatory mediator and/or signaling pathway, which may explain why most drug therapies, in this context, are ineffective. Instead, a pan-anti-inflammatory approach is more likely to have success in mitigating obesity-associated mammary tissue inflammatory paracrine interactions and subsequent tumorigenesis, and in this context, *n*-3 PUFA may have utility. *n*-3 PUFA have been shown to concurrently target multiple aspects of the obese BC phenotype including reduction of macrophage AT infiltration and CLS formation and down-regulation of critical adipokine production. Collectively, increased *n*-3 PUFA intake could attenuate obesity-associated BC. Considering the current state of obesity world-wide, further studies in human at risk populations should be made a priority.

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References

1. Wang, Y.C.; McPherson, K.; Marsh, T.; Gortmaker, S.L.; Brown, M. Health and economic burden of the projected obesity trends in the USA and the UK. *Lancet* **2011**, *378*, 815–825. [CrossRef] [PubMed]
2. Lumeng, C.N.; Saltiel, A.R. Inflammatory links between obesity and metabolic disease. *J. Clin. Investig.* **2011**, *121*, 2111–2117. [CrossRef] [PubMed]
3. Calle, E.E.; Rodriguez, C.; Walker-Thurmond, K.; Thun, M.J. Overweight, obesity, and mortality from cancer in a prospectively studied cohort of U.S. adults. *N. Engl. J. Med.* **2003**, *348*, 1625–1638. [CrossRef] [PubMed]
4. Van Kruijsdijk, R.C.; van der Wall, E.; Visseren, F.L. Obesity and cancer: The role of dysfunctional adipose tissue. *Cancer Epidemiol. Biomark. Prev.* **2009**, *18*, 2569–2578. [CrossRef]
5. Cleary, M.P.; Grossmann, M.E. Minireview: Obesity and breast cancer: The estrogen connection. *Endocrinology* **2009**, *150*, 2537–2542. [CrossRef] [PubMed]
6. Key, T.J.; Appleby, P.N.; Reeves, G.K.; Roddam, A.; Dorgan, J.F.; Longcope, C.; Stanczyk, F.Z.; Stephenson, H.E., Jr.; Falk, R.T.; Miller, R.; *et al.* Body mass index, serum sex hormones, and breast cancer risk in postmenopausal women. *J. Natl. Cancer Inst.* **2003**, *95*, 1218–1226. [CrossRef] [PubMed]
7. van den Brandt, P.A.; Spiegelman, D.; Yaun, S.S.; Adami, H.O.; Beeson, L.; Folsom, A.R.; Fraser, G.; Goldbohm, R.A.; Graham, S.; Kushi, L.; *et al.* Pooled analysis of prospective cohort studies on height, weight, and breast cancer risk. *Am. J. Epidemiol.* **2000**, *152*, 514–527.
8. Li, C.I.; Daling, J.R.; Malone, K.E. Incidence of invasive breast cancer by hormone receptor status from 1992 to 1998. *J. Clin. Oncol.* **2003**, *21*, 28–34. [CrossRef] [PubMed]
9. Biglia, N.; Peano, E.; Sgandurra, P.; Moggio, G.; Pecchio, S.; Maggiorotto, F.; Sismondi, P. Body mass index (bmi) and breast cancer: Impact on tumor histopathologic features, cancer subtypes and recurrence rate in pre and postmenopausal women. *Gynecol. Endocrinol.* **2013**, *29*, 263–267. [CrossRef] [PubMed]
10. Garrisi, V.M.; Tufaro, A.; Trerotoli, P.; Bongarzone, I.; Quaranta, M.; Ventrella, V.; Tommasi, S.; Giannelli, G.; Paradiso, A. Body mass index and serum proteomic profile in breast cancer and healthy women: A prospective study. *PLoS One* **2012**, *7*, e49631. [CrossRef] [PubMed]
11. Kamineni, A.; Anderson, M.L.; White, E.; Taplin, S.H.; Porter, P.; Ballard-Barbash, R.; Malone, K.; Buist, D.S. Body mass index, tumor characteristics, and prognosis following diagnosis of early-stage breast cancer in a mammographically screened population. *Cancer Causes Control* **2013**, *24*, 305–312. [CrossRef] [PubMed]
12. Rinaldi, S.; Key, T.J.; Peeters, P.H.; Lahmann, P.H.; Lukanova, A.; Dossus, L.; Biessy, C.; Vineis, P.; Sacerdote, C.; Berrino, F.; *et al.* Anthropometric measures, endogenous sex steroids and breast cancer risk in postmenopausal women: A study within the epic cohort. *Int. J. Cancer* **2006**, *118*, 2832–2839. [CrossRef] [PubMed]
13. John, E.M.; Phipps, A.I.; Sangaramoorthy, M. Body size, modifying factors, and postmenopausal breast cancer risk in a multiethnic population: The San Francisco bay area breast cancer study. *SpringerPlus* **2013**, *2*, 239. [CrossRef] [PubMed]
14. Krishnan, K.; Bassett, J.K.; MacInnis, R.J.; English, D.R.; Hopper, J.L.; McLean, C.; Giles, G.G.; Baglietto, L. Associations between weight in early adulthood, change in weight, and breast cancer risk in postmenopausal women. *Cancer Epidemiol. Biomark. Prev.* **2013**, *22*, 1409–1416. [CrossRef]
15. Cheraghi, Z.; Poorolajal, J.; Hashem, T.; Esmailnasab, N.; Doosti Irani, A. Effect of body mass index on breast cancer during premenopausal and postmenopausal periods: A meta-analysis. *PLoS One* **2012**, *7*, e51446. [CrossRef] [PubMed]
16. Pierobon, M.; Frankenfeld, C.L. Obesity as a risk factor for triple-negative breast cancers: A systematic review and meta-analysis. *Breast Cancer Res. Treat.* **2013**, *137*, 307–314. [CrossRef] [PubMed]
17. Amadou, A.; Ferrari, P.; Muwonge, R.; Moskal, A.; Biessy, C.; Romieu, I.; Hainaut, P. Overweight, obesity and risk of premenopausal breast cancer according to ethnicity: A systematic review and dose-response meta-analysis. *Obes. Rev.* **2013**, *14*, 665–678. [CrossRef] [PubMed]
18. Brown, K.A. Impact of obesity on mammary gland inflammation and local estrogen production. *J. Mammary Gland Biol. Neoplasia* **2014**, *19*, 183–189. [CrossRef] [PubMed]
19. Coutinho, T.; Goel, K.; Correa de Sa, D.; Carter, R.E.; Hodge, D.O.; Kragelund, C.; Kanaya, A.M.; Zeller, M.; Park, J.S.; Kober, L.; *et al.* Combining body mass index with measures of central obesity in the assessment of mortality in subjects with coronary disease: Role of "Normal weight central obesity". *J. Am. Coll. Cardiol.* **2013**, *61*, 553–560. [CrossRef] [PubMed]

20. Kimura, K.; Tanaka, S.; Iwamoto, M.; Fujioka, H.; Takahashi, Y.; Satou, N.; Uchiyama, K. Association between body mass index and breast cancer intrinsic subtypes in Japanese women. *Exp. Ther. Med.* **2012**, *4*, 391–396. [PubMed]
21. Daling, J.R.; Malone, K.E.; Doody, D.R.; Johnson, L.G.; Gralow, J.R.; Porter, P.L. Relation of body mass index to tumor markers and survival among young women with invasive ductal breast carcinoma. *Cancer* **2001**, *92*, 720–729. [CrossRef] [PubMed]
22. Fagherazzi, G.; Chabbert-Buffet, N.; Fabre, A.; Guillas, G.; Boutron-Ruault, M.C.; Mesrine, S.; Clavel-Chapelon, F. Hip circumference is associated with the risk of premenopausal er-/pr- breast cancer. *Int. J. Obes.* **2012**, *36*, 431–439. [CrossRef]
23. Olefsky, J.M.; Glass, C.K. Macrophages, inflammation, and insulin resistance. *Annu. Rev. Physiol.* **2010**, *72*, 219–246. [CrossRef]
24. Pierce, B.L.; Ballard-Barbash, R.; Bernstein, L.; Baumgartner, R.N.; Neuhouser, M.L.; Wener, M.H.; Baumgartner, K.B.; Gilliland, F.D.; Sorensen, B.E.; McTiernan, A.; *et al.* Elevated biomarkers of inflammation are associated with reduced survival among breast cancer patients. *J. Clin. Oncol.* **2009**, *27*, 3437–3444. [CrossRef] [PubMed]
25. Woodward, W.A.; Cristofanilli, M. Inflammatory breast cancer. *Semin. Radiat. Oncol.* **2009**, *19*, 256–265. [CrossRef] [PubMed]
26. Cariati, M.; Bennett-Britton, T.M.; Pinder, S.E.; Purushotham, A.D. "Inflammatory" Breast cancer. *Surg. Oncol.* **2005**, *14*, 133–143. [CrossRef] [PubMed]
27. Rossmeisl, M.; Jilkova, Z.M.; Kuda, O.; Jelenik, T.; Medrikova, D.; Stankova, B.; Kristinsson, B.; Haraldsson, G.G.; Svensen, H.; Stoknes, I.; *et al.* Metabolic effects of *n*-3 pufa as phospholipids are superior to triglycerides in mice fed a high-fat diet: Possible role of endocannabinoids. *PLoS One* **2012**, *7*, e38834. [CrossRef] [PubMed]
28. Rossmeisl, M.; Medrikova, D.; van Schothorst, E.M.; Pavlisova, J.; Kuda, O.; Hensler, M.; Bardova, K.; Flachs, P.; Stankova, B.; Vecka, M.; *et al.* Omega-3 phospholipids from fish suppress hepatic steatosis by integrated inhibition of biosynthetic pathways in dietary obese mice. *Biochim. Biophys. Acta* **2014**, *1841*, 267–278. [CrossRef] [PubMed]
29. Kalupahana, N.S.; Claycombe, K.; Newman, S.J.; Stewart, T.; Siriwardhana, N.; Matthan, N.; Lichtenstein, A.H.; Moustaid-Moussa, N. Eicosapentaenoic acid prevents and reverses insulin resistance in high-fat diet-induced obese mice via modulation of adipose tissue inflammation. *J. Nutr.* **2010**, *140*, 1915–1922. [CrossRef] [PubMed]
30. Flachs, P.; Mohamed-Ali, V.; Horakova, O.; Rossmeisl, M.; Hosseinzadeh-Attar, M.J.; Hensler, M.; Ruzickova, J.; Kopecky, J. Polyunsaturated fatty acids of marine origin induce adiponectin in mice fed a high-fat diet. *Diabetologia* **2006**, *49*, 394–397. [CrossRef] [PubMed]
31. Neschen, S.; Morino, K.; Rossbacher, J.C.; Pongratz, R.L.; Cline, G.W.; Sono, S.; Gillum, M.; Shulman, G.I. Fish oil regulates adiponectin secretion by a peroxisome proliferator-activated receptor-gamma-dependent mechanism in mice. *Diabetes* **2006**, *55*, 924–928. [CrossRef] [PubMed]
32. Itoh, M.; Suganami, T.; Satoh, N.; Tanimoto-Koyama, K.; Yuan, X.; Tanaka, M.; Kawano, H.; Yano, T.; Aoe, S.; Takeya, M.; *et al.* Increased adiponectin secretion by highly purified eicosapentaenoic acid in rodent models of obesity and human obese subjects. *Arterioscler. Thromb. Vasc. Biol.* **2007**, *27*, 1918–1925. [CrossRef] [PubMed]
33. Nomura, S.; Shouzu, A.; Omoto, S.; Inami, N.; Ueba, T.; Urase, F.; Maeda, Y. Effects of eicosapentaenoic acid on endothelial cell-derived microparticles, angiopoietins and adiponectin in patients with type 2 diabetes. *J. Atheroscler. Thromb.* **2009**, *16*, 83–90. [CrossRef] [PubMed]
34. Gammelmark, A.; Madsen, T.; Varming, K.; Lundbye-Christensen, S.; Schmidt, E.B. Low-dose fish oil supplementation increases serum adiponectin without affecting inflammatory markers in overweight subjects. *Nutr. Res.* **2012**, *32*, 15–23. [CrossRef]
35. Todoric, J.; Loffler, M.; Huber, J.; Bilban, M.; Reimers, M.; Kadl, A.; Zeyda, M.; Waldhausl, W.; Stulnig, T.M. Adipose tissue inflammation induced by high-fat diet in obese diabetic mice is prevented by *n*-3 polyunsaturated fatty acids. *Diabetologia* **2006**, *49*, 2109–2119. [CrossRef] [PubMed]
36. Takahashi, Y.; Ide, T. Dietary *n*-3 fatty acids affect mrna level of brown adipose tissue uncoupling protein 1, and white adipose tissue leptin and glucose transporter 4 in the rat. *Br. J. Nutr.* **2000**, *84*, 175–184. [PubMed]

37. Ruzickova, J.; Rossmeisl, M.; Prazak, T.; Flachs, P.; Sponarova, J.; Veck, M.; Tvrzicka, E.; Bryhn, M.; Kopecky, J. Omega-3 pufa of marine origin limit diet-induced obesity in mice by reducing cellularity of adipose tissue. *Lipids* **2004**, *39*, 1177–1185. [CrossRef] [PubMed]
38. Winnicki, M.; Somers, V.K.; Accurso, V.; Phillips, B.G.; Puato, M.; Palatini, P.; Pauletto, P. Fish-rich diet, leptin, and body mass. *Circulation* **2002**, *106*, 289–291. [CrossRef] [PubMed]
39. Mori, T.A.; Burke, V.; Puddey, I.B.; Shaw, J.E.; Beilin, L.J. Effect of fish diets and weight loss on serum leptin concentration in overweight, treated-hypertensive subjects. *J. Hypertens.* **2004**, *22*, 1983–1990. [CrossRef] [PubMed]
40. Perez-Echarri, N.; Perez-Matute, P.; Marcos-Gomez, B.; Baena, M.J.; Marti, A.; Martinez, J.A.; Moreno-Aliaga, M.J. Differential inflammatory status in rats susceptible or resistant to diet-induced obesity: Effects of epa ethyl ester treatment. *Eur. J. Nutr.* **2008**, *47*, 380–386. [CrossRef] [PubMed]
41. Puglisi, M.J.; Hasty, A.H.; Saraswathi, V. The role of adipose tissue in mediating the beneficial effects of dietary fish oil. *J. Nutr. Biochem.* **2011**, *22*, 101–108. [CrossRef] [PubMed]
42. Connolly, J.M.; Gilhooly, E.M.; Rose, D.P. Effects of reduced dietary linoleic acid intake, alone or combined with an algal source of docosahexaenoic acid, on mda-mb-231 breast cancer cell growth and apoptosis in nude mice. *Nutr. Cancer* **1999**, *35*, 44–49. [CrossRef] [PubMed]
43. Hardman, W.E.; Munoz, J., Jr.; Cameron, I.L. Role of lipid peroxidation and antioxidant enzymes in omega 3 fatty acids induced suppression of breast cancer xenograft growth in mice. *Cancer Cell Int.* **2002**, *2*, 10. [CrossRef] [PubMed]
44. Hardman, W.E.; Sun, L.; Short, N.; Cameron, I.L. Dietary omega-3 fatty acids and ionizing irradiation on human breast cancer xenograft growth and angiogenesis. *Cancer Cell Int.* **2005**, *5*, 12. [CrossRef] [PubMed]
45. Howe, L.R.; Subbaramaiah, K.; Hudis, C.A.; Dannenberg, A.J. Molecular pathways: Adipose inflammation as a mediator of obesity-associated cancer. *Clin. Cancer Res.* **2013**, *19*, 6074–6083. [CrossRef] [PubMed]
46. Leslie, M.A.; Abdelmagid, S.A.; Perez, K.; Muller, W.J.; Ma, D.W. Mammary tumour development is dose-dependently inhibited by *n*-3 polyunsaturated fatty acids in the mmtv-neu(ndl)-yd5 transgenic mouse model. *Lipids Health Dis.* **2014**, *13*, 96. [CrossRef] [PubMed]
47. Ma, D.W.; Ngo, V.; Huot, P.S.; Kang, J.X. *n*-3 polyunsaturated fatty acids endogenously synthesized in fat-1 mice are enriched in the mammary gland. *Lipids* **2006**, *41*, 35–39. [CrossRef] [PubMed]
48. MacLennan, M.B.; Clarke, S.E.; Perez, K.; Wood, G.A.; Muller, W.J.; Kang, J.X.; Ma, D.W. Mammary tumor development is directly inhibited by lifelong *n*-3 polyunsaturated fatty acids. *J. Nutr. Biochem.* **2013**, *24*, 388–395. [CrossRef] [PubMed]
49. Mandal, C.C.; Ghosh-Choudhury, T.; Yoneda, T.; Choudhury, G.G.; Ghosh-Choudhury, N. Fish oil prevents breast cancer cell metastasis to bone. *Biochem. Biophys. Res. Commun.* **2010**, *402*, 602–607. [CrossRef] [PubMed]
50. Manna, S.; Chakraborty, T.; Ghosh, B.; Chatterjee, M.; Panda, A.; Srivastava, S.; Rana, A.; Chatterjee, M. Dietary fish oil associated with increased apoptosis and modulated expression of bax and bcl-2 during 7,12-dimethylbenz(alpha)anthracene-induced mammary carcinogenesis in rats. *Prostaglandins Leukot. Essent. Fatty Acids* **2008**, *79*, 5–14. [CrossRef] [PubMed]
51. Manna, S.; Janarthan, M.; Ghosh, B.; Rana, B.; Rana, A.; Chatterjee, M. Fish oil regulates cell proliferation, protect DNA damages and decrease her-2/neu and c-myc protein expression in rat mammary carcinogenesis. *Clin. Nutr.* **2010**, *29*, 531–537. [CrossRef] [PubMed]
52. Menendez, J.A.; Lupu, R.; Colomer, R. Exogenous supplementation with omega-3 polyunsaturated fatty acid docosahexaenoic acid (dha; 22:6*n*-3) synergistically enhances taxane cytotoxicity and downregulates her-2/neu (c-erbb-2) oncogene expression in human breast cancer cells. *Eur. J. Cancer Prev.* **2005**, *14*, 263–270. [CrossRef] [PubMed]
53. Menendez, J.A.; Vazquez-Martin, A.; Ropero, S.; Colomer, R.; Lupu, R. Her2 (erbb-2)-targeted effects of the omega-3 polyunsaturated fatty acid, alpha-linolenic acid (ala; 18:3*n*-3), in breast cancer cells: The "Fat features" Of the "Mediterranean diet" As an "Anti-her2 cocktail". *Clin. Transl. Oncol.* **2006**, *8*, 812–820. [CrossRef] [PubMed]
54. Mizuno, N.K.; Rogozina, O.P.; Seppanen, C.M.; Liao, D.J.; Cleary, M.P.; Grossmann, M.E. Combination of intermittent calorie restriction and eicosapentaenoic acid for inhibition of mammary tumors. *Cancer Prev. Res.* **2013**, *6*, 540–547. [CrossRef]

55. Yee, L.D.; Young, D.C.; Rosol, T.J.; Vanbuskirk, A.M.; Clinton, S.K. Dietary (*n*-3) polyunsaturated fatty acids inhibit her-2/neu-induced breast cancer in mice independently of the ppargamma ligand rosiglitazone. *J. Nutr.* **2005**, *135*, 983–988. [PubMed]
56. Zou, Z.; Bellenger, S.; Massey, K.A.; Nicolaou, A.; Geissler, A.; Bidu, C.; Bonnotte, B.; Pierre, A.S.; Minville-Walz, M.; Rialland, M.; *et al.* Inhibition of the her2 pathway by *n*-3 polyunsaturated fatty acids prevents breast cancer in fat-1 transgenic mice. *J. Lipid Res.* **2013**, *54*, 3453–3463. [CrossRef] [PubMed]
57. Kershaw, E.E.; Flier, J.S. Adipose tissue as an endocrine organ. *J. Clin. Endocrinol. Metab.* **2004**, *89*, 2548–2556. [CrossRef] [PubMed]
58. Trayhurn, P.; Wood, I.S. Adipokines: Inflammation and the pleiotropic role of white adipose tissue. *Br. J. Nutr.* **2004**, *92*, 347–355. [CrossRef] [PubMed]
59. Balistreri, C.R.; Caruso, C.; Candore, G. The role of adipose tissue and adipokines in obesity-related inflammatory diseases. *Mediat. Inflamm.* **2010**, *2010*, 802078. [CrossRef]
60. Surmi, B.K.; Hasty, A.H. Macrophage infiltration into adipose tissue: Initiation, propagation and remodeling. *Future Lipidol.* **2008**, *3*, 545–556. [CrossRef] [PubMed]
61. Yu, J.G.; Javorschi, S.; Hevener, A.L.; Kruszynska, Y.T.; Norman, R.A.; Sinha, M.; Olefsky, J.M. The effect of thiazolidinediones on plasma adiponectin levels in normal, obese, and type 2 diabetic subjects. *Diabetes* **2002**, *51*, 2968–2974. [CrossRef] [PubMed]
62. Peyron-Caso, E.; Fluteau-Nadler, S.; Kabir, M.; Guerre-Millo, M.; Quignard-Boulange, A.; Slama, G.; Rizkalla, S.W. Regulation of glucose transport and transporter 4 (glut-4) in muscle and adipocytes of sucrose-fed rats: Effects of *n*-3 poly- and monounsaturated fatty acids. *Horm. Metab. Res.* **2002**, *34*, 360–366. [CrossRef] [PubMed]
63. Tishinsky, J.M.; de Boer, A.A.; Dyck, D.J.; Robinson, L.E. Modulation of visceral fat adipokine secretion by dietary fatty acids and ensuing changes in skeletal muscle inflammation. *Appl. Physiol. Nutr. Metab.* **2014**, *39*, 28–37. [CrossRef] [PubMed]
64. Gonzalez-Periz, A.; Horrillo, R.; Ferre, N.; Gronert, K.; Dong, B.; Moran-Salvador, E.; Titos, E.; Martinez-Clemente, M.; Lopez-Parra, M.; Arroyo, V.; *et al.* Obesity-induced insulin resistance and hepatic steatosis are alleviated by omega-3 fatty acids: A role for resolvins and protectins. *FASEB J.* **2009**, *23*, 1946–1957. [CrossRef] [PubMed]
65. Oster, R.T.; Tishinsky, J.M.; Yuan, Z.; Robinson, L.E. Docosahexaenoic acid increases cellular adiponectin mRNA and secreted adiponectin protein, as well as ppargamma mrna, in 3t3-l1 adipocytes. *Appl. Physiol. Nutr. Metab.* **2010**, *35*, 783–789. [CrossRef] [PubMed]
66. Tishinsky, J.M.; Ma, D.W.; Robinson, L.E. Eicosapentaenoic acid and rosiglitazone increase adiponectin in an additive and ppargamma-dependent manner in human adipocytes. *Obesity* **2011**, *19*, 262–268. [CrossRef] [PubMed]
67. Glass, C.K.; Saijo, K. Nuclear receptor transrepression pathways that regulate inflammation in macrophages and t cells. *Nat. Rev. Immunol.* **2010**, *10*, 365–376. [CrossRef] [PubMed]
68. Szanto, A.; Nagy, L. The many faces of ppargamma: Anti-inflammatory by any means? *Immunobiology* **2008**, *213*, 789–803. [CrossRef]
69. Oh, D.Y.; Talukdar, S.; Bae, E.J.; Imamura, T.; Morinaga, H.; Fan, W.; Li, P.; Lu, W.J.; Watkins, S.M.; Olefsky, J.M. Gpr120 is an omega-3 fatty acid receptor mediating potent anti-inflammatory and insulin-sensitizing effects. *Cell* **2010**, *142*, 687–698. [CrossRef] [PubMed]
70. Stulnig, T.M.; Huber, J.; Leitinger, N.; Imre, E.M.; Angelisova, P.; Nowotny, P.; Waldhausl, W. Polyunsaturated eicosapentaenoic acid displaces proteins from membrane rafts by altering raft lipid composition. *J. Biol. Chem.* **2001**, *276*, 37335–37340. [CrossRef] [PubMed]
71. Calder, P.C. Feeding the immune system. *Proc. Nutr. Soc.* **2013**, *72*, 299–309. [CrossRef] [PubMed]
72. Calder, P.C. *n*-3 fatty acids, inflammation and immunity: New mechanisms to explain old actions. *Proc. Nutr. Soc.* **2013**, *72*, 326–336. [CrossRef] [PubMed]
73. Liu, J.; Ma, D.W.L. The role of *n*-3 polyunsaturated fatty acids in the prevention and treatment of breast cancer. *Nutrients* **2014**, in press.
74. Conquer, J.A.; Holub, B.J. Effect of supplementation with different doses of dha on the levels of circulating dha as non-esterified fatty acid in subjects of asian indian background. *J. Lipid Res.* **1998**, *39*, 286–292. [PubMed]

75. Kris-Etherton, P.M.; Taylor, D.S.; Yu-Poth, S.; Huth, P.; Moriarty, K.; Fishell, V.; Hargrove, R.L.; Zhao, G.; Etherton, T.D. Polyunsaturated fatty acids in the food chain in the United States. *Am. J. Clin. Nutr.* **2000**, *71*, 179S–188S. [PubMed]
76. Damsgaard, C.T.; Frokiaer, H.; Lauritzen, L. The effects of fish oil and high or low linoleic acid intake on fatty acid composition of human peripheral blood mononuclear cells. *Br. J. Nutr.* **2008**, *99*, 147–154. [CrossRef] [PubMed]
77. Feskens, E.J.; Kromhout, D. Epidemiologic studies on eskimos and fish intake. *Ann. N. Y. Acad. Sci.* **1993**, *683*, 9–15. [CrossRef] [PubMed]
78. Yee, L.D.; Agarwal, D.; Rosol, T.J.; Lehman, A.; Tian, M.; Hatton, J.; Heestand, J.; Belury, M.A.; Clinton, S.K. The inhibition of early stages of her-2/neu-mediated mammary carcinogenesis by dietary *n*-3 pufas. *Mol. Nutr. Food Res.* **2013**, *57*, 320–327. [CrossRef] [PubMed]
79. Chen, Z.; Zhang, Y.; Jia, C.; Wang, Y.; Lai, P.; Zhou, X.; Wang, Y.; Song, Q.; Lin, J.; Ren, Z.; *et al.* Mtorc1/2 targeted by *n*-3 polyunsaturated fatty acids in the prevention of mammary tumorigenesis and tumor progression. *Oncogene* **2014**, *33*, 4548–4557. [CrossRef] [PubMed]
80. Chung, H.; Lee, Y.S.; Mayoral, R.; Oh, D.Y.; Siu, J.T.; Webster, N.J.; Sears, D.D.; Olefsky, J.M.; Ellies, L.G. Omega-3 fatty acids reduce obesity-induced tumor progression independent of gpr120 in a mouse model of postmenopausal breast cancer. *Oncogene* **2014**. [CrossRef]
81. Bouwens, M.; van de Rest, O.; Dellschaft, N.; Bromhaar, M.G.; de Groot, L.C.; Geleijnse, J.M.; Muller, M.; Afman, L.A. Fish-oil supplementation induces antiinflammatory gene expression profiles in human blood mononuclear cells. *Am. J. Clin. Nutr.* **2009**, *90*, 415–424. [CrossRef] [PubMed]
82. Zheng, J.S.; Hu, X.J.; Zhao, Y.M.; Yang, J.; Li, D. Intake of fish and marine *n*-3 polyunsaturated fatty acids and risk of breast cancer: Meta-analysis of data from 21 independent prospective cohort studies. *BMJ* **2013**, *346*, f3706. [CrossRef] [PubMed]
83. Chajes, V.; Torres-Mejia, G.; Biessy, C.; Ortega-Olvera, C.; Angeles-Llerenas, A.; Ferrari, P.; Lazcano-Ponce, E.; Romieu, I. Omega-3 and omega-6 polyunsaturated fatty acid intakes and the risk of breast cancer in mexican women: Impact of obesity status. *Cancer Epidemiol. Biomark. Prev.* **2012**, *21*, 319–326. [CrossRef]
84. Goodstine, S.L.; Zheng, T.; Holford, T.R.; Ward, B.A.; Carter, D.; Owens, P.H.; Mayne, S.T. Dietary (*n*-3)/(*n*-6) fatty acid ratio: Possible relationship to premenopausal but not postmenopausal breast cancer risk in U.S. Women. *J. Nutr.* **2003**, *133*, 1409–1414. [PubMed]
85. Maillard, V.; Bougnoux, P.; Ferrari, P.; Jourdan, M.L.; Pinault, M.; Lavillonniere, F.; Body, G.; le Floch, O.; Chajes, V. *n*-3 and *n*-6 fatty acids in breast adipose tissue and relative risk of breast cancer in a case-control study in tours, France. *Int. J. Cancer* **2002**, *98*, 78–83. [CrossRef] [PubMed]
86. Bagga, D.; Anders, K.H.; Wang, H.J.; Glaspy, J.A. Long-chain *n*-3-to-*n*-6 polyunsaturated fatty acid ratios in breast adipose tissue from women with and without breast cancer. *Nutr. Cancer* **2002**, *42*, 180–185. [CrossRef] [PubMed]
87. Klein, V.; Chajes, V.; Germain, E.; Schulgen, G.; Pinault, M.; Malvy, D.; Lefrancq, T.; Fignon, A.; le Floch, O.; Lhuillery, C.; *et al.* Low alpha-linolenic acid content of adipose breast tissue is associated with an increased risk of breast cancer. *Eur. J. Cancer* **2000**, *36*, 335–340. [CrossRef] [PubMed]
88. Kim, J.; Lim, S.Y.; Shin, A.; Sung, M.K.; Ro, J.; Kang, H.S.; Lee, K.S.; Kim, S.W.; Lee, E.S. Fatty fish and fish omega-3 fatty acid intakes decrease the breast cancer risk: A case-control study. *BMC Cancer* **2009**, *9*, 216. [CrossRef] [PubMed]
89. Witt, P.M.; Christensen, J.H.; Schmidt, E.B.; Dethlefsen, C.; Tjonneland, A.; Overvad, K.; Ewertz, M. Marine *n*-3 polyunsaturated fatty acids in adipose tissue and breast cancer risk: A case-cohort study from Denmark. *Cancer Causes Control* **2009**, *20*, 1715–1721. [CrossRef] [PubMed]
90. Pike, L.J. Lipid rafts: Heterogeneity on the high seas. *Biochem. J.* **2004**, *378*, 281–292. [CrossRef] [PubMed]
91. Staubach, S.; Hanisch, F.G. Lipid rafts: Signaling and sorting platforms of cells and their roles in cancer. *Expert Rev. Proteomics* **2011**, *8*, 263–277. [CrossRef] [PubMed]
92. Hofman, E.G.; Ruonala, M.O.; Bader, A.N.; van den Heuvel, D.; Voortman, J.; Roovers, R.C.; Verkleij, A.J.; Gerritsen, H.C.; van Bergen En Henegouwen, P.M. Egf induces coalescence of different lipid rafts. *J. Cell Sci.* **2008**, *121*, 2519–2528. [CrossRef] [PubMed]
93. Lingwood, D.; Simons, K. Lipid rafts as a membrane-organizing principle. *Science* **2010**, *327*, 46–50. [CrossRef] [PubMed]

94. Patra, S.K. Dissecting lipid raft facilitated cell signaling pathways in cancer. *Biochim. Biophys. Acta* **2008**, *1785*, 182–206. [PubMed]
95. Pike, L.J.; Han, X.; Chung, K.N.; Gross, R.W. Lipid rafts are enriched in arachidonic acid and plasmenylethanolamine and their composition is independent of caveolin-1 expression: A quantitative electrospray ionization/mass spectrometric analysis. *Biochemistry* **2002**, *41*, 2075–2088. [CrossRef] [PubMed]
96. Simons, K.; Sampaio, J.L. Membrane organization and lipid rafts. *Cold Spring Harb. Perspect. Biol.* **2011**, *3*, a004697. [CrossRef] [PubMed]
97. Brzustowicz, M.R.; Cherezov, V.; Caffrey, M.; Stillwell, W.; Wassall, S.R. Molecular organization of cholesterol in polyunsaturated membranes: Microdomain formation. *Biophys. J.* **2002**, *82*, 285–298. [CrossRef] [PubMed]
98. Wassall, S.R.; Brzustowicz, M.R.; Shaikh, S.R.; Cherezov, V.; Caffrey, M.; Stillwell, W. Order from disorder, corralling cholesterol with chaotic lipids. The role of polyunsaturated lipids in membrane raft formation. *Chem. Phys. Lipids* **2004**, *132*, 79–88. [PubMed]
99. Nicolau, D.V., Jr.; Burrage, K.; Parton, R.G.; Hancock, J.F. Identifying optimal lipid raft characteristics required to promote nanoscale protein-protein interactions on the plasma membrane. *Mol. Cell. Biol.* **2006**, *26*, 313–323. [CrossRef] [PubMed]
100. Corsetto, P.A.; Cremona, A.; Montorfano, G.; Jovenitti, I.E.; Orsini, F.; Arosio, P.; Rizzo, A.M. Chemical-physical changes in cell membrane microdomains of breast cancer cells after omega-3 pufa incorporation. *Cell Biochem. Biophys.* **2012**, *64*, 45–59. [CrossRef] [PubMed]
101. Li, H.; Sugimura, K.; Kaji, Y.; Kitamura, Y.; Fujii, M.; Hara, I.; Tachibana, M. Conventional MRI capabilities in the diagnosis of prostate cancer in the transition zone. *AJR Am. J. Roentgenol.* **2006**, *186*, 729–742. [CrossRef] [PubMed]
102. Lee, E.J.; Yun, U.J.; Koo, K.H.; Sung, J.Y.; Shim, J.; Ye, S.K.; Hong, K.M.; Kim, Y.N. Down-regulation of lipid raft-associated onco-proteins via cholesterol-dependent lipid raft internalization in docosahexaenoic acid-induced apoptosis. *Biochim. Biophys. Acta* **2014**, *1841*, 190–203. [CrossRef] [PubMed]
103. Schley, P.D.; Brindley, D.N.; Field, C.J. (*n*-3) pufa alter raft lipid composition and decrease epidermal growth factor receptor levels in lipid rafts of human breast cancer cells. *J. Nutr.* **2007**, *137*, 548–553. [PubMed]
104. Ravacci, G.R.; Brentani, M.M.; Tortelli, T., Jr.; Torrinhas, R.S.; Saldanha, T.; Torres, E.A.; Waitzberg, D.L. Lipid raft disruption by docosahexaenoic acid induces apoptosis in transformed human mammary luminal epithelial cells harboring her-2 overexpression. *J. Nutr. Biochem.* **2013**, *24*, 505–515. [CrossRef] [PubMed]
105. Altenburg, J.D.; Siddiqui, R.A. Omega-3 polyunsaturated fatty acids down-modulate cxcr4 expression and function in mda-mb-231 breast cancer cells. *Mol. Cancer Res.* **2009**, *7*, 1013–1020. [CrossRef] [PubMed]
106. Fulda, S.; Strauss, G.; Meyer, E.; Debatin, K.M. Functional cd95 ligand and cd95 death-inducing signaling complex in activation-induced cell death and doxorubicin-induced apoptosis in leukemic T cells. *Blood* **2000**, *95*, 301–308. [PubMed]
107. Ewaschuk, J.B.; Newell, M.; Field, C.J. Docosahexanoic acid improves chemotherapy efficacy by inducing cd95 translocation to lipid rafts in er(-) breast cancer cells. *Lipids* **2012**, *47*, 1019–1030. [CrossRef] [PubMed]
108. Bollinger, C.R.; Teichgraber, V.; Gulbins, E. Ceramide-enriched membrane domains. *Biochim. Biophys. Acta* **2005**, *1746*, 284–294. [CrossRef] [PubMed]
109. Kim, H.S.; Lee, Y.S.; Kim, D.K. Doxorubicin exerts cytotoxic effects through cell cycle arrest and fas-mediated cell death. *Pharmacology* **2009**, *84*, 300–309. [CrossRef] [PubMed]
110. Lorincz, A.M.; Sukumar, S. Molecular links between obesity and breast cancer. *Endocr. Relat. Cancer* **2006**, *13*, 279–292. [CrossRef] [PubMed]
111. Korner, A.; Pazaitou-Panayiotou, K.; Kelesidis, T.; Kelesidis, I.; Williams, C.J.; Kaprara, A.; Bullen, J.; Neuwirth, A.; Tseleni, S.; Mitsiades, N.; *et al.* Total and high-molecular-weight adiponectin in breast cancer: *In vitro* and *in vivo* studies. *J. Clin. Endocrinol. Metab.* **2007**, *92*, 1041–1048. [CrossRef] [PubMed]
112. Iyengar, P.; Combs, T.P.; Shah, S.J.; Gouon-Evans, V.; Pollard, J.W.; Albanese, C.; Flanagan, L.; Tenniswood, M.P.; Guha, C.; Lisanti, M.P.; *et al.* Adipocyte-secreted factors synergistically promote mammary tumorigenesis through induction of anti-apoptotic transcriptional programs and proto-oncogene stabilization. *Oncogene* **2003**, *22*, 6408–6423. [CrossRef] [PubMed]
113. Grivennikov, S.I.; Karin, M. Inflammatory cytokines in cancer: Tumour necrosis factor and interleukin 6 take the stage. *Ann. Rheum. Dis.* **2011**, *70* (Suppl. 1), i104–i108. [CrossRef] [PubMed]
114. Horng, T.; Hotamisligil, G.S. Linking the inflammasome to obesity-related disease. *Nat. Med.* **2011**, *17*, 164–165. [CrossRef] [PubMed]

115. Hotamisligil, G.S. Inflammation and metabolic disorders. *Nature* **2006**, *444*, 860–867. [CrossRef] [PubMed]
116. Dalamaga, M.; Diakopoulos, K.N.; Mantzoros, C.S. The role of adiponectin in cancer: A review of current evidence. *Endocr. Rev.* **2012**, *33*, 547–594. [CrossRef] [PubMed]
117. Fain, J.N. Release of inflammatory mediators by human adipose tissue is enhanced in obesity and primarily by the nonfat cells: A review. *Mediat. Inflamm.* **2010**, *2010*, 513948. [CrossRef]
118. Fain, J.N.; Madan, A.K.; Hiler, M.L.; Cheema, P.; Bahouth, S.W. Comparison of the release of adipokines by adipose tissue, adipose tissue matrix, and adipocytes from visceral and subcutaneous abdominal adipose tissues of obese humans. *Endocrinology* **2004**, *145*, 2273–2282. [CrossRef] [PubMed]
119. Cinti, S.; Mitchell, G.; Barbatelli, G.; Murano, I.; Ceresi, E.; Faloia, E.; Wang, S.; Fortier, M.; Greenberg, A.S.; Obin, M.S. Adipocyte death defines macrophage localization and function in adipose tissue of obese mice and humans. *J. Lipid Res.* **2005**, *46*, 2347–2355. [CrossRef] [PubMed]
120. Subbaramaiah, K.; Howe, L.R.; Bhardwaj, P.; Du, B.; Gravaghi, C.; Yantiss, R.K.; Zhou, X.K.; Blaho, V.A.; Hla, T.; Yang, P.; *et al.* Obesity is associated with inflammation and elevated aromatase expression in the mouse mammary gland. *Cancer Prev. Res.* **2011**, *4*, 329–346. [CrossRef]
121. Morris, P.G.; Hudis, C.A.; Giri, D.; Morrow, M.; Falcone, D.J.; Zhou, X.K.; Du, B.; Brogi, E.; Crawford, C.B.; Kopelovich, L.; *et al.* Inflammation and increased aromatase expression occur in the breast tissue of obese women with breast cancer. *Cancer Prev. Res.* **2011**, *4*, 1021–1029. [CrossRef]
122. Bhardwaj, P.; Du, B.; Zhou, X.K.; Sue, E.; Harbus, M.D.; Falcone, D.J.; Giri, D.; Hudis, C.A.; Kopelovich, L.; Subbaramaiah, K.; *et al.* Caloric restriction reverses obesity-induced mammary gland inflammation in mice. *Cancer Prev. Res.* **2013**, *6*, 282–289. [CrossRef]
123. Ibrahim, M.M. Subcutaneous and visceral adipose tissue: Structural and functional differences. *Obes. Rev.* **2010**, *11*, 11–18. [CrossRef] [PubMed]
124. Dalamaga, M. Obesity, insulin resistance, adipocytokines and breast cancer: New biomarkers and attractive therapeutic targets. *World J. Exp. Med.* **2013**, *3*, 34–42. [PubMed]
125. Ferguson, R.D.; Gallagher, E.J.; Scheinman, E.J.; Damouni, R.; LeRoith, D. The epidemiology and molecular mechanisms linking obesity, diabetes, and cancer. *Vitam. Horm.* **2013**, *93*, 51–98. [PubMed]
126. Rose, D.P.; Vona-Davis, L. Biochemical and molecular mechanisms for the association between obesity, chronic inflammation, and breast cancer. *BioFactors* **2014**, *40*, 1–12. [CrossRef] [PubMed]
127. Baglietto, L.; English, D.R.; Hopper, J.L.; MacInnis, R.J.; Morris, H.A.; Tilley, W.D.; Krishnan, K.; Giles, G.G. Circulating steroid hormone concentrations in postmenopausal women in relation to body size and composition. *Breast Cancer Res. Treat.* **2009**, *115*, 171–179. [CrossRef] [PubMed]
128. Yager, J.D.; Davidson, N.E. Estrogen carcinogenesis in breast cancer. *N. Engl. J. Med.* **2006**, *354*, 270–282. [CrossRef] [PubMed]
129. Key, T.; Appleby, P.; Barnes, I.; Reeves, G.; Endogenous, H.; Breast Cancer Collaborative, G. Endogenous sex hormones and breast cancer in postmenopausal women: Reanalysis of nine prospective studies. *J. Natl. Cancer Inst.* **2002**, *94*, 606–616. [CrossRef] [PubMed]
130. Haffner, S.M.; Katz, M.S.; Dunn, J.F. Increased upper body and overall adiposity is associated with decreased sex hormone binding globulin in postmenopausal women. *Int. J. Obes.* **1991**, *15*, 471–478. [PubMed]
131. Vona-Davis, L.; Rose, D.P. The obesity-inflammation-eicosanoid axis in breast cancer. *J. Mammary Gland Biol. Neoplasia* **2013**, *18*, 291–307. [CrossRef] [PubMed]
132. Suzuki, T.; Miki, Y.; Ohuchi, N.; Sasano, H. Intratumoral estrogen production in breast carcinoma: Significance of aromatase. *Breast Cancer* **2008**, *15*, 270–277. [CrossRef] [PubMed]
133. Irahara, N.; Miyoshi, Y.; Taguchi, T.; Tamaki, Y.; Noguchi, S. Quantitative analysis of aromatase mRNA expression derived from various promoters (i.4, i.3, pii and i.7) and its association with expression of TNF-alpha, il-6 and cox-2 mRNAs in human breast cancer. *Int. J. Cancer* **2006**, *118*, 1915–1921. [CrossRef] [PubMed]
134. Brodie, A.; Lu, Q.; Nakamura, J. Aromatase in the normal breast and breast cancer. *J. Steroid Biochem. Mol. Biol.* **1997**, *61*, 281–286. [CrossRef] [PubMed]
135. Macaulay, V.M.; Nicholls, J.E.; Gledhill, J.; Rowlands, M.G.; Dowsett, M.; Ashworth, A. Biological effects of stable overexpression of aromatase in human hormone-dependent breast cancer cells. *Br. J. Cancer* **1994**, *69*, 77–83. [CrossRef] [PubMed]
136. Simpson, E.R.; Mahendroo, M.S.; Nichols, J.E.; Bulun, S.E. Aromatase gene expression in adipose tissue: Relationship to breast cancer. *Int. J. Fertil. Menopausal Stud.* **1994**, *39* (Suppl. 2), S75–S83.

137. Pasqualini, J.R.; Chetrite, G.S. Recent insight on the control of enzymes involved in estrogen formation and transformation in human breast cancer. *J. Steroid Biochem. Mol. Biol.* **2005**, *93*, 221–236. [CrossRef] [PubMed]
138. Simpson, E.R.; Ackerman, G.E.; Smith, M.E.; Mendelson, C.R. Estrogen formation in stromal cells of adipose tissue of women: Induction by glucocorticosteroids. *Proc. Natl. Acad. Sci. USA* **1981**, *78*, 5690–5694. [CrossRef] [PubMed]
139. O'Neill, J.S.; Elton, R.A.; Miller, W.R. Aromatase activity in adipose tissue from breast quadrants: A link with tumour site. *Br. Med. J.* **1988**, *296*, 741–743. [CrossRef]
140. Bulun, S.E.; Price, T.M.; Aitken, J.; Mahendroo, M.S.; Simpson, E.R. A link between breast cancer and local estrogen biosynthesis suggested by quantification of breast adipose tissue aromatase cytochrome p450 transcripts using competitive polymerase chain reaction after reverse transcription. *J. Clin. Endocrinol. Metab.* **1993**, *77*, 1622–1628. [PubMed]
141. Bulun, S.E.; Chen, D.; Moy, I.; Brooks, D.C.; Zhao, H. Aromatase, breast cancer and obesity: A complex interaction. *Trends Endocrinol. Metab.* **2012**, *23*, 83–89. [CrossRef] [PubMed]
142. Bulun, S.E.; Simpson, E.R. Regulation of aromatase expression in human tissues. *Breast Cancer Res. Treat.* **1994**, *30*, 19–29. [CrossRef] [PubMed]
143. Simpson, E.R.; Brown, K.A. Obesity and breast cancer: Role of inflammation and aromatase. *J. Mol. Endocrinol.* **2013**, *51*, T51–T59. [CrossRef] [PubMed]
144. Subbaramaiah, K.; Morris, P.G.; Zhou, X.K.; Morrow, M.; Du, B.; Giri, D.; Kopelovich, L.; Hudis, C.A.; Dannenberg, A.J. Increased levels of cox-2 and prostaglandin e2 contribute to elevated aromatase expression in inflamed breast tissue of obese women. *Cancer Discov.* **2012**, *2*, 356–365. [CrossRef] [PubMed]
145. Pender-Cudlip, M.C.; Krag, K.J.; Martini, D.; Yu, J.; Guidi, A.; Skinner, S.S.; Zhang, Y.; Qu, X.; He, C.; Xu, Y.; *et al.* Delta-6-desaturase activity and arachidonic acid synthesis are increased in human breast cancer tissue. *Cancer Sci.* **2013**, *104*, 760–764. [CrossRef] [PubMed]
146. Chen, D.; Reierstad, S.; Lin, Z.; Lu, M.; Brooks, C.; Li, N.; Innes, J.; Bulun, S.E. Prostaglandin e(2) induces breast cancer related aromatase promoters via activation of p38 and c-jun nh(2)-terminal kinase in adipose fibroblasts. *Cancer Res.* **2007**, *67*, 8914–8922. [CrossRef] [PubMed]
147. Zhao, Y.; Agarwal, V.R.; Mendelson, C.R.; Simpson, E.R. Estrogen biosynthesis proximal to a breast tumor is stimulated by pge2 via cyclic amp, leading to activation of promoter II of the cyp19 (aromatase) gene. *Endocrinology* **1996**, *137*, 5739–5742. [PubMed]
148. Larsson, S.C.; Kumlin, M.; Ingelman-Sundberg, M.; Wolk, A. Dietary long-chain *n*-3 fatty acids for the prevention of cancer: A review of potential mechanisms. *Am. J. Clin. Nutr.* **2004**, *79*, 935–945. [PubMed]
149. Shackelford, D.B.; Shaw, R.J. The lkb1-ampk pathway: Metabolism and growth control in tumour suppression. *Nat. Rev. Cancer* **2009**, *9*, 563–575. [CrossRef] [PubMed]
150. Brown, K.A.; McInnes, K.J.; Hunger, N.I.; Oakhill, J.S.; Steinberg, G.R.; Simpson, E.R. Subcellular localization of cyclic amp-responsive element binding protein-regulated transcription coactivator 2 provides a link between obesity and breast cancer in postmenopausal women. *Cancer Res.* **2009**, *69*, 5392–5399. [CrossRef] [PubMed]
151. Purohit, A.; Newman, S.P.; Reed, M.J. The role of cytokines in regulating estrogen synthesis: Implications for the etiology of breast cancer. *Breast Cancer Res.* **2002**, *4*, 65–69. [CrossRef] [PubMed]
152. Zhao, Y.; Nichols, J.E.; Valdez, R.; Mendelson, C.R.; Simpson, E.R. Tumor necrosis factor-alpha stimulates aromatase gene expression in human adipose stromal cells through use of an activating protein-1 binding site upstream of promoter 1.4. *Mol. Endocrinol.* **1996**, *10*, 1350–1357. [PubMed]
153. To, S.Q.; Knower, K.C.; Clyne, C.D. Nfkappab and mapk signalling pathways mediate tnfalpha-induced early growth response gene transcription leading to aromatase expression. *Biochem. Biophys. Res. Commun.* **2013**, *433*, 96–101. [CrossRef] [PubMed]
154. Honma, S.; Shimodaira, K.; Shimizu, Y.; Tsuchiya, N.; Saito, H.; Yanaihara, T.; Okai, T. The influence of inflammatory cytokines on estrogen production and cell proliferation in human breast cancer cells. *Endocr. J.* **2002**, *49*, 371–377. [CrossRef] [PubMed]
155. Panigrahy, D.; Kaipainen, A.; Greene, E.R.; Huang, S. Cytochrome p450-derived eicosanoids: The neglected pathway in cancer. *Cancer Metastasis Rev.* **2010**, *29*, 723–735. [CrossRef] [PubMed]
156. Arnold, C.; Konkel, A.; Fischer, R.; Schunck, W.H. Cytochrome p450-dependent metabolism of omega-6 and omega-3 long-chain polyunsaturated fatty acids. *Pharmacol. Rep.* **2010**, *62*, 536–547. [CrossRef] [PubMed]

157. Bannenberg, G.; Arita, M.; Serhan, C.N. Endogenous receptor agonists: Resolving inflammation. *Sci. World J.* **2007**, *7*, 1440–1462. [CrossRef]
158. Bannenberg, G.L.; Chiang, N.; Ariel, A.; Arita, M.; Tjonahen, E.; Gotlinger, K.H.; Hong, S.; Serhan, C.N. Molecular circuits of resolution: Formation and actions of resolvins and protectins. *J. Immunol.* **2005**, *174*, 4345–4355. [CrossRef] [PubMed]
159. Rose, D.P.; Connolly, J.M. Omega-3 fatty acids as cancer chemopreventive agents. *Pharmacol. Ther.* **1999**, *83*, 217–244. [CrossRef] [PubMed]
160. Calder, P.C. The role of marine omega-3 (*n*-3) fatty acids in inflammatory processes, atherosclerosis and plaque stability. *Mol. Nutr. Food Res.* **2012**, *56*, 1073–1080. [CrossRef] [PubMed]
161. Wei, N.; Wang, B.; Zhang, Q.Y.; Mi, M.T.; Zhu, J.D.; Yu, X.P.; Yuan, J.L.; Chen, K.; Wang, J.; Chang, H. Effects of different dietary fatty acids on the fatty acid compositions and the expression of lipid metabolic-related genes in mammary tumor tissues of rats. *Nutr. Cancer* **2008**, *60*, 810–825. [CrossRef] [PubMed]
162. Kaur, B.; Jorgensen, A.; Duttaroy, A.K. Fatty acid uptake by breast cancer cells (mda-mb-231): Effects of insulin, leptin, adiponectin, and tnfalpha. *Prostaglandins Leukot. Essent. Fatty Acids* **2009**, *80*, 93–99. [CrossRef] [PubMed]
163. Liu, X.H.; Connolly, J.M.; Rose, D.P. The 12-lipoxygenase gene-transfected mcf-7 human breast cancer cell line exhibits estrogen-independent, but estrogen and omega-6 fatty acid-stimulated proliferation *in vitro*, and enhanced growth in athymic nude mice. *Cancer Lett.* **1996**, *109*, 223–230. [CrossRef] [PubMed]
164. Connolly, J.M.; Rose, D.P. Enhanced angiogenesis and growth of 12-lipoxygenase gene-transfected mcf-7 human breast cancer cells in athymic nude mice. *Cancer Lett.* **1998**, *132*, 107–112. [CrossRef] [PubMed]
165. Avis, I.; Hong, S.H.; Martinez, A.; Moody, T.; Choi, Y.H.; Trepel, J.; Das, R.; Jett, M.; Mulshine, J.L. Five-lipoxygenase inhibitors can mediate apoptosis in human breast cancer cell lines through complex eicosanoid interactions. *FASEB J.* **2001**, *15*, 2007–2009. [PubMed]
166. Tong, W.G.; Ding, X.Z.; Adrian, T.E. The mechanisms of lipoxygenase inhibitor-induced apoptosis in human breast cancer cells. *Biochem. Biophys. Res. Commun.* **2002**, *296*, 942–948. [CrossRef] [PubMed]
167. Rose, D.P.; Connolly, J.M. Effects of fatty acids and inhibitors of eicosanoid synthesis on the growth of a human breast cancer cell line in culture. *Cancer Res.* **1990**, *50*, 7139–7144. [PubMed]
168. Navarro-Tito, N.; Soto-Guzman, A.; Castro-Sanchez, L.; Martinez-Orozco, R.; Salazar, E.P. Oleic acid promotes migration on mda-mb-231 breast cancer cells through an arachidonic acid-dependent pathway. *Int. J. Biochem. Cell Biol.* **2010**, *42*, 306–317. [CrossRef] [PubMed]
169. Hu, N.; Li, Y.; Zhao, Y.; Wang, Q.; You, J.C.; Zhang, X.D.; Ye, L.H. A novel positive feedback loop involving fasn/p-erk1/2/5-lox/ltb4/fasn sustains high growth of breast cancer cells. *Acta Pharmacol. Sin.* **2011**, *32*, 921–929. [CrossRef] [PubMed]
170. Rose, D.P.; Connolly, J.M.; Rayburn, J.; Coleman, M. Influence of diets containing eicosapentaenoic or docosahexaenoic acid on growth and metastasis of breast cancer cells in nude mice. *J. Natl. Cancer Inst.* **1995**, *87*, 587–592. [CrossRef] [PubMed]
171. Erickson, K.L.; Hubbard, N.E. Fatty acids and breast cancer: The role of stem cells. *Prostaglandins Leukot. Essent. Fatty Acids* **2010**, *82*, 237–241. [CrossRef] [PubMed]
172. Sethi, S.; Sarkar, F.H.; Ahmed, Q.; Bandyopadhyay, S.; Nahleh, Z.A.; Semaan, A.; Sakr, W.; Munkarah, A.; Ali-Fehmi, R. Molecular markers of epithelial-to-mesenchymal transition are associated with tumor aggressiveness in breast carcinoma. *Transl. Oncol.* **2011**, *4*, 222–226. [CrossRef] [PubMed]
173. Zhou, Y.; Yau, C.; Gray, J.W.; Chew, K.; Dairkee, S.H.; Moore, D.H.; Eppenberger, U.; Eppenberger-Castori, S.; Benz, C.C. Enhanced NF kappa b and ap-1 transcriptional activity associated with antiestrogen resistant breast cancer. *BMC Cancer* **2007**, *7*, 59. [CrossRef] [PubMed]
174. Naugler, W.E.; Karin, M. NF-kappab and cancer-identifying targets and mechanisms. *Curr. Opin. Genet. Dev.* **2008**, *18*, 19–26. [CrossRef] [PubMed]
175. Calder, P.C. Lipids for intravenous nutrition in hospitalised adult patients: A multiple choice of options. *Proc. Nutr. Soc.* **2013**, *72*, 263–276. [CrossRef] [PubMed]
176. Kurtzman, S.H.; Anderson, K.H.; Wang, Y.; Miller, L.J.; Renna, M.; Stankus, M.; Lindquist, R.R.; Barrows, G.; Kreutzer, D.L. Cytokines in human breast cancer: Il-1alpha and il-1beta expression. *Oncol. Rep.* **1999**, *6*, 65–70. [PubMed]

177. Pantschenko, A.G.; Pushkar, I.; Anderson, K.H.; Wang, Y.; Miller, L.J.; Kurtzman, S.H.; Barrows, G.; Kreutzer, D.L. The interleukin-1 family of cytokines and receptors in human breast cancer: Implications for tumor progression. *Int. J. Oncol.* **2003**, *23*, 269–284. [PubMed]
178. Chavey, C.; Bibeau, F.; Gourgou-Bourgade, S.; Burlinchon, S.; Boissiere, F.; Laune, D.; Roques, S.; Lazennec, G. Oestrogen receptor negative breast cancers exhibit high cytokine content. *Breast Cancer Res.* **2007**, *9*, R15. [CrossRef] [PubMed]
179. Candido, J.; Hagemann, T. Cancer-related inflammation. *J. Clin. Immunol.* **2013**, *33* (Suppl. 1), S79–S84. [CrossRef]
180. Kim, S.; Choi, J.H.; Kim, J.B.; Nam, S.J.; Yang, J.H.; Kim, J.H.; Lee, J.E. Berberine suppresses TNF-alpha-induced mmp-9 and cell invasion through inhibition of ap-1 activity in mda-mb-231 human breast cancer cells. *Molecules* **2008**, *13*, 2975–2985. [CrossRef] [PubMed]
181. Wu, Y.; Deng, J.; Rychahou, P.G.; Qiu, S.; Evers, B.M.; Zhou, B.P. Stabilization of snail by NF-kappab is required for inflammation-induced cell migration and invasion. *Cancer Cell* **2009**, *15*, 416–428. [CrossRef] [PubMed]
182. Cho, S.G.; Li, D.; Stafford, L.J.; Luo, J.; Rodriguez-Villanueva, M.; Wang, Y.; Liu, M. Kiss1 suppresses tnfalpha-induced breast cancer cell invasion via an inhibition of rhoa-mediated NF-kappab activation. *J. Cell Biochem.* **2009**, *107*, 1139–1149. [CrossRef] [PubMed]
183. Kushiro, K.; Nunez, N.P. Ob/ob serum promotes a mesenchymal cell phenotype in b16bl6 melanoma cells. *Clin. Exp. Metastasis* **2011**, *28*, 877–886. [CrossRef] [PubMed]
184. Soria, G.; Ben-Baruch, A. The inflammatory chemokines ccl2 and ccl5 in breast cancer. *Cancer Lett.* **2008**, *267*, 271–285. [CrossRef] [PubMed]
185. Ueno, T.; Toi, M.; Saji, H.; Muta, M.; Bando, H.; Kuroi, K.; Koike, M.; Inadera, H.; Matsushima, K. Significance of macrophage chemoattractant protein-1 in macrophage recruitment, angiogenesis, and survival in human breast cancer. *Clin. Cancer Res.* **2000**, *6*, 3282–3289. [PubMed]
186. Ye, J.; Gao, Z.; Yin, J.; He, Q. Hypoxia is a potential risk factor for chronic inflammation and adiponectin reduction in adipose tissue of ob/ob and dietary obese mice. *Am. J. Physiol. Endocrinol. Metab.* **2007**, *293*, E1118–E1128. [CrossRef] [PubMed]
187. Cancello, R.; Henegar, C.; Viguerie, N.; Taleb, S.; Poitou, C.; Rouault, C.; Coupaye, M.; Pelloux, V.; Hugol, D.; Bouillot, J.L.; *et al.* Reduction of macrophage infiltration and chemoattractant gene expression changes in white adipose tissue of morbidly obese subjects after surgery-induced weight loss. *Diabetes* **2005**, *54*, 2277–2286. [CrossRef] [PubMed]
188. Itariu, B.K.; Zeyda, M.; Hochbrugger, E.E.; Neuhofer, A.; Prager, G.; Schindler, K.; Bohdjalian, A.; Mascher, D.; Vangala, S.; Schranz, M.; *et al.* Long-chain *n*-3 pufas reduce adipose tissue and systemic inflammation in severely obese nondiabetic patients: A randomized controlled trial. *Am. J. Clin. Nutr.* **2012**, *96*, 1137–1149. [CrossRef] [PubMed]
189. Awada, M.; Meynier, A.; Soulage, C.O.; Hadji, L.; Geloen, A.; Viau, M.; Ribourg, L.; Benoit, B.; Debard, C.; Guichardant, M.; *et al. n*-3 pufa added to high-fat diets affect differently adiposity and inflammation when carried by phospholipids or triacylglycerols in mice. *Nutr. Metab.* **2013**, *10*, 23. [CrossRef]
190. Titos, E.; Rius, B.; Gonzalez-Periz, A.; Lopez-Vicario, C.; Moran-Salvador, E.; Martinez-Clemente, M.; Arroyo, V.; Claria, J. Resolvin d1 and its precursor docosahexaenoic acid promote resolution of adipose tissue inflammation by eliciting macrophage polarization toward an m2-like phenotype. *J. Immunol.* **2011**, *187*, 5408–5418. [CrossRef] [PubMed]
191. Oliver, E.; McGillicuddy, F.C.; Harford, K.A.; Reynolds, C.M.; Phillips, C.M.; Ferguson, J.F.; Roche, H.M. Docosahexaenoic acid attenuates macrophage-induced inflammation and improves insulin sensitivity in adipocytes-specific differential effects between lc *n*-3 pufa. *J. Nutr. Biochem.* **2012**, *23*, 1192–1200. [CrossRef] [PubMed]
192. De Boer, A.A.; Monk, J.M.; Robinson, L.E. Docosahexaenoic acid decreases pro-inflammatory mediators in an *in vitro* murine adipocyte macrophage co-culture model. *PLoS One* **2014**, *9*, e85037. [CrossRef] [PubMed]
193. Novak, T.E.; Babcock, T.A.; Jho, D.H.; Helton, W.S.; Espat, N.J. Nf-kappa b inhibition by omega-3 fatty acids modulates lps-stimulated macrophage tnf-alpha transcription. *Am. J. Physiol. Lung Cell. Mol. Physiol.* **2003**, *284*, L84–L89. [PubMed]

194. Weldon, S.M.; Mullen, A.C.; Loscher, C.E.; Hurley, L.A.; Roche, H.M. Docosahexaenoic acid induces an anti-inflammatory profile in lipopolysaccharide-stimulated human thp-1 macrophages more effectively than eicosapentaenoic acid. *J. Nutr. Biochem.* **2007**, *18*, 250–258. [CrossRef] [PubMed]
195. Moon, H.S.; Dalamaga, M.; Kim, S.Y.; Polyzos, S.A.; Hamnvik, O.P.; Magkos, F.; Paruthi, J.; Mantzoros, C.S. Leptin's role in lipodystrophic and nonlipodystrophic insulin-resistant and diabetic individuals. *Endocr. Rev.* **2013**, *34*, 377–412. [CrossRef] [PubMed]
196. Widjaja, A.; Stratton, I.M.; Horn, R.; Holman, R.R.; Turner, R.; Brabant, G. Ukpds 20: Plasma leptin, obesity, and plasma insulin in type 2 diabetic subjects. *J. Clin. Endocrinol. Metab.* **1997**, *82*, 654–657. [CrossRef] [PubMed]
197. Jarde, T.; Caldefie-Chezet, F.; Damez, M.; Mishellany, F.; Penault-Llorca, F.; Guillot, J.; Vasson, M.P. Leptin and leptin receptor involvement in cancer development: A study on human primary breast carcinoma. *Oncol. Rep.* **2008**, *19*, 905–911. [PubMed]
198. Revillion, F.; Charlier, M.; Lhotellier, V.; Hornez, L.; Giard, S.; Baranzelli, M.C.; Djiane, J.; Peyrat, J.P. Messenger RNA expression of leptin and leptin receptors and their prognostic value in 322 human primary breast cancers. *Clin. Cancer Res.* **2006**, *12*, 2088–2094. [CrossRef] [PubMed]
199. Neville, M.C.; McFadden, T.B.; Forsyth, I. Hormonal regulation of mammary differentiation and milk secretion. *J. Mammary Gland Biol. Neoplasia* **2002**, *7*, 49–66. [CrossRef] [PubMed]
200. Garofalo, C.; Surmacz, E. Leptin and cancer. *J. Cell. Physiol.* **2006**, *207*, 12–22. [CrossRef] [PubMed]
201. Caldefie-Chezet, F.; Damez, M.; de Latour, M.; Konska, G.; Mishellani, F.; Fusillier, C.; Guerry, M.; Penault-Llorca, F.; Guillot, J.; Vasson, M.P. Leptin: A proliferative factor for breast cancer? Study on human ductal carcinoma. *Biochem. Biophys. Res. Commun.* **2005**, *334*, 737–741. [CrossRef] [PubMed]
202. Dieudonne, M.N.; Machinal-Quelin, F.; Serazin-Leroy, V.; Leneveu, M.C.; Pecquery, R.; Giudicelli, Y. Leptin mediates a proliferative response in human mcf7 breast cancer cells. *Biochem. Biophys. Res. Commun.* **2002**, *293*, 622–628. [CrossRef] [PubMed]
203. Garofalo, C.; Koda, M.; Cascio, S.; Sulkowska, M.; Kanczuga-Koda, L.; Golaszewska, J.; Russo, A.; Sulkowski, S.; Surmacz, E. Increased expression of leptin and the leptin receptor as a marker of breast cancer progression: Possible role of obesity-related stimuli. *Clin. Cancer Res.* **2006**, *12*, 1447–1453. [CrossRef]
204. Hu, X.; Juneja, S.C.; Maihle, N.J.; Cleary, M.P. Leptin—A growth factor in normal and malignant breast cells and for normal mammary gland development. *J. Natl. Cancer Inst.* **2002**, *94*, 1704–1711. [CrossRef] [PubMed]
205. Ishikawa, M.; Kitayama, J.; Nagawa, H. Enhanced expression of leptin and leptin receptor (ob-r) in human breast cancer. *Clin. Cancer Res.* **2004**, *10*, 4325–4331. [CrossRef] [PubMed]
206. Laud, K.; Gourdou, I.; Pessemesse, L.; Peyrat, J.P.; Djiane, J. Identification of leptin receptors in human breast cancer: Functional activity in the t47-d breast cancer cell line. *Mol. Cell. Endocrinol.* **2002**, *188*, 219–226. [CrossRef] [PubMed]
207. Yin, N.; Wang, D.; Zhang, H.; Yi, X.; Sun, X.; Shi, B.; Wu, H.; Wu, G.; Wang, X.; Shang, Y. Molecular mechanisms involved in the growth stimulation of breast cancer cells by leptin. *Cancer Res.* **2004**, *64*, 5870–5875. [CrossRef] [PubMed]
208. Nyante, S.J.; Gammon, M.D.; Kaufman, J.S.; Bensen, J.T.; Lin, D.Y.; Barnholtz-Sloan, J.S.; Hu, Y.; He, Q.; Luo, J.; Millikan, R.C. Common genetic variation in adiponectin, leptin, and leptin receptor and association with breast cancer subtypes. *Breast Cancer Res. Treat.* **2011**, *129*, 593–606. [CrossRef] [PubMed]
209. Miyoshi, Y.; Funahashi, T.; Tanaka, S.; Taguchi, T.; Tamaki, Y.; Shimomura, I.; Noguchi, S. High expression of leptin receptor mRNA in breast cancer tissue predicts poor prognosis for patients with high, but not low, serum leptin levels. *Int. J. Cancer* **2006**, *118*, 1414–1419. [CrossRef] [PubMed]
210. Catalano, S.; Mauro, L.; Marsico, S.; Giordano, C.; Rizza, P.; Rago, V.; Montanaro, D.; Maggiolini, M.; Panno, M.L.; Ando, S. Leptin induces, via erk1/erk2 signal, functional activation of estrogen receptor alpha in mcf-7 cells. *J. Biol. Chem.* **2004**, *279*, 19908–19915. [CrossRef] [PubMed]
211. Grossmann, M.E.; Cleary, M.P. The balance between leptin and adiponectin in the control of carcinogenesis—Focus on mammary tumorigenesis. *Biochimie* **2012**, *94*, 2164–2171. [CrossRef] [PubMed]
212. Jarde, T.; Perrier, S.; Vasson, M.P.; Caldefie-Chezet, F. Molecular mechanisms of leptin and adiponectin in breast cancer. *Eur. J. Cancer* **2011**, *47*, 33–43. [CrossRef] [PubMed]
213. Ray, A. Adipokine leptin in obesity-related pathology of breast cancer. *J. Biosci.* **2012**, *37*, 289–294. [CrossRef] [PubMed]

214. Okumura, M.; Yamamoto, M.; Sakuma, H.; Kojima, T.; Maruyama, T.; Jamali, M.; Cooper, D.R.; Yasuda, K. Leptin and high glucose stimulate cell proliferation in mcf-7 human breast cancer cells: Reciprocal involvement of pkc-alpha and ppar expression. *Biochim. Biophys. Acta* **2002**, *1592*, 107–116. [PubMed]
215. Chen, C.; Chang, Y.C.; Liu, C.L.; Chang, K.J.; Guo, I.C. Leptin-induced growth of human zr-75-1 breast cancer cells is associated with up-regulation of cyclin d1 and c-myc and down-regulation of tumor suppressor p53 and p21waf1/cip1. *Breast Cancer Res. Treat.* **2006**, *98*, 121–132. [CrossRef] [PubMed]
216. Seilkop, S.K. The effect of body weight on tumor incidence and carcinogenicity testing in b6c3f1 mice and f344 rats. *Fundam. Appl. Toxicol.* **1995**, *24*, 247–259. [CrossRef] [PubMed]
217. Klurfeld, D.M.; Lloyd, L.M.; Welch, C.B.; Davis, M.J.; Tulp, O.L.; Kritchevsky, D. Reduction of enhanced mammary carcinogenesis in la/n-cp (corpulent) rats by energy restriction. *Proc. Soc. Exp. Biol. Med.* **1991**, *196*, 381–384. [CrossRef] [PubMed]
218. Cleary, M.P.; Juneja, S.C.; Phillips, F.C.; Hu, X.; Grande, J.P.; Maihle, N.J. Leptin receptor-deficient mmtv-tgf-alpha/lepr(db)lepr(db) female mice do not develop oncogene-induced mammary tumors. *Exp. Biol. Med.* **2004**, *229*, 182–193.
219. Cleary, M.P.; Phillips, F.C.; Getzin, S.C.; Jacobson, T.L.; Jacobson, M.K.; Christensen, T.A.; Juneja, S.C.; Grande, J.P.; Maihle, N.J. Genetically obese mmtv-tgf-alpha/lep(ob)lep(ob) female mice do not develop mammary tumors. *Breast Cancer Res. Treat.* **2003**, *77*, 205–215. [CrossRef] [PubMed]
220. Otvos, L., Jr.; Kovalszky, I.; Riolfi, M.; Ferla, R.; Olah, J.; Sztodola, A.; Nama, K.; Molino, A.; Piubello, Q.; Wade, J.D.; *et al.* Efficacy of a leptin receptor antagonist peptide in a mouse model of triple-negative breast cancer. *Eur. J. Cancer* **2011**, *47*, 1578–1584. [CrossRef] [PubMed]
221. Gonzalez, R.R.; Cherfils, S.; Escobar, M.; Yoo, J.H.; Carino, C.; Styer, A.K.; Sullivan, B.T.; Sakamoto, H.; Olawaiye, A.; Serikawa, T.; *et al.* Leptin signaling promotes the growth of mammary tumors and increases the expression of vascular endothelial growth factor (vegf) and its receptor type two (vegf-r2). *J. Biol. Chem.* **2006**, *281*, 26320–26328. [CrossRef] [PubMed]
222. Catalano, S.; Marsico, S.; Giordano, C.; Mauro, L.; Rizza, P.; Panno, M.L.; Ando, S. Leptin enhances, via ap-1, expression of aromatase in the mcf-7 cell line. *J. Biol. Chem.* **2003**, *278*, 28668–28676. [CrossRef] [PubMed]
223. Garofalo, C.; Sisci, D.; Surmacz, E. Leptin interferes with the effects of the antiestrogen ici 182,780 in mcf-7 breast cancer cells. *Clin. Cancer Res.* **2004**, *10*, 6466–6475. [CrossRef] [PubMed]
224. Machinal-Quelin, F.; Dieudonne, M.N.; Pecquery, R.; Leneveu, M.C.; Giudicelli, Y. Direct *in vitro* effects of androgens and estrogens on ob gene expression and leptin secretion in human adipose tissue. *Endocrine* **2002**, *18*, 179–184. [CrossRef] [PubMed]
225. O'Neil, J.S.; Burow, M.E.; Green, A.E.; McLachlan, J.A.; Henson, M.C. Effects of estrogen on leptin gene promoter activation in mcf-7 breast cancer and jeg-3 choriocarcinoma cells: Selective regulation via estrogen receptors alpha and beta. *Mol. Cell. Endocrinol.* **2001**, *176*, 67–75. [CrossRef] [PubMed]
226. Liu, X.; Wu, Y.M.; Xu, L.; Tang, C.; Zhong, Y.B. [Influence of hypoxia on leptin and leptin receptor gene expression of c57bl/6j mice]. *Zhonghua Jie He He Hu Xi Za Zhi* **2005**, *28*, 173–175. [PubMed]
227. Ambrosini, G.; Nath, A.K.; Sierra-Honigmann, M.R.; Flores-Riveros, J. Transcriptional activation of the human leptin gene in response to hypoxia. Involvement of hypoxia-inducible factor 1. *J. Biol. Chem.* **2002**, *277*, 34601–34609. [CrossRef] [PubMed]
228. Grosfeld, A.; Andre, J.; Hauguel-De Mouzon, S.; Berra, E.; Pouyssegur, J.; Guerre-Millo, M. Hypoxia-inducible factor 1 transactivates the human leptin gene promoter. *J. Biol. Chem.* **2002**, *277*, 42953–42957. [CrossRef] [PubMed]
229. Fan, C.; Liu, X.; Shen, W.; Deckelbaum, R.J.; Qi, K. The regulation of leptin, leptin receptor and pro-opiomelanocortin expression by *n*-3 pufas in diet-induced obese mice is not related to the methylation of their promoters. *Nutr. Metab.* **2011**, *8*, 31. [CrossRef]
230. Zeidan, A.; Javadov, S.; Chakrabarti, S.; Karmazyn, M. Leptin-induced cardiomyocyte hypertrophy involves selective caveolae and rhoa/rock-dependent p38 mapk translocation to nuclei. *Cardiovasc. Res.* **2008**, *77*, 64–72. [CrossRef] [PubMed]
231. Kadowaki, T.; Yamauchi, T. Adiponectin and adiponectin receptors. *Endocr. Rev.* **2005**, *26*, 439–451. [CrossRef] [PubMed]
232. Waki, H.; Yamauchi, T.; Kamon, J.; Kita, S.; Ito, Y.; Hada, Y.; Uchida, S.; Tsuchida, A.; Takekawa, S.; Kadowaki, T. Generation of globular fragment of adiponectin by leukocyte elastase secreted by monocytic cell line thp-1. *Endocrinology* **2005**, *146*, 790–796. [CrossRef] [PubMed]

233. Yamauchi, T.; Kamon, J.; Ito, Y.; Tsuchida, A.; Yokomizo, T.; Kita, S.; Sugiyama, T.; Miyagishi, M.; Hara, K.; Tsunoda, M.; *et al.* Cloning of adiponectin receptors that mediate antidiabetic metabolic effects. *Nature* **2003**, *423*, 762–769. [CrossRef] [PubMed]
234. Ryan, A.S.; Berman, D.M.; Nicklas, B.J.; Sinha, M.; Gingerich, R.L.; Meneilly, G.S.; Egan, J.M.; Elahi, D. Plasma adiponectin and leptin levels, body composition, and glucose utilization in adult women with wide ranges of age and obesity. *Diabetes Care* **2003**, *26*, 2383–2388. [CrossRef] [PubMed]
235. Vona-Davis, L.; Rose, D.P. Adipokines as endocrine, paracrine, and autocrine factors in breast cancer risk and progression. *Endocr. Relat. Cancer* **2007**, *14*, 189–206. [CrossRef] [PubMed]
236. Kern, P.A.; di Gregorio, G.B.; Lu, T.; Rassouli, N.; Ranganathan, G. Adiponectin expression from human adipose tissue: Relation to obesity, insulin resistance, and tumor necrosis factor-alpha expression. *Diabetes* **2003**, *52*, 1779–1785. [CrossRef] [PubMed]
237. Miyoshi, Y.; Funahashi, T.; Kihara, S.; Taguchi, T.; Tamaki, Y.; Matsuzawa, Y.; Noguchi, S. Association of serum adiponectin levels with breast cancer risk. *Clin. Cancer Res.* **2003**, *9*, 5699–5704. [PubMed]
238. Mantzoros, C.; Petridou, E.; Dessypris, N.; Chavelas, C.; Dalamaga, M.; Alexe, D.M.; Papadiamantis, Y.; Markopoulos, C.; Spanos, E.; Chrousos, G.; *et al.* Adiponectin and breast cancer risk. *J. Clin. Endocrinol. Metab.* **2004**, *89*, 1102–1107. [CrossRef] [PubMed]
239. Macis, D.; Guerrieri-Gonzaga, A.; Gandini, S. Circulating adiponectin and breast cancer risk: A systematic review and meta-analysis. *Int. J. Epidemiol.* **2014**, *43*, 1226–1236. [CrossRef] [PubMed]
240. Ye, J.; Jia, J.; Dong, S.; Zhang, C.; Yu, S.; Li, L.; Mao, C.; Wang, D.; Chen, J.; Yuan, G. Circulating adiponectin levels and the risk of breast cancer: A meta-analysis. *Eur. J. Cancer Prev.* **2014**, *23*, 158–165. [CrossRef] [PubMed]
241. Liu, L.Y.; Wang, M.; Ma, Z.B.; Yu, L.X.; Zhang, Q.; Gao, D.Z.; Wang, F.; Yu, Z.G. The role of adiponectin in breast cancer: A meta-analysis. *PLoS One* **2013**, *8*, e73183. [CrossRef] [PubMed]
242. Grossmann, M.E.; Ray, A.; Nkhata, K.J.; Malakhov, D.A.; Rogozina, O.P.; Dogan, S.; Cleary, M.P. Obesity and breast cancer: Status of leptin and adiponectin in pathological processes. *Cancer Metastasis Rev.* **2010**, *29*, 641–653. [CrossRef] [PubMed]
243. Takahata, C.; Miyoshi, Y.; Irahara, N.; Taguchi, T.; Tamaki, Y.; Noguchi, S. Demonstration of adiponectin receptors 1 and 2 mRNA expression in human breast cancer cells. *Cancer Lett.* **2007**, *250*, 229–236. [CrossRef] [PubMed]
244. Jarde, T.; Caldefie-Chezet, F.; Damez, M.; Mishellany, F.; Perrone, D.; Penault-Llorca, F.; Guillot, J.; Vasson, M.P. Adiponectin and leptin expression in primary ductal breast cancer and in adjacent healthy epithelial and myoepithelial tissue. *Histopathology* **2008**, *53*, 484–487. [CrossRef] [PubMed]
245. Lam, J.B.; Chow, K.H.; Xu, A.; Lam, K.S.; Liu, J.; Wong, N.S.; Moon, R.T.; Shepherd, P.R.; Cooper, G.J.; Wang, Y. Adiponectin haploinsufficiency promotes mammary tumor development in mmtv-pyvt mice by modulation of phosphatase and tensin homolog activities. *PLoS One* **2009**, *4*, e4968. [CrossRef] [PubMed]
246. Wang, Y.; Lam, J.B.; Lam, K.S.; Liu, J.; Lam, M.C.; Hoo, R.L.; Wu, D.; Cooper, G.J.; Xu, A. Adiponectin modulates the glycogen synthase kinase-3beta/beta-catenin signaling pathway and attenuates mammary tumorigenesis of mda-mb-231 cells in nude mice. *Cancer Res.* **2006**, *66*, 11462–11470. [CrossRef] [PubMed]
247. Nakayama, S.; Miyoshi, Y.; Ishihara, H.; Noguchi, S. Growth-inhibitory effect of adiponectin via adiponectin receptor 1 on human breast cancer cells through inhibition of s-phase entry without inducing apoptosis. *Breast Cancer Res. Treat.* **2008**, *112*, 405–410. [CrossRef] [PubMed]
248. Dos Santos, E.; Benaitreau, D.; Dieudonne, M.N.; Leneveu, M.C.; Serazin, V.; Giudicelli, Y.; Pecquery, R. Adiponectin mediates an antiproliferative response in human mda-mb 231 breast cancer cells. *Oncol. Rep.* **2008**, *20*, 971–977. [PubMed]
249. Pfeiler, G.; Hudelist, G.; Wulfing, P.; Mattsson, B.; Konigsberg, R.; Kubista, E.; Singer, C.F. Impact of adipor1 expression on breast cancer development. *Gynecol. Oncol.* **2010**, *117*, 134–138. [CrossRef] [PubMed]
250. Jarde, T.; Caldefie-Chezet, F.; Goncalves-Mendes, N.; Mishellany, F.; Buechler, C.; Penault-Llorca, F.; Vasson, M.P. Involvement of adiponectin and leptin in breast cancer: Clinical and *in vitro* studies. *Endocr. Relat. Cancer* **2009**, *16*, 1197–1210. [CrossRef] [PubMed]
251. Dieudonne, M.N.; Bussiere, M.; dos Santos, E.; Leneveu, M.C.; Giudicelli, Y.; Pecquery, R. Adiponectin mediates antiproliferative and apoptotic responses in human mcf7 breast cancer cells. *Biochem. Biophys. Res. Commun.* **2006**, *345*, 271–279. [CrossRef] [PubMed]

252. Taliaferro-Smith, L.; Nagalingam, A.; Zhong, D.; Zhou, W.; Saxena, N.K.; Sharma, D. Lkb1 is required for adiponectin-mediated modulation of ampk-s6k axis and inhibition of migration and invasion of breast cancer cells. *Oncogene* **2009**, *28*, 2621–2633. [CrossRef] [PubMed]
253. Nkhata, K.J.; Ray, A.; Schuster, T.F.; Grossmann, M.E.; Cleary, M.P. Effects of adiponectin and leptin co-treatment on human breast cancer cell growth. *Oncol. Rep.* **2009**, *21*, 1611–1619. [PubMed]
254. Villarreal-Molina, M.T.; Antuna-Puente, B. Adiponectin: Anti-inflammatory and cardioprotective effects. *Biochimie* **2012**, *94*, 2143–2149. [CrossRef] [PubMed]
255. Ouchi, N.; Walsh, K. Adiponectin as an anti-inflammatory factor. *Clin. Chim. Acta* **2007**, *380*, 24–30. [CrossRef] [PubMed]
256. Folco, E.J.; Rocha, V.Z.; Lopez-Ilasaca, M.; Libby, P. Adiponectin inhibits pro-inflammatory signaling in human macrophages independent of interleukin-10. *J. Biol. Chem.* **2009**, *284*, 25569–25575. [CrossRef] [PubMed]

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The Anti-Proliferative Effects of Enterolactone in Prostate Cancer Cells: Evidence for the Role of DNA Licencing Genes, mi-R106b Cluster Expression, and PTEN Dosage

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Abstract: The mammalian lignan, enterolactone, has been shown to reduce the proliferation of the earlier stages of prostate cancer at physiological concentrations *in vitro*. However, efficacy in the later stages of the disease occurs at concentrations difficult to achieve through dietary modification. We have therefore investigated what concentration(s) of enterolactone can restrict proliferation in multiple stages of prostate cancer using an *in vitro* model system of prostate disease. We determined that enterolactone at 20 μM significantly restricted the proliferation of mid and late stage models of prostate disease. These effects were strongly associated with changes in the expression of the DNA licencing genes (GMNN, CDT1, MCM2 and 7), in reduced expression of the miR-106b cluster (miR-106b, miR-93, and miR-25), and in increased expression of the PTEN tumour suppressor gene. We have shown anti-proliferative effects of enterolactone in earlier stages of prostate disease than previously reported and that these effects are mediated, in part, by microRNA-mediated regulation.

Keywords: enterolactone; lignan; prostate; proliferation; PTEN; miR-106b cluster

1. Introduction

Prostate cancer is the second most common cancer in men worldwide with seventy percent of annual diagnoses occurring in Westernised societies [1]. The incidence of the disease is considerably higher in the EU, North America and New Zealand than in China (14, 22 and 25-fold, respectively) [2–5] Whilst risk factors for prostate cancer such as age, ethnic origin and heredity are important, geographical and economic differences in diet and lifestyle appear to influence prostate disease risk to a greater extent [6–8]. A clear link between diet and prostate cancer is yet to be shown, due in part to a lack of understanding of the effects, or absence of effect of dietary components on the mechanisms of prostate tumourigenesis.

Enterolactone (ENL) is a weakly-oestrogenic (100 to 1000-fold less compared to natural oestradiol) mammalian metabolite that is produced by the metabolism of plant lignans by intestinal bacteria, but may also be present in low amounts in dairy foods and meat as a consequence of ruminant intestinal metabolism (reviewed [9–11]), [12–16]. ENL has been reported to have anti-cancer, anti-oxidant,

anti-inflammatory and anti-angiogenic properties [10,11,17–21], but ecological studies examining ENL exposure and disease risk, especially with regard to prostate cancer, have been inconclusive [9–11]. This is due, in part, to a lack of understanding of how the inter-individual response to ENL may be affected by diet and lifestyle, genetic and/or epigenetic factors and intestinal microbiota composition.

Serum or urinary ENL levels, a biomarker of exposure, vary considerably by population and dietary preference, and typically ranges from 0.1 to 10 μM [9–11]. There is, however, some evidence that ENL can accumulate to higher levels (up to 25-fold higher) in prostate tissue and fluid, suggesting a biological function for ENL in the prostate [22]. Although there are human, animal and *in vitro* studies showing that purified ENL, or foods rich in ENL, can inhibit the development and progression of prostate cancer for example by reducing proliferation [18–21] or affecting steroid metabolism and activity [23], it is not yet clear if these effects occur at concentrations achievable through dietary intake alone [9–11]. There is a distinct lack of data available on the concentration of ENL in prostate tissue pre and post-intervention with ENL precursors, which restricts our understanding of how bio-available ENL is in the prostate. We have recently shown that physiologically-relevant concentrations of ENL can reduce the proliferation of early-stage prostate disease *in vitro* and that these effects are associated with alterations in the expression of DNA replication licencing genes [19].

The correct initiation of DNA replication requires the licencing of origin of replications by the minichromosome maintenance complex (MCM) [24]. The loading of this MCM complex is facilitated, in part, by chromatin licensing and DNA replication factor 1 (CDT1), which is itself negatively regulated by geminin (GMMN). Abnormal expression of GMNN, CDT1, and MCM2 and 7 have been linked with the malignant progression of prostate cancer [25–31]. Another key signalling pathway disrupted in prostate cancer is the phosphoinositide-3-kinase (PI3K)-AKT signalling pathway. The phosphatase and tensin homolog (PTEN) tumour suppressor gene negatively regulates the PI3K/AKT pathway and PTEN is one of the most common tumour suppressor genes whose appropriate function is compromised in prostate cancer (~70% of cases) [32–34], which leads to abnormal proliferation and cell death. Initiation of DNA replication and PTEN tumour suppression are transcriptionally-linked as one of the MCMs (MCM7) has a microRNA cluster (miR-106b, -93, and 25) in one of its introns that suppresses PTEN translation and dysregulation of the cluster is also linked to cancer [35].

Cancer is not composed of abnormal cells at the same stage of disease; rather it is a series of abnormal cells at differing stages of disease that collectively compromise the appropriate function of the tissue. Previous research has shown anti-proliferative effects of ENL in one or in limited stages of disease, rather than efficacy in a range of disease states [18–21]. Therefore, we hypothesised that ENL could restrict the proliferation of more than just the later stages of prostate disease. To investigate our hypothesis we used an *in vitro* model system of six prostate cell lines representing the early (RWPE-1 and WPE1-NA22), mid (WPE1-NB14 and WPE1-NB11) and later (WPE1-NB26 and LNCaP) stages of prostate tumourigenesis [36,37]. The LNCaP cell line is a model of the switch between androgen sensitivity and insensitivity during prostate disease that occurs in the later stages of carcinogenesis [37]. This *in vitro* model system was used to assess how the metabolic activity, growth rate, cell cycle progression changes with ENL exposure over 24 and 48 h. Based on these data we explored potential mechanisms for the anti-proliferative activity by measuring the expression of the GMMN, CDT1, MCM2 and MCM7, miR-106b cluster, and PTEN genes.

2. Experimental Section

2.1. Cell Culture and Enterolactone Preparation

Authenticated RWPE-1 (P52), WPE1-NA22 (P20), WPE1-NB14 (P16), WPE1-NB11 (P24), WPE1-NB26 (P15), and LNCaP (P22) cell lines were purchased from the American Type Culture Collection (Manassas, VA, USA) to test the effects of ENL. All cell culture reagents were obtained from Life Technologies (Auckland, New Zealand) unless otherwise stated. The cell lines were cultured and

maintained as described previously [19], with all experiments were completed within ten sub-cultures from the original ATCC stock.

A stock solution of ENL (45199, Sigma-Aldrich, Auckland, New Zealand) at 16.76 mM (100% DMSO) was prepared and used to prepare test concentrations of ENL in cell-line specific medium. Etoposide (Sigma-Aldrich, Auckland New Zealand) was used as a positive control for proliferation as it blocks DNA synthesis resulting in apoptosis. The negative control for all experiments was cell-line specific medium adjusted to contain 0.36% v/v of DMSO.

2.2. Cell Viability—Mitochondrial Activity Assay

The effect of 10 to 100 μM ENL on the metabolic activity of the six cell lines over 48 h was measured using the water soluble tetrazolium cytotoxicity assay (WST-1, Clontech, Mountain View, CA, USA) as described previously [19]. The negative control and each concentration of ENL were tested on twenty-four biological replicates for both time points. The absorbance of the formazan dye produced was measured at 450 and 650 nm using a SpectraMax 250 spectrophotometer (Molecular Devices, Sunnyvale, CA, USA). For all measurements (including the blank), the background absorbance (650 nm) was subtracted from the detection wavelength (450 nm) and these corrected values were used for analysis.

2.3. Cell Viability—Growth Kinetics Assay

The effect of 10 to 60 μM ENL on the viability of each cell line over 48 h was measured using trypan blue staining and the number of non-blue (viable) cells counted as described previously [19]. For each of three separate assays for each time point, each concentration was tested on two technical replicates with an initial seeding density of 5×10^5 cells. From these data the effect of ENL on the doubling time of the cell lines was calculated.

2.4. Cell Viability—Cell Cycle Profile Assay

The effect of 20 μM ENL on the cell cycle profile of six cell lines over 48 h was measured with the Dead Cell Apoptosis Kit with Annexin V Alexa Fluor® 488 & Propidium Iodide from Life Technologies (Auckland, New Zealand). This kit is a flow cytometry kit used to measure early apoptosis by detecting phosphatidyl serine expression and membrane permeability [18,38].

Each assay was completed according to the manufacturer's instructions. For each of three separate assays for each time point, each concentration was tested on three technical replicates with an initial seeding density of 5×10^5 cells. The fluorescence intensities of Alexa Fluor 488 and Propidium Iodide in each sample, at 585 nm, were measured using a FACSCalibur flow cytometer with CELLQuest Pro Software (BD Biosciences, Auckland, New Zealand), and analysed using FlowJo V7.6.3 (TreeStar, Ashland, OR, USA).

2.5. Quantification of Gene Expression—mRNA and miRNA Genes

The expression of GMNN, CDT1, MCMs 2 and 7, PTEN, hsa-miR-106b, hsa-miR-93, and hsa-miR-25 by the six cell lines treated with 20 μM ENL over 48 h was quantified using probe-based real-time PCR. All reagents were obtained from Life Technologies (Auckland, New Zealand) unless otherwise stated. For each gene and time point, two biological replicates (with triplicate qPCR measurements) of 5×10^5 cells were used.

The NucleoSpin® miRNA kit (Macherey-Nagel, Düren, Germany) was used to extract large and small RNA in separate fractions from each of the samples according to the manufacturer's instructions. RNA quantity and integrity was determined based on A260:280 and A260:230 nm ratios using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Melbourne, Australia) and a Agilent 2100 bioanalyser (Agilent, Santa Clara, CA, USA). Only RNA with both absorbance ratios of 1.8 to 2.1 and with a RIN value of 9 or greater were considered to be of sufficient quality and integrity.

For the large RNA fractions, 500 ng was reverse transcribed into cDNA using a high capacity RNA-to-cDNA kit according to the manufacturer's instructions. The expression levels of the mRNA transcripts of the GMNN, CDT1, MCMs 2 and 7, and PTEN genes were quantified using pre-validated PrimeTime Nuclease assays (Hs.PT.51.14706721.g, Hs.PT.53.27448129.gs, Hs.PT.53.25820936, Hs.PT.53.23112694.g, and Hs.PT.51.14706721.g) (Integrated DNA Technologies, Singapore, Singapore). The HPRT1 (Hs.PT.39a.22214821) reference gene was used to normalise for RNA content.

For the small RNA fractions, 10 ng was reverse transcribed into cDNA with gene specific primers using the TaqMan microRNA RT kit according to the manufacturer's instructions. The expression levels of the mature hsa-miR-106, hsa-miR-93, and hsa-miR-25 genes were quantified using pre-validated TaqMan assays (000442, 002139, and 002442). The RNU6B reference gene (001093) was used to normalise for RNA content.

All real-time PCR assays were prepared as triplicate 10 μL reactions comprising a 9.0 μL aliquot of master mix (5.0 μL of 2x Kapa Fast Probe mix (Kapa Biosystems, Wilmington, DE, USA), 0.5 μL of 20x mRNA or miRNA gene assay, 3.5 μL of nuclease-free water, and 1 μL of cDNA (10-fold dilution in nuclease-free water). The thermal profile used was: 95 °C for 20 s, followed by 40 cycles of 95 °C for 3 s and 60 °C for 30 s. The experiment was completed using a RotorGene 6000 qPCR instrument (Qiagen, Hilden, Germany). Data were normalised to the appropriate reference gene and analysed for expression level changes (ratio compared to untreated) using the ΔCq method with efficiency correction. The efficiencies for all PCRs ranged between 1.91 and 2.03, where 2.0 represents 100% efficiency.

2.6. Statistical Analyses

All data were analysed for statistical significance using a one-way ANOVA with SigmaStat 12.3 (Systat Software Inc., San Jose, CA, USA). The normality of the data was tested using the Shapiro-Wilk method and the equality of variance using the Leven Median test. Non-normally distributed data was ranked and analysed using the Kruskal-Wallis ANOVA method. Following ANOVA, significantly different means were identified using the Dunnett's post-hoc test. A probability (p) value of less than 0.05 was considered to show a significant difference.

3. Results

3.1. ENL Reduces the Viability of Mid to Later Stage Prostate Disease Cell Lines

ENL exerted differential effects on the mitochondrial metabolic activity and growth kinetics of the prostate cell lines at 24 and 48 h of exposure (Figures 1 and 2).

At 24 h, 20 μM ENL or greater significantly reduced the metabolic activity of the WPE1-NB14, WPE1-NB11, WPE1-NB26, and LNCaP cell lines. At 48 h, 40 μM ENL or greater reduced the activity of all cell lines. However, the activity of the WPE1-NA22 (10 and 20 μM), WPE1-NB14 (10 and 20 μM), WPE1-NB11 (10 and 20 μM), WPE1-NB26 (10 and 20 μM), and LNCaP (20 μM) cell lines was reduced at this time point. The RWPE-1 cell line tolerated up 40 μM ENL without significant alterations in metabolic activity. The metabolic activity of the WPE1-NB44, WPE1-NB11, WPE1-NB26, and LNCaP cell lines were significantly reduced in a dose dependent manner at concentrations of 20 μM of greater at both time points. The WPE1-NB14 and WPE1-NB11 cells were particularly sensitive to ENL at 24 and 48 h.

These data indicate that the lowest concentration of ENL that affects the metabolic activity in the "diseased" cell lines, but does not affect the "normal" cell line is 20 μM. As 40 to 100 μM ENL clearly affected all cell lines, these concentrations were excluded from further analysis. As changes in metabolic activity may result in altered growth rates, *i.e.*, a change in the time taken for a population of cells to double in number, we measured the doubling times of the cell lines in response to 10 and 20 μM ENL over 48 h.

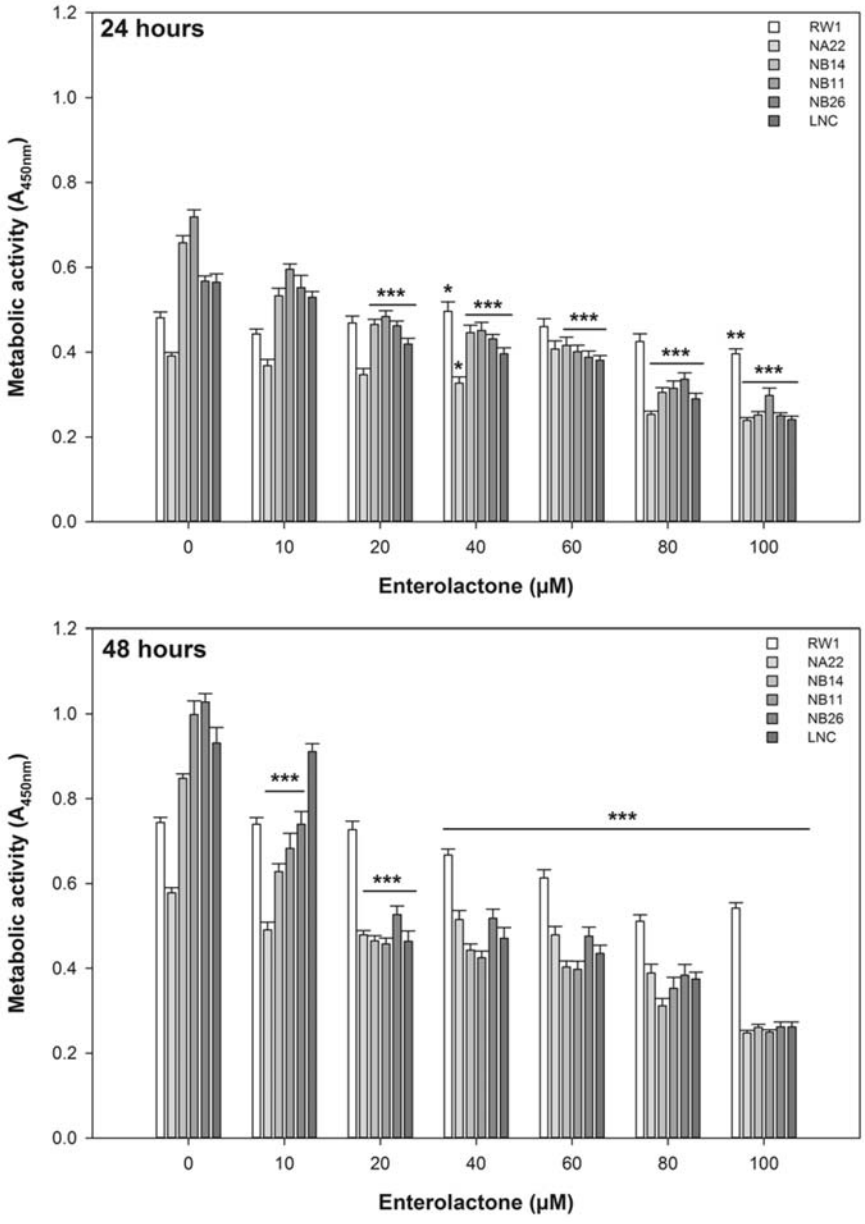

Figure 1. The effect of ENL on the metabolic activity of prostate cell lines over 48 h. The data are expressed as the mean absorbance ±SEM (n = 24). A statistical difference between untreated and treated samples is indicated by * ($p < 0.05$), ** ($p < 0.01$), or *** ($p < 0.002$).

The growth kinetics, based on the time for the population to double in number, of the RWPE-1 and WPE1-NA22 cell lines were unaltered by ENL. The positive control, 20 μM etoposide, significantly ($p < 0.018$) decreased the metabolic activity and increased in the doubling time of the cell lines over 48 h. The WPE1-NB14, WPE1-NB11, and WPE1-NB26 cell lines were the most sensitive to the ENL-induced

increased doubling time (*i.e.*, slower growth) of these cell lines. The doubling time of the LNCaP cell line was only affected by 20 μM ENL.

As 20 μM ENL was the lowest concentration that affected both the metabolic activity and doubling times of the WPE1 and LNCaP cell lines, but not the RWPE-1 cell line (the least diseased cell line in our model and an approximation of a "normal" cell line) this concentration was selected for further study.

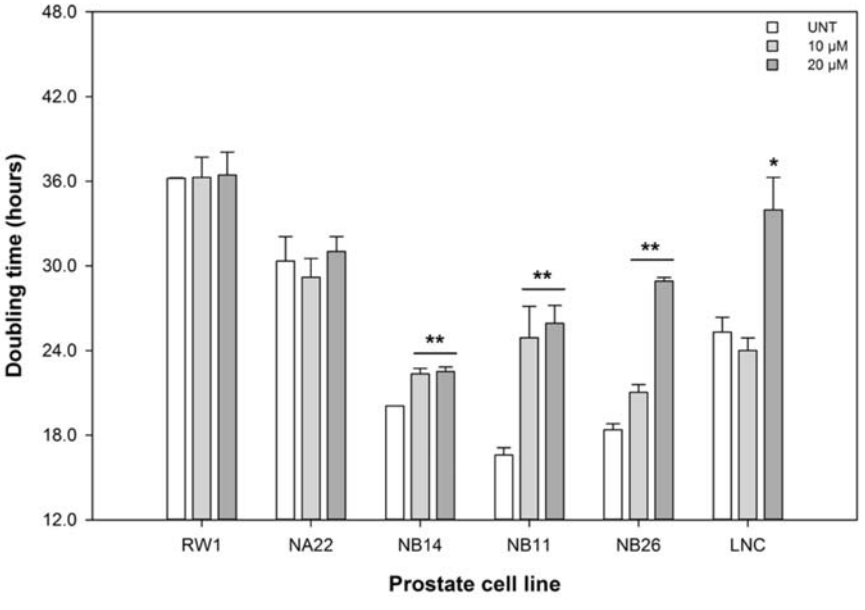

Figure 2. The effect of ENL on the doubling times of prostate cell lines over 48 h. The data are expressed as the mean doubling time ±SEM ($n = 3$). A statistical difference between untreated (UNT) and treated samples is indicated by * ($p < 0.05$), or ** ($p < 0.01$).

3.2. ENL Restricts the Cell Cycle of and Induces Apoptosis in Mid to Later Stage Prostate Disease Cell Lines

The restriction of cell cycle progression in the cell lines with 20 μM ENL over 48 h is shown in Figure 3 to Figure 4. The positive control, 20 μM etoposide, significantly increased the S-phase and level of apoptosis of the cell lines over 48 h ($p < 0.014$).

At 24 h, there was an increase in the percentage of cells in the G_0/G_1 phase of the cell cycle for the RWPE-1, WPE1-NB14, WPE1-NB11, WPE1-NB26, and LNCaP cell lines in response to 20 μM ENL. For the LNCaP cell line there was also decrease in the percentage of cells in the G_2/M phase. At 48 h, the cell cycle of the WPE1-NB14 and WPE1-NB11 cell lines remained altered (NB14: increased G_0/G_1, decreased S, and NB11: decreased G_0/G_1, increased S, decreased G_2/M) by ENL. The G_0/G_1 and G_2/M phases of the LNCaP cell line were also restricted, both reduced, after 48 h.

The data in Figures 3 and 4 also show that 20 μM ENL induces apoptosis in the WPE1-NB14, WPE1-NB11, and WPE1-NB26 after 24 and 48 h. At 48 h, the WPE1-NA22 and LNCaP cell lines also had increased levels of apoptosis in response to ENL.

These data indicate that the disrupted viability (metabolic activity and doubling times), shown in Figures 1 and 2, of the cell lines is due, in part, to alterations in cell cycling and cell death. Given the alterations shown Figures 3 and 4, the effect of 20 μM ENL on the expression of genes involved in two key pathways during abnormal growth and carcinogenesis was quantified to explore potential mechanisms of action.

3.3. ENL Alters the Expression of DNA Licencing Genes in Mid to Later Stage Prostate Disease Cell Lines

The expression of the DNA licencing genes in response to 20 μM ENL by the six cell lines are shown in Figure 5. The co-efficient of variation for the HPRT1 reference gene amongst the untreated cell lines was 9% and 5%, at 24 and 48 h respectively. These data show that the expression of GMNN (CDT1 inhibitor) is increased approximately 2 to 3 fold in the WPE1-NB14 and WPE1-NB11 cell lines after 24 and 48 h. The expression of CDT1 was reduced in these cell lines by approximately 2 fold. The expression of the MCM2 and 7 genes was reduced in the majority of cell lines at 24 h, but only in the WPE1-NA2, WPE1-NB14, and WPE1-NB11 cell lines at 48 h. These changes in expression imply that the licencing of DNA for replication is reduced and would results in cell cycle restrictions (particularly in the G_0/G_1 and S phases), reduced proliferation, and/or increased cell death.

The reduced expression of MCM7 suggests that the miR-106b cluster (located in one of the introns of MCM7) may also be influenced by ENL and if so this may affect the expression of the PTEN gene.

3.4. ENL Alters the Expression of the miR-106b Cluster Leading to Increased PTEN Expression

The expression of the miR-106b cluster and PTEN genes in response to 20 μM ENL by the six cell lines are shown in Figure 6. The co-efficient of variation for the rnu6b reference gene in the untreated cell lines was 2.3% and 2%, at 24 and 48 h respectively. These data show that the expression of miR-106b, miR-93, and miR-25 are decreased in the WPE1-NB14, WPE1-NB11, WPE1-NB26, and LNCaP cell lines after 24 and 48 h. The expression of PTEN is substantially increased in the WPE1 and LNCaP cell lines.

These data suggest that the repression of the miR-106b cluster leads, in part, to increased PTEN expression. However, the expression of PTEN was increased by ENL in the WPE1-NA22 cell line despite no substantial change in the expression of the miR-106b cluster.

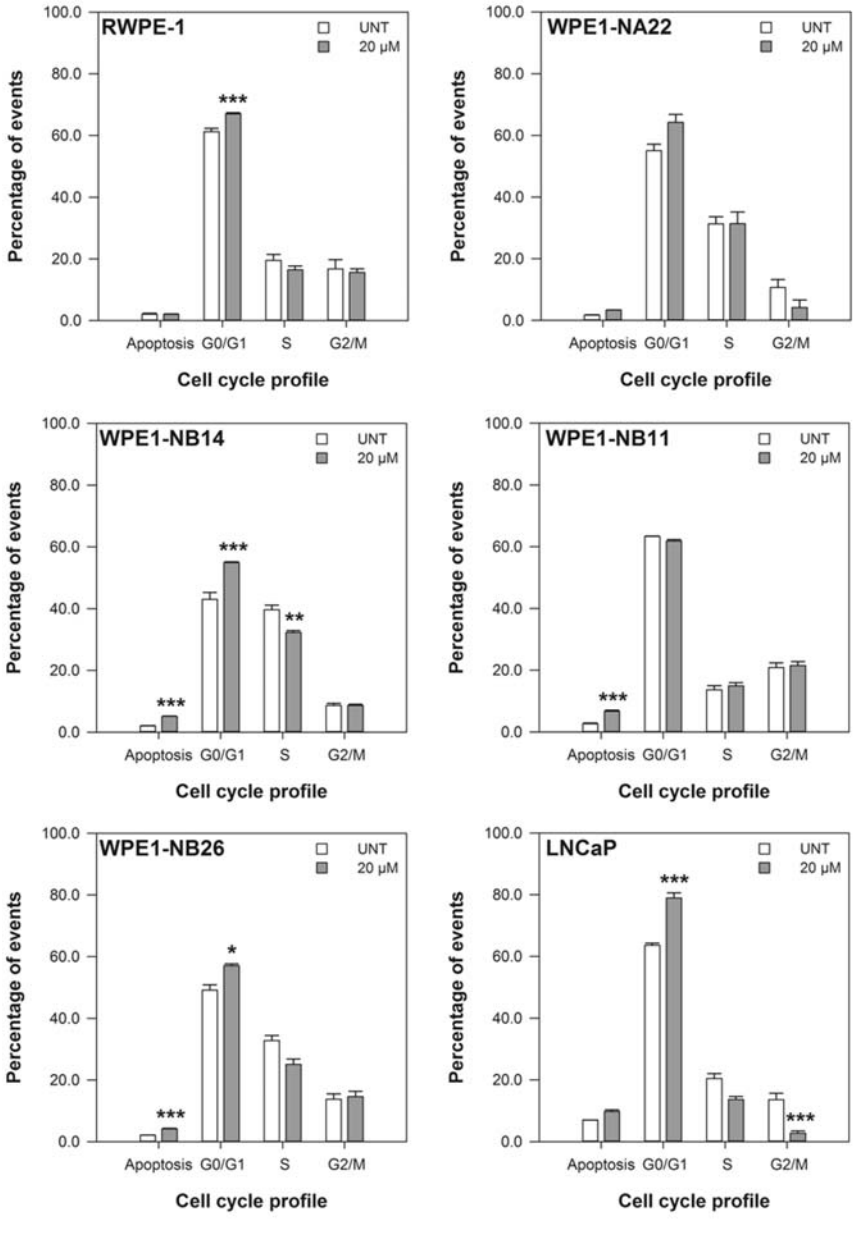

Figure 3. The effect of 20 μM ENL on the cell cycle profile of prostate cell lines after 24 h. The data are expressed as the mean percentage of events in each phase ±SEM ($n = 3$). A statistical difference between untreated and treated samples is indicated by * ($p < 0.05$), ** ($p < 0.01$), or *** ($p < 0.002$).

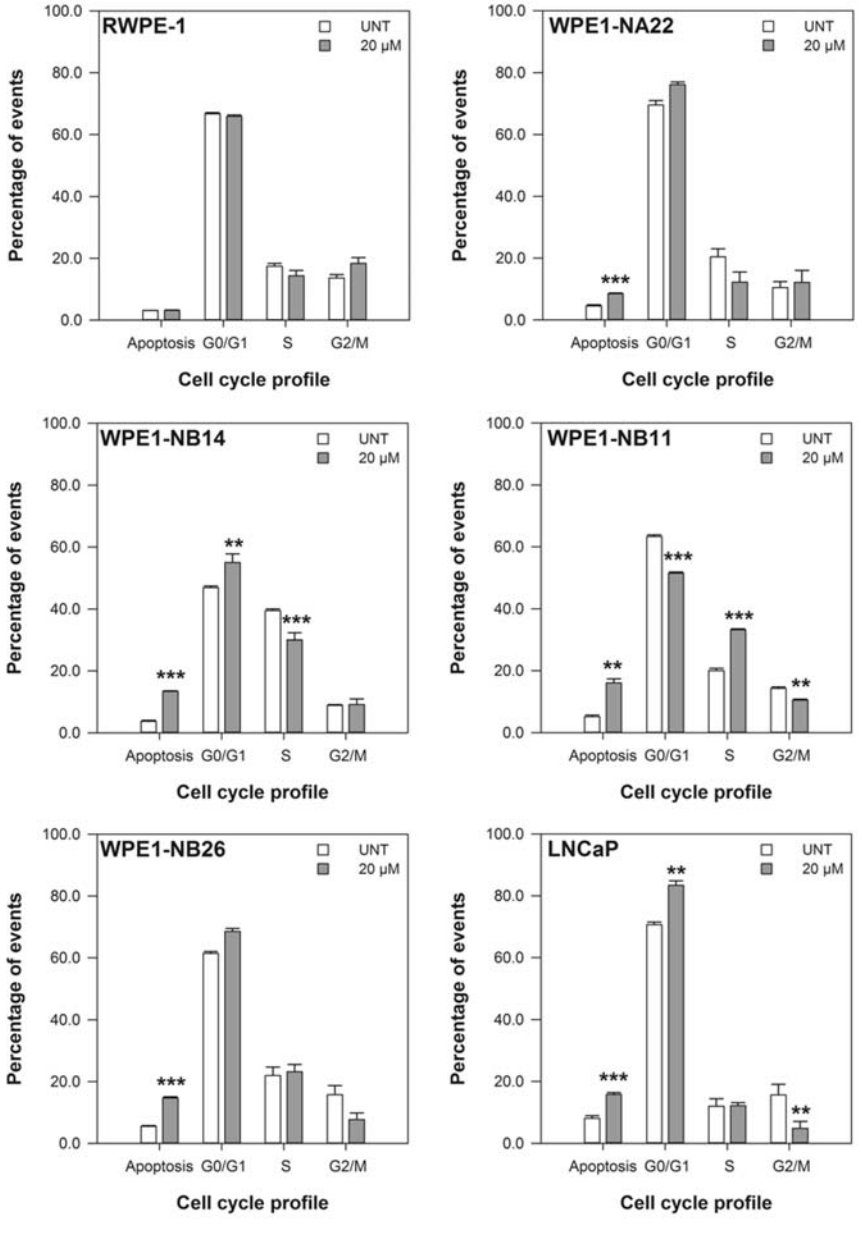

Figure 4. The effect of 20 μM ENL on the cell cycle profile of prostate cell lines after 48 h. The data are expressed as the mean percentage of events in each phase ±SEM ($n = 3$). A statistical difference between untreated and treated samples is indicated by * ($p < 0.05$), ** ($p < 0.01$), or *** ($p < 0.002$).

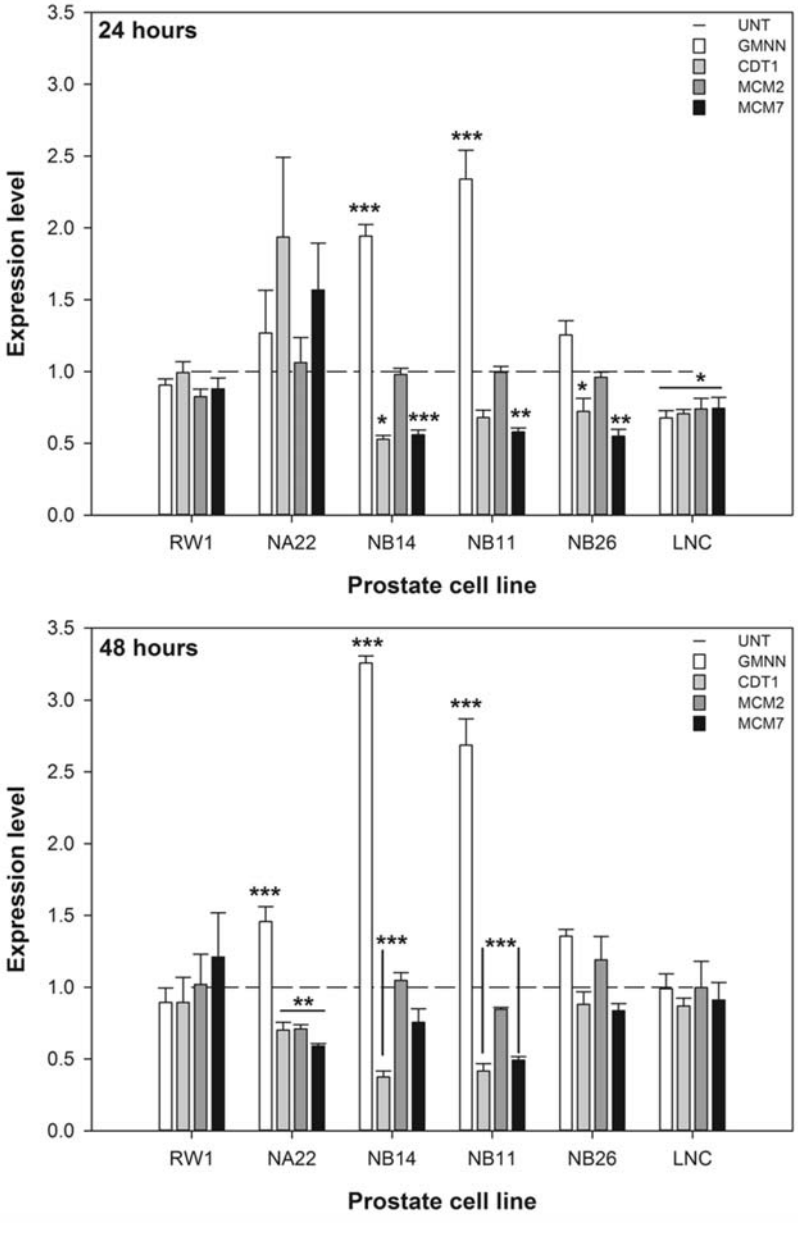

Figure 5. The effect of 20 μM ENL on the expression of DNA licencing genes by prostate cell lines over 48 h. The data are expressed as the mean expression level ±SEM ($n = 3$). For each cell line, a difference between untreated and treated samples is indicated by * ($p < 0.05$), ** ($p < 0.01$), or *** ($p < 0.002$).

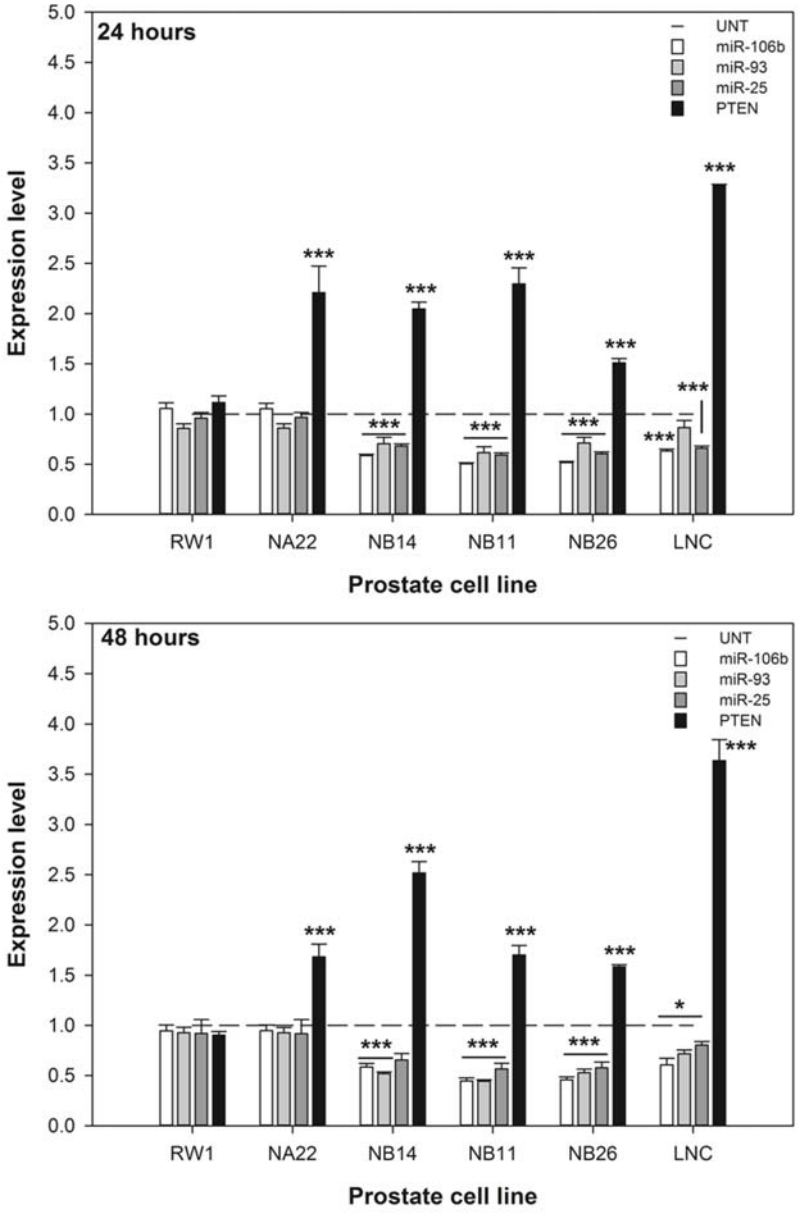

Figure 6. The effect of ENL on the expression of the PTEN gene and miR-106b cluster genes by prostate cell lines over 48 h. The data are expressed as the mean expression level ±SEM ($n = 3$). For each cell line, a difference between untreated and treated samples is indicated by * ($p < 0.05$), or *** ($p < 0.002$).

4. Discussion

The present study provides further evidence that a pure mammalian lignan inhibits the *in vitro* proliferation of prostate cell lines. To our knowledge, this is the first study to examine the correlation

between the expression of genes associated with DNA licencing and miR-106b cluster mediated PTEN and biological end-points of proliferation *in vitro*.

Previous *in vitro* studies examining how the prostate cell lines respond to ENL have reported anti-proliferative effects for concentrations ranging from 25 to 100 μM and our data are in agreement with these findings [18,20]. In contrast to the previous studies we have studied the effects of ENL on the proliferation of a range of prostate cell models, rather than the late-stage models generally used, and explored potential mechanisms of action. We have shown that ENL at 20 μM over 48 h is sufficient to restrict the proliferation of primarily mid to later stage prostate cancer cells without any effects on the approximately "normal" RWPE-1 cell line. However, this cell line is immortalised and is not truly normal. Additionally we have shown that the anti-proliferative effects of ENL are strongly associated with: (1) improved negative regulation of abnormal DNA licencing (increased GMNN expression and decreased CDT1 expression); and (2) inhibition of miR-106b cluster expression leading to increased expression of the tumour suppressive gene PTEN.

The ENL-induced changes in genes required for DNA replication initiation may explain the effects of ENL on cell cycle control and consequently proliferation in the prostate cell lines. However, the effect of altering the GMNN/CDT1 balance in tumourigenic cells (which express higher levels of these genes compared to normal cells [31]) is unclear as there is debate about how the GMNN/CDT1 balance influences the development and progression of cancer [26–28,39,40]. Additionally we have also shown that the expression of MCM2 and MCM7 is reduced by ENL in the prostate cell lines used, and this may be linked altered CDT1 expression as CDT1 is required for the loading of the MCM complex during the initiation of DNA replication [24]. MCM7, in particular, is known to be oncogenic [25,29,41] not only for its role in DNA licencing, but also due to other interactions such as: (1) MCM7 overexpression can inhibit the retinoblastoma-controlled cell G_1/S cell cycle block [42,43]; (2) MCM7 interacts with the androgen receptor [44], appropriate androgen signalling and the consequences of androgen insensitivity are key factors in prostate carcinogenesis and relapse [45,46]; and (3) one of the introns of MCM7 contains the mir-106b cluster [35] which targets two key tumour suppressor genes implicated in the prostate cancer, PTEN and CDKN1A (p21) [47–49].

Reduced PTEN expression can contribute to cancer development due to decreased negative regulation of the PI3K/AKT pathway (known as quasi-sufficiency) [50]. Unlike the classic "two-hit" model of tumour suppression, it is the amount of functional PTEN (which can be affected by several factors not just transcription) that determines its tumour suppressive capacity. We have shown that ENL can increase PTEN expression, which may restore the appropriate regulation by PTEN of the PI3K/AKT pathway. However, the LNCaP cell line has a mutated and non-functional PTEN gene [32] and therefore the consequence of its increased expression by ENL is unclear.

The concentrations used in this study have yet to be shown to be achievable in the prostate *in vivo* either through dietary or pharmacological intervention. We also have only demonstrated a link between gene expression and proliferation markers—further work is needed to establish whether the expression changes result in functional changes at the post-transcriptional level. We have shown that there appears to be a relationship between ENL-mediated expression of the PTEN gene, perhaps via suppression of the miR-106b cluster, and proliferation in prostate cancer cell lines. If these are confirmed at the proteomic and functional level, it may represent a novel mechanism for the anti-proliferative activity of ENL.

5. Conclusions

In conclusion we have provided evidence for the anti-proliferative effects of ENL in mid and late prostate cell lines, and have shown that changes in the transcription of DNA licencing, miR-106b cluster, and PTEN genes may be involved in these effects. This is important as we have shown that ENL is effective in earlier stages of prostate cancer than previously reported and two important pathways in prostate tumourigenesis are linked through miRNA effects.

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Author Contributions: Mark J. McCann wrote the manuscript and completed the experimental work. All of the authors reviewed the manuscript and contributed to the scientific content of this paper.

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References

1. Jemal, A.; Bray, F.; Center, M.M.; Ferlay, J.; Ward, E.; Forman, D. Global cancer statistics. *CA Cancer J. Clin.* **2011**, *61*, 69–90. [CrossRef]
2. Center, M.M.; Jemal, A.; Lortet-Tieulent, J.; Ward, E.; Ferlay, J.; Brawley, O.; Bray, F. International variation in prostate cancer incidence and mortality rates. *Eur. Urol.* **2012**, *61*, 1079–1092. [CrossRef] [PubMed]
3. Ferlay, J.; Shin, H.R.; Bray, F.; Forman, D.; Mathers, C.; Parkin, D.M. Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. *Int. J. Cancer* **2010**, *127*, 2893–2917. [CrossRef] [PubMed]
4. Baade, P.D.; Youlden, D.R.; Krnjacki, L.J. International epidemiology of prostate cancer: Geographical distribution and secular trends. *Mol. Nutr. Food Res.* **2009**, *53*, 171–184. [CrossRef] [PubMed]
5. Cullen, J.; Elsamanoudi, S.; Brassell, S.A.; Chen, Y.; Colombo, M.; Srivastava, A.; McLeod, D.G. The burden of prostate cancer in Asian nations. *J. Carcinog.* **2012**, *11*, 7. [CrossRef] [PubMed]
6. Venkateswaran, V.; Klotz, L.H. Diet and prostate cancer: Mechanisms of action and implications for chemoprevention. *Nat. Rev. Urol.* **2010**, *7*, 442–453. [CrossRef] [PubMed]
7. Khan, N.; Afaq, F.; Mukhtar, H. Lifestyle as risk factor for cancer: Evidence from human studies. *Cancer Lett.* **2010**, *293*, 133–143. [CrossRef] [PubMed]
8. Muller, D.C.; Severi, G.; Baglietto, L.; Krishnan, K.; English, D.R.; Hopper, J.L.; Giles, G.G. Dietary patterns and prostate cancer risk. *Cancer Epidemiol. Biomark. Prev.* **2009**, *18*, 3126–3129. [CrossRef]
9. Saarinen, N.M.; Tuominen, J.; Pylkkanen, L.; Santti, R. Assessment of information to substantiate a health claim on the prevention of prostate cancer by lignans. *Nutrients* **2010**, *2*, 99–115. [CrossRef] [PubMed]
10. Adlercreutz, H. Lignans and human health. *Crit. Rev. Clin. Lab. Sci.* **2007**, *44*, 483–525. [CrossRef] [PubMed]
11. McCann, M.J.; Gill, C.I.; McGlynn, H.; Rowland, I.R. Role of mammalian lignans in the prevention and treatment of prostate cancer. *Nutr. Cancer* **2005**, *52*, 1–14. [CrossRef] [PubMed]
12. Kuhnle, G.G.; Dell'Aquila, C.; Aspinall, S.M.; Runswick, S.A.; Mulligan, A.A.; Bingham, S.A. Phytoestrogen content of foods of animal origin: Dairy products, eggs, meat, fish, and seafood. *J. Agric. Food Chem.* **2008**, *56*, 10099–10104. [CrossRef] [PubMed]
13. Setchell, K.D.; Lawson, A.M.; Mitchell, F.L.; Adlercreutz, H.; Kirk, D.N.; Axelson, M. Lignans in man and in animal species. *Nature* **1980**, *287*, 740–742. [CrossRef] [PubMed]
14. Woting, A.; Clavel, T.; Loh, G.; Blaut, M. Bacterial transformation of dietary lignans in gnotobiotic rats. *FEMS Microbiol. Ecol.* **2010**, *72*, 507–514. [CrossRef] [PubMed]
15. Clavel, T.; Borrmann, D.; Braune, A.; Dore, J.; Blaut, M. Occurrence and activity of human intestinal bacteria involved in the conversion of dietary lignans. *Anaerobe* **2006**, *12*, 140–147. [CrossRef] [PubMed]
16. Clavel, T.; Henderson, G.; Engst, W.; Dore, J.; Blaut, M. Phylogeny of human intestinal bacteria that activate the dietary lignan secoisolariciresinol diglucoside. *FEMS Microbiol. Ecol.* **2006**, *55*, 471–478. [CrossRef] [PubMed]
17. Saarinen, N.M.; Thompson, L.U. Prolonged administration of secoisolariciresinol diglycoside increases lignan excretion and alters lignan tissue distribution in adult male and female rats. *Br. J. Nutr.* **2010**, *104*, 833–841. [CrossRef] [PubMed]
18. McCann, M.J.; Gill, C.I.; Linton, T.; Berrar, D.; McGlynn, H.; Rowland, I.R. Enterolactone restricts the proliferation of the LNCaP human prostate cancer cell line *in vitro*. *Mol. Nutr. Food Res.* **2008**, *52*, 567–580. [CrossRef] [PubMed]
19. McCann, M.J.; Rowland, I.R.; Roy, N.C. Anti-proliferative effects of physiological concentrations of enterolactone in models of prostate tumourigenesis. *Mol. Nutr. Food Res.* **2013**, *57*, 212–224. [CrossRef] [PubMed]
20. Chen, L.H.; Fang, J.; Li, H.; Demark-Wahnefried, W.; Lin, X. Enterolactone induces apoptosis in human prostate carcinoma LNCaP cells via a mitochondrial-mediated, caspase-dependent pathway. *Mol. Cancer Ther.* **2007**, *6*, 2581–2590. [CrossRef] [PubMed]

21. Lin, X.; Switzer, B.R.; Demark-Wahnefried, W. Effect of mammalian lignans on the growth of prostate cancer cell lines. *Anticancer Res.* **2001**, *21*, 3995–3999. [PubMed]
22. Morton, M.S.; Chan, P.S.; Cheng, C.; Blacklock, N.; Matos-Ferreira, A.; Abranches-Monteiro, L.; Correia, R.; Lloyd, S.; Griffiths, K. Lignans and isoflavonoids in plasma and prostatic fluid in men: Samples from Portugal, Hong Kong, and the United Kingdom. *Prostate* **1997**, *32*, 122–128. [CrossRef] [PubMed]
23. Adlercreutz, H.; Bannwart, C.; Wahala, K.; Makela, T.; Brunow, G.; Hase, T.; Arosemena, P.J.; Kellis, J.T., Jr.; Vickery, L.E. Inhibition of human aromatase by mammalian lignans and isoflavonoid phytoestrogens. *J. Steroid Biochem. Mol. Biol.* **1993**, *44*, 147–153. [CrossRef] [PubMed]
24. Masai, H.; Matsumoto, S.; You, Z.; Yoshizawa-Sugata, N.; Oda, M. Eukaryotic chromosome DNA replication: Where, when, and how? *Annu. Rev. Biochem.* **2010**, *79*, 89–130. [CrossRef]
25. Luo, J.H. Oncogenic activity of MCM7 transforming cluster. *World J. Clin. Oncol.* **2011**, *2*, 120–124. [CrossRef] [PubMed]
26. Hook, S.S.; Lin, J.J.; Dutta, A. Mechanisms to control rereplication and implications for cancer. *Curr. Opin. Cell Biol.* **2007**, *19*, 663–671. [CrossRef] [PubMed]
27. Lau, E.; Tsuji, T.; Guo, L.; Lu, S.H.; Jiang, W. The role of pre-replicative complex (pre-RC) components in oncogenesis. *FASEB J.* **2007**, *21*, 3786–3794. [CrossRef] [PubMed]
28. Montanari, M.; Macaluso, M.; Cittadini, A.; Giordano, A. Role of geminin: from normal control of DNA replication to cancer formation and progression? *Cell Death Differ.* **2006**, *13*, 1052–1056. [CrossRef] [PubMed]
29. Ren, B.; Yu, G.; Tseng, G.C.; Cieply, K.; Gavel, T.; Nelson, J.; Michalopoulos, G.; Yu, Y.P.; Luo, J.H. MCM7 amplification and overexpression are associated with prostate cancer progression. *Oncogene* **2006**, *25*, 1090–1098. [CrossRef] [PubMed]
30. Honeycutt, K.A.; Chen, Z.; Koster, M.I.; Miers, M.; Nuchtern, J.; Hicks, J.; Roop, D.R.; Shohet, J.M. Deregulated minichromosomal maintenance protein MCM7 contributes to oncogene driven tumorigenesis. *Oncogene* **2006**, *25*, 4027–4032. [CrossRef] [PubMed]
31. Xouri, G.; Lygerou, Z.; Nishitani, H.; Pachnis, V.; Nurse, P.; Taraviras, S. Cdt1 and geminin are down-regulated upon cell cycle exit and are over-expressed in cancer-derived cell lines. *Eur. J. Biochem.* **2004**, *271*, 3368–3378. [CrossRef] [PubMed]
32. Chen, Z.; Trotman, L.C.; Shaffer, D.; Lin, H.K.; Dotan, Z.A.; Niki, M.; Koutcher, J.A.; Scher, H.I.; Ludwig, T.; Gerald, W.; *et al.* Crucial role of p53-dependent cellular senescence in suppression of Pten-deficient tumorigenesis. *Nature* **2005**, *436*, 725–730. [CrossRef] [PubMed]
33. Pourmand, G.; Ziaee, A.A.; Abedi, A.R.; Mehrsai, A.; Alavi, H.A.; Ahmadi, A.; Saadati, H.R. Role of PTEN gene in progression of prostate cancer. *Urol. J.* **2007**, *4*, 95–100. [PubMed]
34. Vlietstra, R.J.; van Alewijk, D.C.; Hermans, K.G.; van Steenbrugge, G.J.; Trapman, J. Frequent inactivation of PTEN in prostate cancer cell lines and xenografts. *Cancer Res.* **1998**, *58*, 2720–2723. [PubMed]
35. Poliseno, L.; Salmena, L.; Riccardi, L.; Fornari, A.; Song, M.S.; Hobbs, R.M.; Sportoletti, P.; Varmeh, S.; Egia, A.; Fedele, G.; *et al.* Identification of the miR-106b~25 microRNA cluster as a proto-oncogenic PTEN-targeting intron that cooperates with its host gene MCM7 in transformation. *Sci. Signal.* **2010**, *3*, ra29. [CrossRef] [PubMed]
36. Webber, M.M.; Quader, S.T.; Kleinman, H.K.; Bello-DeOcampo, D.; Storto, P.D.; Bice, G.; DeMendonca-Calaca, W.; Williams, D.E. Human cell lines as an *in vitro/in vivo* model for prostate carcinogenesis and progression. *Prostate* **2001**, *47*, 1–13. [CrossRef] [PubMed]
37. Horoszewicz, J.S.; Leong, S.S.; Chu, T.M.; Wajsman, Z.L.; Friedman, M.; Papsidero, L.; Kim, U.; Chai, L.S.; Kakati, S.; Arya, S.K.; *et al.* The LNCaP cell line—A new model for studies on human prostatic carcinoma. *Prog. Clin. Biol. Res.* **1980**, *37*, 115–132. [PubMed]
38. Van Engeland, M.; Nieland, L.J.; Ramaekers, F.C.; Schutte, B.; Reutelingsperger, C.P. Annexin V-affinity assay: A review on an apoptosis detection system based on phosphatidylserine exposure. *Cytometry* **1998**, *31*, 1–9. [CrossRef] [PubMed]
39. Arentson, E.; Faloon, P.; Seo, J.; Moon, E.; Studts, J.M.; Fremont, D.H.; Choi, K. Oncogenic potential of the DNA replication licensing protein CDT1. *Oncogene* **2002**, *21*, 1150–1158. [CrossRef] [PubMed]
40. Blow, J.J.; Gillespie, P.J. Replication licensing and cancer—A fatal entanglement? *Nat. Rev. Cancer* **2008**, *8*, 799–806. [CrossRef]
41. Lei, M. The MCM complex: Its role in DNA replication and implications for cancer therapy. *Curr. Cancer Drug Targets* **2005**, *5*, 365–380. [CrossRef] [PubMed]

42. Sterner, J.M.; Dew-Knight, S.; Musahl, C.; Kornbluth, S.; Horowitz, J.M. Negative regulation of DNA replication by the retinoblastoma protein is mediated by its association with MCM7. *Mol. Cell. Biol.* **1998**, *18*, 2748–2757. [PubMed]
43. Mukherjee, P.; Winter, S.L.; Alexandrow, M.G. Cell cycle arrest by transforming growth factor beta1 near G1/S is mediated by acute abrogation of prereplication complex activation involving an Rb-MCM interaction. *Mol. Cell. Biol.* **2010**, *30*, 845–856. [CrossRef] [PubMed]
44. Shi, Y.K.; Yu, Y.P.; Zhu, Z.H.; Han, Y.C.; Ren, B.; Nelson, J.B.; Luo, J.H. MCM7 interacts with androgen receptor. *Am. J. Pathol.* **2008**, *173*, 1758–1767. [CrossRef] [PubMed]
45. Evans, C.P.; Lara, P.N., Jr. Prostate cancer: Predicting response to androgen receptor signalling inhibition. *Nat. Rev. Urol.* **2014**, *11*, 433–435. [CrossRef] [PubMed]
46. Balk, S.P. Androgen receptor functions in prostate cancer development and progression. *Asian J. Androl.* **2014**, *16*, 561–564. [CrossRef] [PubMed]
47. Song, M.S.; Salmena, L.; Pandolfi, P.P. The functions and regulation of the PTEN tumour suppressor. *Nat. Rev. Mol. Cell Biol.* **2012**, *13*, 283–296. [PubMed]
48. Sarker, D.; Reid, A.H.; Yap, T.A.; de Bono, J.S. Targeting the PI3K/AKT pathway for the treatment of prostate cancer. *Clin. Cancer Res.* **2009**, *15*, 4799–4805. [CrossRef] [PubMed]
49. Abbas, T.; Dutta, A. p21 in cancer: Intricate networks and multiple activities. *Nat. Rev. Cancer* **2009**, *9*, 400–414. [CrossRef] [PubMed]
50. Berger, A.H.; Knudson, A.G.; Pandolfi, P.P. A continuum model for tumour suppression. *Nature* **2011**, *476*, 163–169. [CrossRef] [PubMed]

nutrients

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Review

The Role of *n*-3 Polyunsaturated Fatty Acids in the Prevention and Treatment of Breast Cancer

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Abstract: Breast cancer (BC) is the most common cancer among women worldwide. Dietary fatty acids, especially *n*-3 polyunsaturated fatty acids (PUFA), are believed to play a role in reducing BC risk. Evidence has shown that fish consumption or intake of long-chain *n*-3 PUFA, such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), are beneficial for inhibiting mammary carcinogenesis. The evidence regarding α-linolenic acid (ALA), however, remains equivocal. It is essential to clarify the relation between ALA and cancer since ALA is the principal source of *n*-3 PUFA in the Western diet and the conversion of ALA to EPA and DHA is not efficient in humans. In addition, the specific anticancer roles of individual *n*-3 PUFA, alone, have not yet been identified. Therefore, the present review evaluates ALA, EPA and DHA consumed individually as well as in *n*-3 PUFA mixtures. Also, their role in the prevention of BC and potential anticancer mechanisms of action are examined. Overall, this review suggests that each *n*-3 PUFA has promising anticancer effects and warrants further research.

Keywords: *n*-3 polyunsaturated fatty acids (PUFA); eicosapentaenoic acid (EPA); docosahexaenoic acid (DHA); α-linolenic acid (ALA); mammary gland; breast cancer (BC)

1. Introduction

Breast cancer (BC) is a major health problem among women worldwide, and is the second leading cause of death for women in Canada and the United States [1,2]. On average, 65 Canadian women will be diagnosed with BC per day, with 1 in 9 females expected to develop BC in their lifetime [1,2]. Both genetic and environmental factors are believed to play a role in a woman's risk of developing BC [3,4]. Most anticancer drugs, developed to date, aim to kill cancer cells and decrease tumor burden but are relatively ineffective against some phases of tumorigenesis [5,6]. Thus, alternate strategies to prevent tumorigenesis are urgently required. In the past few decades, epidemiological studies have suggested that a healthy diet and lifestyle are critical for the prevention of BC. Dietary fatty acids are one of the most intensively studied dietary factors [7–9].

Saturated fatty acids (SFA), monounsaturated fatty acids (MUFA) and trans fatty acids (TFA) have been found to increase cancer risk; while specific polyunsaturated fatty acids (PUFA) are indicated to have anticancer effects [9,10]. There are two major classes of PUFA: *n*-6 PUFA and *n*-3 PUFA. In mammals, *n*-6 and *n*-3 PUFA are both essential fatty acids for health and must be consumed as part of the diet because they cannot be endogenously synthesized [11]. Linoleic acid (LA, 18:2*n*-6) and arachidonic acid (AA, 20:4*n*-6) are the two most common *n*-6 PUFA in typical Western diets; LA can be found in some plant oils such as corn and safflower oils, and AA usually comes from dietary animal sources or can be synthesized from LA [12,13]. α-linolenic acid (ALA, 18:3*n*-3) is the precursor of the *n*-3 PUFA family which can be further elongated and desaturated to two important long chain *n*-3

PUFA, eicosapentaenoic acid (EPA, 20:5*n*-3) and docosahexaenoic acid (DHA, 22:6*n*-3) [11]. ALA is a plant-derived *n*-3 PUFA, which is present in flaxseed, canola and soybean oils [14]. The longer chain *n*-3 PUFA, EPA and DHA can be obtained directly from marine sources such as seafood and fish oils, and are widely known for their cardioprotective benefit [13]. ALA is the major *n*-3 PUFA consumed in the Western diet, whereas intakes of EPA and DHA are typically low. It is estimated that the typical North American diet provides approximately 1.4 g of ALA and 0.1–0.2 g of EPA plus DHA per day [15]. With regard to dietary reference intake of *n*-3 PUFA, the Institute of Medicine (IOM) recommends since 2005 a daily intake 1.1 g ALA for women and 1.6 g ALA for men to prevent some chronic diseases, and up to ten percent of this can be consumed as EPA and/or DHA [16]. While current intakes meet IOM recommendations, in a 2014 report by the Academy of Nutrition and Dietetics, 500 mg EPA plus DHA per day is required for the general healthy adult population [17].

Results from both *in vivo* and *in vitro* studies suggest that *n*-6 PUFA accelerate tumorigenesis, in contrast, *n*-3 PUFA may have anticancer effects [18–22]. Western diets are typically deficient in *n*-3 PUFA and high in *n*-6 PUFA compared with traditional Asian diets [8,23,24]. Migration studies have shown that Asian women, who typically have a lower rate of BC and higher fish consumption exhibited an increased incidence of BC within one generation after migration to the Western countries [24,25]. Historically, the intake of *n*-6 and *n*-3 PUFA has been estimated to be approximately equal. However, in recent years, the content of Western diets has significantly increased in *n*-6 PUFA resulting in an increase in the *n*-6/*n*-3 PUFA ratio [8]. Excessive amounts of *n*-6 PUFA and a very high *n*-6/*n*-3 ratio (16:1 or higher), as is currently found in Western diets, have been suggested to promote the pathogenesis of many diseases such as cardiovascular disease, autoimmune diseases and some types of cancer; whereas increased levels of *n*-3 PUFA (a low *n*-6/*n*-3 ratio) have been shown to exert suppressive effects [8]. However, it remains to be resolved whether it is simply the reduced *n*-3 PUFA or the changing *n*-6/*n*-3 ratio that is relevant to these outcomes.

Substantial evidence from cell culture and rodent studies indicate that increased fish consumption or intake of *n*-3 PUFA inhibits BC cell proliferation and reduces BC risk relative to *n*-6 PUFA [26–30]. Nevertheless, there has been longstanding controversy in epidemiological and observational studies regarding the potential anticancer effects of *n*-3 PUFA due to the inability to show causality. Previous published reviews have focused on the role of fish and marine *n*-3 fatty acids in BC prevention, whereas the evidence for ALA and individual effects of long chain *n*-3 PUFA is lacking [3,8,11,23,31–34]. Since a typical North American diet is mainly comprised of ALA as the source of *n*-3 PUFA, it is necessary to elucidate the specific effects of ALA and cancer risk. Therefore, the purpose of the present review is to evaluate the preventative role of ALA, EPA and DHA in BC development when consumed individually, as well as in *n*-3 PUFA mixtures through dietary and supplemental forms. In addition, the potential mechanisms by which they exert anticancer effects will also be discussed.

2. The Effects of *n*-3 PUFA in Human BC Studies

Dietary *n*-3 PUFA may influence breast cancer (BC) progression and prognosis. In the past ten years, six prospective cohort studies (Table 1) and nine case-control studies (Table 2) have examined the association between the consumption of either fish or fish oil supplements and BC risk, showing a protective effect of *n*-3 PUFA. These studies were conducted in many different geographic areas with mixed findings. In general, Asian populations with a low total fat intake and high fish consumption, associated *n*-3 PUFA intake with a reduced risk of BC [35–39]. There was also a weak association with reduced BC risk in US studies involving women whose diets had higher *n*-6 PUFA content combined with fish oil supplements [18,40,41]. European studies were less consistent and somewhat contradictory [42–45].

In the Japan Collaborative Cohort (JACC) study, a significant decrease in the risk of BC was detected in women with the highest dietary intake of fish fat and the long-chain *n*-3 PUFA [35]. Similar results were observed in a large prospective study of 35,298 Singapore women, indicating an inverse association between dietary *n*-3 PUFA from marine sources and BC risk [36]. Relative to the lowest

quartile of *n*-3 PUFA intake, individuals in the top three quartiles exhibited a 26% reduction in BC risk (relative risk = 0.74; 95% confidence interval = 0.58–0.94). Additionally, a recent analysis from the VITamins And Lifestyle (VITAL) cohort carried out in US indicated that the current use of fish oil supplements was associated with a decreased risk of localized invasive ductal carcinomas in postmenopausal women [40].

Table 1. *n*-3 PUFA and breast cancer risk: Prospective cohort studies.

Year	Country	Subjects	Method of Assessment	*n*-3/*n*-6 PUFA Source	BC Risk	Reference
2005	Japan	26,291 women 40–79 years 129 BC cases	FFQ [1]	Animal and fish fat, vegetable oil, SFA, MUFA and PUFA	↑ fish fat, EPA + DHA ↓ BC risk	[35]
2003	Singapore	35,298 women 45–74 years 342 BC cases	FFQ	Fish/shellfish, saturated, monounsaturated and polyunsaturated fat	↑ *n*-3 PUFA from fish/shellfish ↓ BC risk ↑ *n*-6 PUFA (low marine *n*-3) ↑ BC risk	[36]
2010	US	35,016 postmenopausal 50–76 years 880 BC cases	FFQ	Dietary fish oil supplement	↑ fish oil ↓ risk of invasive ductal carcinomas	[40]
2009	France	56,007 women 40–65 years 1650 BC case	FFQ	ALA and *n*-6 PUFA from fruit, nuts and vegetable oils; Long chain *n*-3 PUFA from meals	no association between total *n*-3 and BC risk ↑ ALA ↓BC risk ↑ long chain *n*-3 PUFA ↓ BC risk (at highest quintile of *n*-6 PUFA)	[42]
2003	Denmark	23,693 postmenopausal 50–64 years 424 BC cases	FFQ	Fish	↑ intake of fish ↑ ER + BC incidence	[43]
2011	China	72,571 women 40–70 years 712 BC cases	FFQ	Fish, marine-derived *n*-3 PUFA red meat	↑ *n*-6/*n*-3 PUFA ratio ↑ BC risk	[37]

[1] FFQ: food frequency questionnaire; ↑: increase; ↓: decrease.

Table 2. *n*-3 PUFA and breast cancer risk: Case-control studies.

Year	Country	Subjects Characteristics	Method of Assessment	*n*-3/*n*-6 PUFA Source	BC Risk	Reference
2007	Japan	103 incident BC cases 309 controls	erythrocyte membrane FFQ	dietary food intake including soy and meat products, fish and other seafood, vegetables	↑ dietary intake of *n*-3 fatty acids ↓ BC risk ↑ long chain *n*-3 PUFA in erythrocyte ↓ BC risk ↑ saturated fatty ↑ BC risk	[46]
2007	China	322 incident BC cases 1030 controls	erythrocyte membrane		↑ total *n*-3 fatty acids and EPA ↓ BC risk	[47]
2009	China	155 NPFC [1] 185 PFC [2] 241 BC, 1030 controls	erythrocyte membrane FFQ	dietary food intake	↑ EPA ↓ risk of NPFC ↓ progression of PFC to BC ↑ γ-linolenic acid ↑ risk of NPFC, PFC and BC	[38]
2002	US	73 BC patients 74 controls	breast adipose tissue		↑ EPA and DHA ↓ *n*-6/*n*-3 PUFA ratio ↓ BC risk ↑ *n*-6 PUFA ↑ BC risk	[18]
2003	US	565 incident BC 554 controls	FFQ	daily fat intake	↓ *n*-6/*n*-3 PUFA ratio ↓ BC risk (premenopausal) ↑EPA, DHA ↓ BC risk (21% and 18%, respectively)	[41]
2009	Denmark	463 BC cases 1098 controls	Gluteal adipose tissue biopsy	dietary food intake	No association between total or individual marine *n*-3 PUFA in adipose tissue and risk of BC	[44]
2002	France	241 invasive BC cases 88 controls-benign breast disease	breast adipose tissue		↑ ALA ↑ DHA ↓ *n*-6/*n*-3 PUFA ratio ↓ BC risk	[45]
2012	Mexican	1000 incident BC cases 1074 controls	Interview and FFQ	dietary food intake	↑ *n*-3 PUFA ↓ BC risk (obese women) ↑ *n*-6 PUFA ↑ BC risk (premenopausal)	[19]
2009	South Korea	358 incident BC patients 360 controls	FFQ	fatty and lean fish	↑ fatty fish consumption ↓ BC risk ↑EPA and DHA derived from fish ↓ BC risk	[39]

[1] Benign proliferative fibrocystic conditions (PFC); [2] non-proliferative fibrocystic conditions (NPFC); ↑: increase; ↓: decrease.

The association between dietary intakes of *n*-3 PUFA or fish oil supplements with overall survival was also examined. Patterson *et al.* demonstrated that women with higher intakes of EPA and DHA from food, but not from fish oil supplements, had a dose-dependent reduction in all-cause mortality [48]. They also showed a reduced risk of additional BC events of approximately 25% when compared with the lowest tertile of intake (tertile 3:hazard ratio = 0.72; 95% confidence interval = 0.57–0.90) [48]. In support of these self-reported intake studies, Zheng *et al.* performed a comprehensive analysis of 21 independent prospective cohort studies and found that marine *n*-3 PUFA were associated with a 14% risk reduction of BC, and the relative risk remained similar whether marine *n*-3 PUFA was measured as dietary intake or as tissue biomarkers [7]. Further, a dose-response analysis indicated a 5% lower risk of BC per 0.1 g/day (0.95, 0.90 to 1.00, I^2 = 52%) increment of dietary marine *n*-3 PUFA [7].

Conversely, a French study comprising over 56,000 women found no association between total *n*-3 or *n*-6 PUFA intake and BC risk [42]. Also, a large study of postmenopausal women in Denmark concluded that increased fish consumption was associated with elevated incidence rates of BC, but this association was present only for development of estrogen positive BC [43]. These null studies were mostly conducted in European populations with relatively low per capita intake of *n*-3 PUFA [32,44].

In order to examine the relationship between *n*-3 PUFA exposure and BC risk, several case-control studies have been conducted using different biomarkers (Table 2). Kuriki *et al.* investigated the fatty acid compositions of erythrocyte membranes as a biomarker and demonstrated that BC risk exhibited a significant inverse association with dietary intake of *n*-3 PUFA derived from fish and high levels of long-chain *n*-3 PUFA in erythrocyte membranes [46]. Another assessment of erythrocyte fatty acid composition found the inverse association significant only for EPA and total *n*-3 PUFA content [47]. Furthermore, Shannon *et al.* evaluated the role of *n*-3 and *n*-6 PUFA in the development of benign proliferative fibrocystic conditions (PFC) and non-proliferative fibrocystic conditions (NPFC) in the breast [38]. They showed that women in the highest quartile of erythrocyte EPA concentrations were 67% less likely to have NPFC alone or with BC, and EPA significantly lowered the risk of progressing from PFC to BC by 43% [38]. However, γ-linolenic acid (*n*-6 PUFA) was found to be positively associated with nearly all conditions [38]. These results were consistent with an earlier meta-analysis showing that total and individual *n*-3 PUFA, especially EPA and DHA, play a protective effect against BC, while total SFA, MUFA, palmitic and oleic acids were associated with increased BC risk [9].

In a Korean case control study, 358 patients with BC and 360 healthy controls underwent dietary assessment by questionnaire and interview to determine their dietary consumption of fish and *n*-3 PUFA derived from fish. Both pre- and postmenopausal women in the highest quartile of fatty-fish intake had a lower incidence of BC (odds ratio OR = 0.23, 95% CI = 0.13–0.42; $p < 0.001$), but the protective effect of EPA and/or DHA intake was only observed for postmenopausal women [39]. These findings were similarly observed in a study of Mexican women where BC risk was lower in obese women (BMI $\geq$ 30) with high *n*-3 PUFA intake, not in women of normal weight [19]. In contrast, a case-cohort study of Danish women did not find any association between either total or individual marine *n*-3 PUFA intake and BC risk [44]. As the total levels of marine *n*-3 PUFA intake were low in Europe, this may account for observed discrepancies relative to populations consuming marine-rich diets [32,44].

Other population studies have investigated interactions between *n*-3 and *n*-6 PUFA. Quantifying consumption of both types of fatty acids is an essential step in isolating *n*-3 PUFA specific effects. The study of Bagga *et al.* showed that excessive intake of *n*-6 PUFA contributed to the high risk of BC in US, while a decreased risk of BC development was accompanied with higher EPA and DHA consumption [18]. A similar inverse relationship was also observed in regard to the *n*-6/*n*-3 PUFA ratio. In another US study, when the analysis was restricted to pre-menopausal women, the consumption of the lowest ratio of *n*-6 to *n*-3 was associated with a 41% reduction of BC risk, although it was not significant [41]. This observation was also observed in studies conducted in China and France, although there was no association between *n*-3 PUFA intake and BC risk, low *n*-3 PUFA intake by women who had the highest *n*-6 PUFA was correlated with elevated BC risk [37,45]. However, based on the ratio, it is not possible to determine whether it is increased *n*-6 or decreased *n*-3 PUFA that is the causal driver of BC risk. Nevertheless, these studies indicate the necessity of higher *n*-3 PUFA intakes given that *n*-6 intakes are adequate in all populations, and thus heightening the potential value of *n*-3 PUFA as effective agents against BC.

Diet intervention by *n*-3 PUFA supplements as a mean of decreasing BC risk in women still needs to be tested clinically. To date, few human intervention studies have assessed the effectiveness of *n*-3 PUFA in BC prevention and treatment. One randomized clinical trial tested the combined effects of *n*-3 PUFA (EPA + DHA = 3.36 g/day, 2 year) and Raloxifene (anti-estrogen) in reducing risk of BC in postmenopausal women [49]. Although the plasma *n*-6/*n*-3 ratio significantly decreased among subjects after *n*-3 PUFA intervention compared with the subjects without intervention, *n*-3 PUFA

administration did not affect any selected biomarkers that associated with BC risk [49]. While in another human intervention study, Thompson *et al.* demonstrated that daily intake of 25 g flaxseed (ALA = 57% of total fatty acids) can significantly reduce cell proliferation and increase cell apoptosis in tumors of postmenopausal BC patients. These limited results provide encouragement for future study of *n*-3 PUFA as an adjuvant therapy in BC [50].

3. PUFA—Potential Mechanisms of Action

For more than 30 years, numerous studies have attempted to establish whether there is a causal relationship between *n*-3 PUFA ingestion and a reduction in mammary carcinogenesis. Mounting evidence shows that dietary *n*-3 PUFA may exert an anti-carcinogenic action by altering the composition of cell membrane phospholipids, inhibiting AA metabolism and decreasing AA derived eicosanoids, as well as modulating the expression and function of numerous receptors, transcription factors and lipid derived signaling molecules. However, studies of the effects of dietary *n*-3 PUFA on BC progression and prognosis are limited in humans. Epidemiological studies do not allow for the analysis of important cellular interactions and the specific molecular pathways which are activated during the course of tumor initiation and cancer progression in the mammary gland. Thus, the use of animal models (*in vivo*) and BC cell lines (*in vitro*) provide crucial avenues for improving our understanding of the underlying biological pathways involved in trigger BC development and potential therapeutic approaches for BC treatment and prevention. Here we first introduce some potential mechanisms and important downstream mediators that involved in the anticancer action of *n*-3 PUFA.

3.1. Influence on Cell Plasma Membrane Composition

Fatty acids play an important role in membrane biogenesis in the form of glycerophospholipids, a major class of lipids found all cell membranes [51]. Dietary PUFA integrate into plasma membrane glycerophosholipids and influence the fatty acid composition [52]. The *sn*-1 position on the glycerol backbone of glycerophospholipids is usually linked to saturated fatty acids, and the *sn*-2 position is linked to an *n*-6 PUFA such as AA. Increased intake of dietary *n*-3 PUFA may replace *n*-6 with *n*-3 fatty acids at the *sn*-2 position of glycerophospholipids [52]. Since *n*-3 PUFA has a greater density compared to *n*-6 PUFA, the aggregation of *n*-3 fatty acids tend to be closer to the lipid-water interface of the membrane. This characteristic can significantly affect plasma membrane fluidity and permeability [53]. In addition, due to the high level unsaturation of long chain *n*-3 PUFA, they have very poor affinity for cholesterol [52,54]. Membrane cholesterol serves as a spacer for the hydrocarbon chains of sphingolipids and maintains the assembled microdomains of lipid rafts [55]. Thus, cholesterol depletion leads to the disorganization of lipid raft structure [54]. Lipid rafts are important membrane domains for cell signaling since it is enriched with many regulatory proteins and some growth factor receptors. [54,55] As a result, the incorporation of *n*-3 PUFA, especially EPA and DHA, can disturb formation of lipid rafts and suppress raft-associated cell signal transduction [22,56,57].

3.2. Inhibition of Arachidonic Acid (AA) Derived Eicosanoid Biosynthesis

One of the key cellular functions of PUFA is related to their enzymatic conversion into eicosanoids. Eicosanoids are short-lived, hormone-like lipids, typically comprised of 20 carbon atoms, which play a critical role in platelet aggregation, cellular growth and cell differentiation [31]. The most salient mechanism by which *n*-3 PUFA reduce tumor development is through inhibiting the synthesis of inflammatory eicosanoids derived from AA [31]. As indicated in Figure 1, firstly, both ALA and LA are initially converted to their long-chain metabolites (EPA and AA, respectively) through the same desaturation/elongation pathway; therefore, there exists potential competition between these two families of fatty acids for desaturases and elongases. The initial conversion of ALA to stearidonic acid (18:4*n*-3) is the rate limiting reaction of the pathway. The affinity of delta 6-desaturase for ALA is greater than for LA [58]. As a result, a higher intake of ALA reduces the synthesis of AA from LA and thus, less AA is available for synthesis of inflammatory eicosanoids [59]. Secondly, increase

consumption of *n*-3 PUFA results in their incorporation into membrane phospholipids, where they partially replace AA, therefore reducing the substrate for AA derived eicosanoids. Healy *et al.* showed that dietary supplements with four different concentrations of fish oil resulted in the incorporation of EPA and DHA into human inflammatory cells occurs in a dose-response fashion at the expense of AA [60]. Thirdly, both AA and EPA are substrate for eicosanoid synthesis such as prostaglandins (PG) and leukotrienes (LT) [31]. AA can be metabolized by two major pathways including the cyclooxyenase (COX) and lipoxygenase (LOX) pathways. COX-2 catalyzes the rate limiting step in the formation of 2-series PGs. 5-LOX catalyzes the first step in oxygenation of AA to produce hydroxyl derivatives and 4-series LTs. The overexpression of COX-2 has been detected in many types of cancers and PGE_2 has been shown to promote cell proliferation in mammary tumor tissues [61]. PGE_2 stimulates the expression and activation of aromatase, the enzyme that converts androgens to estrogens [62]. Therefore, it is hypothesized that estrogen levels can be lowered by decreasing *n*-6 PUFA intake. In contrast, EPA metabolism results in 3-series PGs and 5-series LTs, with a slightly different structure and anti-tumorigenic properties [58]. *n*-3 PUFA supplementation has been shown to lower production of PGE_2 (by 60%) and LTB_4 (by 75%) in human peripheral blood mononuclear cells [63]. Furthermore, *n*-3 PUFA has been suggested to suppress the expression of COX-2 and 5-LOX. Feeding mice with high *n*-3 PUFA diets influences mammary tumor development by down-regulating COX-2 and 5-LOX expression [20,64]. In addition to the inhibitory effects on the generation of inflammatory eicosanoids, recent studies have identified a novel group of lipid mediators, termed resolvins, which are formed from EPA and DHA [65,66]. These mediators appear to exert potent anti-inflammatory actions and are considered as potential therapeutic interventions for some chronic inflammatory diseases and cancer [67,68].

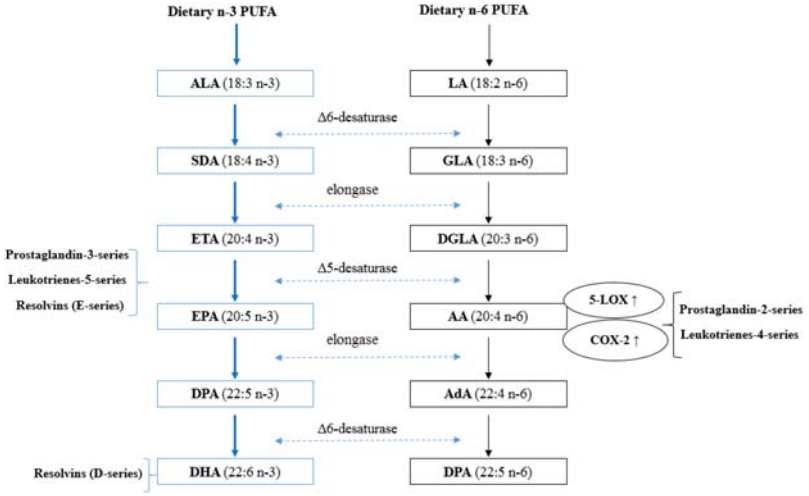

Figure 1. Synthetic pathways of long-chain PUFA and eicosanoids. α-linolenic acid (ALA; 18:3*n*-3) and linoleic acid (LA; 18:2*n*-6) are essential PUFA obtained from the diet, and involve in similar sequential desaturation and elongation steps, give rise to long chain, more unsaturated PUFA eicosapentaenoic acid (EPA; 20:5*n*-3), docosahexaenoic acid (DHA; 22:6*n*-3), and arachidonic acid (AA; 20:4*n*-6). Relevant intermediates in these pathways include SDA (stearidonic acid), ETA (eicosatetraenoic acid), DPA (docosapentaenoic acid), GLA (γ-linolenic acid), DGLA (dihomo-γ-linolenic acid) and AdA (adrenic acid). Both AA and EPA are substrates for the synthesis of eicosanoid products such as prostaglandins (PG) and leukotrienes (LT). The products of *n*-6 PUFA tend to promote cell proliferation while the products of *n*-3 PUFA have anti-tumorigenic properties. *n*-3 PUFA may lower the risk of BC by disrupting the biosynthesis of AA-derived inflammatory eicosanoids.

In general, *n*-3 PUFA can not only block AA metabolism, but can also compete with AA for eicosanoid synthesis. This anti-inflammatory effect of *n*-3 PUFA is of interest since chronic inflammation has been linked to cancer initiation and progression [69].

3.3. Influence on Gene Expression, Transcription Factor Activity and Signal Transduction

BC development is a multi-step process that requires the accumulation of several genetic alterations in a single cell. Many studies have now demonstrated that the regulation of intracellular signaling is a critical aspect of controlling cell functions in various types of cancers. Neoplastic growth and progression are generally regarded as dependent on a high rate of cell proliferation and a low rate of apoptosis. Dietary PUFA and their metabolites may exert some of their anti-cancer or tumor-promoting effects by affecting gene expression or activating signal transduction molecules involved in the control of cell proliferation, differentiation apoptosis and metastasis (Figure 2).

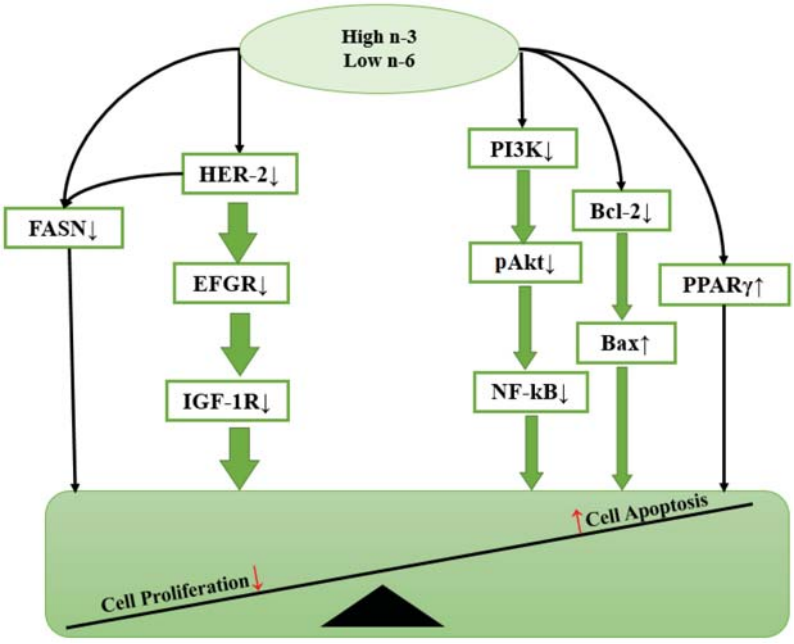

Figure 2. Hypothetical scheme showing how *n*-3 PUFA modulates cell functions via intracellular signaling molecules. Cell proliferation and cell apoptosis are the two important fundamental processes integral to carcinogenesis. *n*-3 PUFA exerts anti-cancer effects by reducing the expression of some growth factors including human epidermal growth factor receptor-2 (HER-2), epidermal growth factor receptor (EGFR) and insulin-like growth factor 1(IGF-1R); inhibiting cell proliferation by either activating PPARγ or decreasing levels of fatty acid synthase (FAS) protein; and promoting cell apoptosis via blocking PI3K/Akt pathways, downregulating phosphorylated Akt, inhibiting NF-κB activity and lowering Bcl-2/Bax ratio.

3.3.1. EGFR and HER-2

Among numerous factors, carcinogenesis involves the activation of oncogenes such as the epidermal growth factor receptor (EGFR) and human epidermal growth factor receptor-2 (HER-2). EGFR is a receptor tyrosine kinase, which plays essential roles in regulating a number of cellular processes including cell proliferation, survival and migration [70]. EGFR is usually activated in response to extracellular ligands (epidermal growth factor [EGF]) by its phosphorylation [71].

Dysregulated EGFR activation is often associated with overexpression of EGFR, which has been observed in several cancer types including breast carcinomas [72]. Marine *n*-3 PUFA were able to inhibit EGFR activity, in particular, DHA was found to induce apoptosis in BC cells by down-regulating EGFR expression [71]. Similar to EGFR, HER-2 (HER-2/neu or erbB-2) is a 185-kD transmembrane receptor tyrosine kinase that is involved in human mammary oncogenesis [73]. The overexpression of HER-2 occurs in 25%–30% of human invasive BCs, and is associated with a more aggressive phenotype and poor patient prognosis [12,74]. HER-2 functions as a co-receptor and forms homodimers or heterodimers with EGFR or insulin-like growth factor 1(IGF-1R) to activate downstream target signaling cascades that involve cell survival and proliferation, such as phosphatidylinositol 3-kinase (PI3K)/Akt, mitogen-activated protein kinase (MAPK) and inhibition of apoptotic pathways such as Bcl-2-associated death promoter protein [75,76]. It should be noted that the HER-2 receptors also activate lipogenic pathways mediated by the fatty acid synthase (FAS) protein [6], a key lipogenic enzyme catalyzing the terminal steps in the de novo biogenesis of fatty acids in cancer pathogenesis [51]. Dietary *n*-3 PUFA were demonstrated to inhibit the early stages of HER-2/neu-mediated mammary carcinogenesis in rats [77]. Notably, ALA alone was able to reduce HER-2 protein expression by 79% in MCF-7 cell lines [78]. Both EGFR and HER-2 are regarded as important therapeutic targets against BC, and *n*-3 PUFA may be a dietary treatment for controlling the growth factor-mediated oncogenesis.

3.3.2. Peroxisome Proliferator-Activated Receptor Gamma (PPARγ)

Peroxisome proliferator-activated receptors (PPARs) are members of the nuclear receptor superfamily and function as ligand-activated transcription factors [79]. PPARγ is a subset of the PPAR family, it is mainly expressed in adipose tissue, mammary gland, colon and the immune system [80,81]. PPARγ regulates the expression of target genes by binding to DNA sequence elements, termed PPAR response elements (PPREs). PPREs have been identified in the regulatory regions of a variety of genes that are involved in lipid metabolism and homeostasis, but recently have appeared to be involved in cell proliferation, cell differentiation, and inflammatory responses [79,82]. PPARγ ligands include naturally occurring compounds such as PUFA and eicosanoids, as well as synthetic activators, such as the hypolipidemic drugs [83]. Clay *et al.* indicated that induction of apoptosis is a biological response resulting from PPARγ activation in some BC cells [84]. *n*-3 PUFA are direct agonists for PPARγ, which have been shown to exert anti-tumorigenic effects via the activation of PPARγ [82,85]. For instance, DHA was found to attenuate MCF-7 cell proliferation by activation of PPARγ [86]. In addition, dietary supplementation with a low ratio of *n*-6/*n*-3 PUFA (1:14.6) was shown to increase PPARγ protein content, which was paralleled with a reduction of tumor burden in rats with induced mammary carcinogenesis [87]. As a result, PPARγ activation is beneficial for controlling BC, which suggests a potential role for PPARγ ligands in the treatment of BC.

3.3.3. Bax/Bcl-2

Apoptosis is a form of cell death triggered during a variety of physiological conditions and is tightly regulated by a number of gene products that promote or block cell death at different stages [88]. Bcl-2 is well-known as an important apoptosis-regulator protein [89], normally blocking apoptosis and its overexpression contributes to BC by prolonging cell survival [90]. Bax is a pro-apoptotic member of the Bcl-2 family of proteins. It is likely to have pore-forming activity to increase mitochondrial membrane permeability, and can also form a homodimer with Bcl-2 to enhance the effects of apoptotic stimuli [90,91]. Raisova *et al.* showed that the Bax/Bcl-2 ratio determines the susceptibility of cells to apoptosis [92]. Thus, a low Bax/Bcl-2 ratio is associated with enhanced survival of BC cells and resistance to apoptosis, and vice versa. It has been proposed that diets rich in *n*-3 PUFA, such as fish and canola oil, reduces the abundance of Bcl-2 and up-regulates Bax expression to induce apoptosis, thereby reducing BC risk [27,93].

3.3.4. PI3K/Akt, NF-κB

Besides Bcl-2, the PI3K/Akt pathway also plays an important role in cell apoptosis. Phosphatidylinositol 3-kinase (PI3K) is a heterodimeric lipid kinase that is composed of a regulatory and catalytic subunit that are encoded by different genes [93]. The primary consequence of PI3K activation is the generation of the second messenger PtdIns (3,4,5) P3 (PIP3) in the membrane, which in turn recruits and activates Akt, a downstream serine/threonine kinase [94]. Akt activation is a dual regulatory mechanism that requires translocation to the plasma membrane and phosphorylation at Thr308 and Ser473 [52,94,95]. Due to this mechanism, Akt functions as an anti-apoptotic signaling molecule. Thus, upregulation of phosphorylated Akt is relevant to tumor cell growth and resistance to cell apoptosis [6,87,96]. HER-2 overexpression constitutively activates survival and proliferation pathways by increasing activation of Akt, however, *n*-3 PUFA was found to either modulate total Akt expression or interact with Akt to down-regulate its phosphorylation [6,97]. Since Akt requires translocation to the plasma membrane for activation, it is possible that tumor cell membrane enrichment of *n*-3 PUFA might affect the phosphorylation of Akt that are recruited to the membrane for activation [96]. In addition, recent studies have demonstrated that PI3K/Akt promoted cell survival is mediated, in part, through the activation of the nuclear factor kappa-B (NF-κB) transcription factor [95,98,99]. NF-κB is a key regulator of genes involved in cell proliferation, migration, and angiogenesis [100,101]. In tumor cells, impaired regulation of NF-κB activation will lead to deregulated expression of the anti-apoptotic genes under the control of NF-κB [100]. For instance, NF-κB has been shown to inhibit the activity of p53, a tumor suppressor known to trigger apoptosis in cells with damaged DNA [102]. As a result, constitutive NF-κB expression may contribute to the development and progression of BC.

3.3.5. Cell Proliferation Marker: Ki-67 and PCNA

Cell proliferation is another fundamental process integral to carcinogenesis. Ki-67 is a nuclear protein, and being widely used as a prognostic or predictive marker in BC and other malignant disease [103]. The human Ki-67 protein is present during all active phases of the cell cycle G(1), S, G(2), and mitosis, but is absent from resting cells G(0), which makes Ki-67 an excellent marker for determining the growth fraction of a given cell population [104]. Ki-67 immunohistochemical staining has been used as an index of tumor growth in numerous of cancer studies, especially prostate and breast carcinomas [26,87]. Treatment with ALA-rich flaxseed oil markedly lowered tumor burden in rats accompanied by reduced Ki-67 level [78,105].

Similarly to Ki-67, Proliferating Cell Nuclear Antigen (PCNA) is also considered a potential prognostic marker in BC [106]. PCNA is a ring-like nuclear protein which functions as the sliding clamp of DNA polymerases [107,108]. Thus, it is involved in DNA replication and repair machinery of the cell [109]. Expression of PCNA is a valid cell proliferation marker since the distribution of PCNA was found to occur during G1, S and G2 phase, but reaches low immunohistochemically detectable levels in M-phase of the cell cycle [106,108,109]. It has been shown that supplementation with *n*-3 PUFA reduces the percentage of proliferating tumor cells by decreasing the expression of PCNA [110].

The overall effect of high *n*-3 PUFA intake on cellular signaling process either inhibits cell proliferation or promotes cell apoptosis (Figure 2). The changes in gene expression, transcription factor activity and signaling transduction will be highlighted in the following sections.

4. The Effect of *n*-3 PUFA Mixtures on BC Development

4.1. Animal Studies

The inhibitory effects of *n*-3 PUFA on tumor growth have been well documented in rodent models of BC. Xenograft, transgenic and chemically induced methods are the three main approaches employed in rodent models.

4.1.1. Breast Cancer Studies in Xenograft Rodent Models

Xenograft rodent models involve the transplantation of human BC cells into immunocompromised mice [78,111]. The type and concentration of dietary PUFA have profound influences on the growth rate of transplantable human BC in rodents (Table 3). In one study, MDA-MB-435 BC cells xenografted into athymic nude mice in order to compare intake of LA alone with diets containing LA and various proportions of EPA/DHA [20]. The diet rich in *n*-6 PUFA stimulated the growth and migration of human BC cells in mice, whereas diets supplemented with EPA or DHA exerted suppressive effects [20]. Similarly, Karmail *et al.* also found an inhibition of R3230AC mammary tumor growth in rat fed with Maxepa, a menhaden oil supplement that contains approximately 18% EPA and 12% DHA [112]. These observations were largely attributed to the high incorporation of *n*-3 PUFA into the tumor phospholipids, which further reduced pro-inflammatory eicosanoid synthesis from AA [20]. More recently, a diet supplemented with 3% w/w fish oil concentrate was shown to stimulate lipid peroxidation in the tumor cells, and thereby slowed down the tumor growth rate in athymic nude mice implanted with MDA-MB-231 [30]. Similar effects were observed in MCF-7 human breast cancer xenografts, although a higher percentage of fish oil (19% w/w) was required to decrease tumor volume in the nude mice [113]. These findings supported an earlier study showed that *n*-6/*n*-3 PUFA consumed in a low ratio resulted in prolonged tumor latency and reduced tumor growth rate in BALB/cAnN mice [114]. Therefore, diet enriched with *n*-3 PUFA can alter murine mammary tumorigenesis.

4.1.2. Breast Cancer Studies in Transgenic Rodent Models

In transgenic mouse models, tumor growth can be initiated in two different ways: gain of function involving oncogenes responsible for cell proliferation or loss of function involving cell apoptotic pathways [115,116]. For example, the mouse mammary tumor virus (MMTV) promoter allows the cancer-causing virus to be activated and expressed in mammary tissue, leading to the development of mammary tumors [117]. Several studies have employed MMTV-neu related models and provided strong evidence for protective effects of *n*-3 PUFA towards HER-2 positive BC (Table 4).

One study examined mammary tumor development in MMTV-Her-2/neu mice fed menhaden fish oil from 7 weeks of age onwards. The tumor incidence was dramatically reduced as well as prolonged tumor latency [115]. Further, Yee *et al.* demonstrated that dietary *n*-3 PUFA downregulated COX-2 and Ki-67 to inhibit cell proliferation, further reducing atypical hyperplasia to prevent HER-2/neu mammary carcinogenesis at early stages [77]. Moreover, we showed that lifelong *n*-3 PUFA exposure can mitigate tumor development in mice expressing MMTV-neu(ndl)-YD5, a more aggressive HER-2-positive BC model [116]. In this study, MMTV-neu(ndl)-YD5 mice were crossed with fat-1 mice, yielding mice that were capable of endogenous synthesis of *n*-3 from *n*-6 PUFA and had the susceptibility to mammary tumor growth [116]. Thus, this study provided a direct evidence for a protective effect of *n*-3 PUFA via both complementary genetic and conventional dietary approaches [116]. In order to understand the dose-dependent effect of *n*-3 PUFA on mammary gland tumor development, Leslie *et al.* further demonstrated a dose-response relationship of 0%, 3% and 9% (w/w) menhaden oil on tumor burden reduction in MMTV-neu-YD5 mice [21]. In line with the previous study, the observed dose-dependent effects of *n*-3 PUFA were associated with a dose-dependent change in the fatty acid profile, reflecting a decreased *n*-6/*n*-3 ratio in the mammary glands and an increased of EPA and DHA in tumor phospholipid classes [21]. This is consistent with an earlier human clinical trial which showed dietary DHA and EPA supplementation caused a dose-dependent increase of *n*-3 PUFA in serum and breast adipose tissues of BC patients [118].

Table 3. *n*-3 PUFA and breast cancer risk: Xenograft rodent models.

Animal Model	*n*-3 PUFA Source	Feeding Period	Main Findings	Mechanism	Reference
Athymic nu/nu mice MDA-MB 231	3% w/w fish oil concentrate (10.2 g/kg EPA, 7.2 g/kg DHA, 3.0 g/kg ALA)	7-week (fed after tumor established)	↓ tumor growth rate ↑ effectiveness of doxorubucin	↑ EPA incorporation into tumor ↑ lipid peroxidation in tumor	[30]
Athymic nu/nu mice (NCr-nu/nu) MDA-MB 435	40 or 80 g/kg EPA, DHA	13-week (fed before transplantation)	↓ tumor growth, size ↓ tumor weight	↑ EPA, DHA in tumor phospholipids ↓ LA, AA in tumor phospholipids ↓ AA-derived eicosanoids	[20]
Inbred F44 rats R3230AC	5% marine oil supplementation (18% EPA, 12% DHA)	4-week (fed before transplantation)	↓ tumor weight, volume	↑ EPA, DHA, AA incorporation into tumor ↓ Prostaglandins 2 series	[112]
BALB/cAnN mice Mouse BC cell	10% or 20% w/w menhaden fish oil	7-week (fed before transplantation)	↑ tumor latency ↓ tumor growth rate	NA	[114]
Athymic nude mice MCF-7	19% w/w menhaden oil (1.9 g/kg ALA, 19.4 g/kg EPA, 24.3 g/kg DHA)	6 or 8-week (fed after tumor established)	↓ tumor volume	↑ lipid peroxidation in tumor	[113]

↑: increase; ↓: decrease; NA: not available.

Table 4. *n*-3 PUFA and breast cancer risk: transgenic rodent models.

Animal Model	*n*-3 PUFA Source	Feeding Period	Main Findings	Mechanism	Reference
MMTV-HER-2/neu	22.50 kcal% menhaden oil (15 g/kg EPA, 10.8 g/kg DHA)	28-week (fed before tumor development)	↓ atypical ductal hyperplasia ↓ cell proliferation prevented HER-2/neu at early stages	↓ Ki-67 expression ↓ COX-2 expression	[77]
MMTV-HER-2/neu	22.50 kcal% menhaden oil (15 g/kg EPA, 10.8 g/kg DHA)	52-week (fed before tumor development)	↓ tumor incidence and multiplicity ↑ tumor latency ↓ mammary gland dysplasia	NA	[115]
MMTV-neu (ndl)-YD5 × fat1	3% w/w menhaden oil (0.5 g/kg ALA, 4.1 g/kg EPA, 3 g/kg DHA)	20-week (lifelong treatment, fed before tumor development)	↓ tumor volume and multiplicity	↑ EPA, DHA and overall *n*-3 in mammary tissues ↓ *n*-6/*n*-3 ratio in tumor phospholipids	[116]
MMTV-neu (ndl)-YD5	3% w/w menhaden oil (0.5 g/kg ALA, 4.1 g/kg EPA, 3 g/kg DHA) 9% w/w menhaden oil (1.3 g/kg ALA, 12.4 g/kg EPA, 9 g/kg DHA)	20-week (lifelong treatment, fed before tumor development)	↓ tumor volume and multiplicity ↑ tumor latency (all in a dose-dependent manner)	↑ EPA, DPA in mammary tissues ↑ EPA, DHA in tumor phospholipids ↓ LA, AA, *n*-6/*n*-3 PUFA ratio in both mammary and tumor tissues in a dose-dependent manner	[21]

↑: increase; ↓: decrease; NA: not available.

Supplementing mice with 24% (w/w) menhaden oil delayed mammary tumor development by 15 weeks relative to mice fed the same amount of corn oil [119]. This result heightened the potential value of *n*-3 as an effective agent against BC, while the timing of exposure to *n*-3 PUFA will also influence future cancer risk [34]. Mammary gland research has identified critical periods of development, including early windows such as in utero, lactation and pubescence. The mammary gland undergoes rapid growth during these periods, and exposure to environmental agents may influence the long-term health of mammary tissue. Thus, supplement with *n*-3 PUFA in early life stages may protect against later BC development. In support of this, Su *et al.* conducted a study exposing rats to maternal high *n*-6 PUFA diet with or without fish oil supplementation during the perinatal period via maternal intake or during puberty or adulthood [29]. They found that fish oil intake during the perinatal period had a greater effect in preventing mammary tumors than fish oil supplementation in later life [29]. The decreased maternal serum estradiol levels in pregnant rats with fish oil supplementation was thought to play a role in reducing susceptibility to later BC development in the female offspring [29].

4.1.3. Breast Cancer Studies in Chemically-Induced Rodent Models

Evidence from chemical-induced BC studies consistently supports the anti-cancer effect of *n*-3 PUFA. Induced mammary carcinoma in rats by the injection of carcinogenic chemicals such as 7,

12-dimethylbenz (α) anthracene (DMBA) and *N*-methyl-*N*-nitrosourea (MNU) have been widely used in various BC chemopreventive studies. When provided in the diet through menhaden or fish oil concentrates, the incorporated *n*-3 PUFA, EPA and DHA in particular, lower tumor incidence as well as retard tumor growth and metastasis of chemically-induced and transplantable mammary tumors (Table 5). Olivo *et al.* compared the effects of low- or high-, *n*-3 or *n*-6 PUFA exposure on mammary tumorigenesis in rats [110]. Feeding rats a low-fat *n*-3 diet significantly lowered the incidence of DMBA-induced mammary tumors compared to *n*-6 fed rats. This was accompanied by reduced cell proliferation and elevated lipid peroxidation. However, the high-fat *n*-3 diet resulted in a significant increase risk of mammary tumorigenesis relative to rats fed with *n*-6 diets [110]. Further, an *in vivo* study demonstrated that low-fat *n*-3 PUFA diets inhibited cell proliferation via activation of PPARγ and/or down-regulation of COX-2 and PCNA expression; whereas high fat *n*-3 PUFA diets stimulated cell proliferation and were positively associated with levels of phosphorylated Akt [110]. Similar suppressive effects of *n*-3 PUFA were observed in another DMBA-induced rat model [27]. Supplementation of EPA and DHA (from Maxepa) effectively suppressed cell proliferation, which was accompanied by down-regulation of Ki-67 and HER-2/neu positive expressions. Meanwhile, EPA and DHA induced cell apoptosis through modulating the expression of Bcl-2 and Bax in mammary tissue of Sprague-Sawley rats [26–28]. These findings suggest that gene-nutrient interactions are of a critical importance in the development of BC.

Table 5. *n*-3 PUFA and breast cancer risk: Chemically-induced rodent models.

Carcinogen	*n*-3 PUFA Source	Feeding Period	Main Findings	Mechanism	Reference
MNU	Fish oil 2%–10% w/w *n*-3 PUFA in diet	18-week (at the same time as MNU administration)	Absolute *n*-3 diet: ↓ body weight, no tumor occurrence (10% w/w *n*-3 PUFA) 1:1*n*-6/*n*-3 diet ↓ tumor incidence and multiplicity (5% w/w *n*-3 PUFA)	↑ EPA, DHA in mammary ↓ FAS, COX-2, 5-LOX	[64]
MNU	Fish oil concentrate Low *n*-6/*n*-3 = 1:14.6 High *n*-6/*n*-3 = 1:0.7	2-week (at the same time as MNU administration)	Low *vs.* high ratio *n*-6/*n*-3 PUFA diet: ↓ tumor incidence (21%), ↓ tumor multiplicity (30%), tumor burden (80%) ↑ apoptotic index (129%)	↓ Ki-67 ↑ Bax, Bax/Bcl2, PPARγ ↓ NF-κB p65, pAkt, IGF-IR	[87]
MNU	EPA/DHA alone: 95 g/kg EPA/DHA EPA + DHA: 47.5 g/kg EPA + 47.5 g/kg DHA	20-week (at the same time as MNU administration)	DHA alone *vs* EPA + DHA *vs* EPA alone: ↓ tumor incidence: 23%, 73%, 65% ↓ tumor multiplicity: 0.23, 1.67, 1.59 DHA is more effectively than EPA	NA	[120]
DMBA	Maxepa (fish oil concentrate): 90 mg EPA + 60 mg DHA per day	24-week study 35-week study (before DMBA injection)	↓ DNA single-strand breaks ↓ cell proliferation	↓ Ki-67, Her-2/neu	[26]
DMBA	Maxepa: 90 mg EPA + 60 mg DHA per day	24-week study 35-week study (before DMBA injection)	↓ tumor incidence (23%), tumor multiplicity (42%) ↑ cell apoptosis ↓cell proliferation	↓ Bcl-2 ↑Bax ↑ p53	[27,28]
DMBA	Fish oil (0.5%ALA, 16% EPA, 1.2% DPA, 8% DHA in fish oil)	NA	↓ tumor incidence with fish oil consumption: adulthood < in utero < puberty < perinatal ↓ tumor multiplicity with fish oil consumption: adulthood > puberty > perinatal > in utero	↓ maternal serum estradiol	[29]
DMBA	Menhaden oil Low-fat *n*-3 PUFA diet: 4.6 g/kg EPA + 3.2 g/kg DHA High fat *n*-3 PUFA diet: 9.1 g/kg EPA + 6.3 g/kg DHA	20-day (before DMBA injection)	Low *n*-3 diet: ↓ tumor incidence ↓ TEBs ↓ cell proliferation ↑ cell apoptosis; High *n*-3 diets exert opposite effects	Low *n*-3 diet: ↓ COX-2, PCNA ↑ PPARγ ↑ lipid peroxidation High *n*-3 diet: ↑ pAkt ↑ lipid peroxidation	[110]

↑: increase; ↓: decrease; NA: not available.

It has been shown that the different dietary fatty acid composition and *n*-6/*n*-3 PUFA ratios can diversely influence the occurrence and progression of BC [8,32,64]. Wei *et al.* fed MNU-induced rats with various *n*-6/*n*-3 PUFA ratios and demonstrated the 1:1 ratio of *n*-6/*n*-3 PUFA in diet was more effective in the prevention of mammary tumor development when compared with diets higher in *n*-6 PUFA [64]. Replacement of *n*-6 by *n*-3 PUFA in diets, reflected an increase in EPA and DHA in mammary tumor tissue, which subsequently downregulated the expression of lipid metabolic-related genes and inhibited cell proliferation [64]. In agreement with this study, Jiang *et al.* reported that low *n*-6/*n*-3 PUFA ratio (1:14.6) caused 80% reduction in tumor burden and 30% decrease in tumor multiplicity in the same rodent model compared to a high *n*-6/*n*-3 PUFA ratio (1:0.7). These observations were mainly mediated by downregulating NF-κB, pAkt and IGF-IR, and increased activity of PPARγ [87].

4.2. Cell Culture Studies

It has been well established that *n*-3 PUFA can suppress the development of cancers by inhibiting cellular proliferation and inducing apoptosis. Cell culture studies investigating the effects of *n*-3 PUFA on murine and human BC cells provide important insights into the mechanisms underlying this inhibitory effect. Although there are few studies describing the individual effects of ALA, EPA and DHA *in vitro*, the available data consistently show that *n*-3 PUFA have direct growth inhibitory effects on several BC cells lines (Table 6).

MDA-MB-231 human BC cell line was used to investigate the effect of various classes of fatty acids (*n*-3, *n*-6 and *n*-9 PUFA) [121]. EPA and DHA exhibited a dose-dependent inhibition of cell growth, whereas LA and oleic acid (OA) stimulated cell growth at very low concentration [121]. Some *in vitro* studies have not included the essential *n*-6 PUFA (*i.e.*, LA) in the growth medium, which would be present *in vivo* and is required for mammary tumorgenesis in animals. These studies are not able to explain the tumor growth inhibition by *n*-3 PUFA in the presence of abundant LA. Schley *et al.* examined the inhibitory effects of *n*-3 PUFA on BC *in vitro* in the presence or absence of LA [22,96]. It was demonstrated that EPA and DHA induced apoptosis and increased DNA fragmentation in MDA-MB-231 cells when provided in combination with LA. Similar effects were observed in the MCF-7 BC cell line [71,86,105,122]. Barascu *et al.* revealed that EPA and DHA decreased MCF-7 cell growth and increased the fraction of apoptotic cells in a concentration-dependent manner, and particularly, a higher efficiency noted for DHA [86]. Specifically, they demonstrated that *n*-3 PUFA inhibited cell proliferation by lengthening the cell cycle between the G2/M transition [86]. In accordance with this finding, a previous study demonstrated that DHA induced marked G2-M and G1-S arrest of the MCF-7 cells [123]. Furthermore, treatment with EPA and DHA was also shown to induce cell differentiation and increase lipid peroxidation product levels in MCF-7 cells [105,124].

Table 6. *n*-3 PUFA and breast cancer risk: cell culture studies.

Cell Type	*n*-3 PUFA Source	Main Finding	Mechanism	Reference
MDA-MB-231	EPA/DHA alone: 75 μM or 100 μM EPA + DHA combination: 45 μM EPA + 30 μM DHA or 60 μM EPA + 40 μM DHA (in presence/absence of LA)	↓ cell viability, cell proliferation ↑ DNA fragmentation, cell apoptosis DHA was more potent than EPA	↓ pAkt ↓ NF-κB and DNA binding activity	[96]
MDA-MB-231	0.5–2.5 μg/mL of EPA, DHA (1.7–8.2 μM EPA, 1.5–7.6 μMDHA)	↓ tumor cells growth (DHA > EPA, dose-dependent)	↓ LA composition in cell lipids ↓ AA-derived eicosanoid synthesis	[121]
MDA-MB-231	EPA/DHA alone: 75 μM or 100 μM EPA + DHA combination: 45 μM EPA + 30 μM DHA or 60 μM EPA + 40 μM DHA (in presence/absence of LA)	↓cell growth (48%–62%)	↑ EPA, DHA, DPA and total *n*-3 in lipid rafts ↓ EGFR levels ↑ pEGFR	[22]
MDA-MB-231 MCF-7	EPA (230 μM), DHA (200 μM)	↓ cell viability ↑ cell apoptosis	↓ Bcl-2 ↑pro-caspase-8 ↓ pEGFR ↓ EGFR (only DHA) ↓ AA ↑ EPA, DPA, DHA in total cell lipids	[71]
MDA-MB-231 MCF-7	3–100 μM of EPA, DHA	At 50 μM EPA, 30 μM DHA ↑ cell apoptosis ↓ cell growth At 50 μM EPA, DHA ↑ G2/M duration DHA was more potent than EPA	↓ phosphorylation of cyclin B1 ↓ activity of CDK1-cyclin B1	[86]
MCF-7	100 μM of EPA, DHA	↓ cell growth (30% by EPA, 54% by DHA) ↑ cell differentiation (30% by EPA, 65% by DHA) No significant effects on cell apoptosis and cell cycle DHA was more potent than EPA	↑ PPARγ (DHA only)	[125]
MCF-7 MCF-10A	6–30 μM of ALA, EPA, DHA	All *n*-3 PUFA ↓ MCF-7 cell growth (EPA, DHA > ALA, dose-dependent) AA ↓ MCF-7 cell growth (similar as ALA)	NA	[122]
ER+ and ER− cells	20 μg/mL of ALA, EPA, DHA (72 μM ALA, 66 μM EPA, 61 μM DHA)	EPA, DHA ↓ cell proliferation (all cell lines) ALA ↓ estrogen independent BC cell proliferation	↑ lipid peroxidation	[124]

↑: increase; ↓: decrease; NA: not available

Chajes *et al.* examined the effect of ALA, EPA, and DHA on the proliferation of human estrogen-positive (ER+) and estrogen-negative (ER−) BC cell lines [124]. EPA and DHA displayed a significant inhibitory effect on the proliferation of all types of tumor cells, whereas ALA significantly inhibited cell growth in (ER−) MDA-MB-231 and HBL-100 human breast tumor cells but not in (ER+) MCF-7 cells [124]. The efficiency of this inhibitory effect was found to be correlated with the generation of lipid peroxidation products [124]. Consistently, another *in vitro* study identified a dose-dependent inhibitory effect of ALA, EPA, and DHA on MCF-7 cells; however, the cells were dramatically inhibited by EPA and DHA, and moderately inhibited by ALA [122].

Rapid tumor cell proliferation is a critical feature for tumor aggressiveness. Treatment with LA and OA increased MDA-MB-231 cell proliferation through production of pro-inflammatory prostaglandins and leukotrienes [121]. EPA and DHA could mimic the effect of indomethacin, an inhibitor of both COX and LOX, which in turn attenuated BC cell proliferation by inhibiting *n*-6 PUFA related eicosanoid synthesis [126]. In addition, treatment with EPA and DHA inhibited EGFR phosphorylation and diminished EFGR levels in lipid rafts [22,71]. These observations were due to the incorporation of *n*-3 PUFA into BC cell membrane, which subsequently altered membrane structure, signal transduction and function of BC cells [71]. Moreover, *n*-3 PUFA can exert anti-proliferative effects by limiting cell cycle progression, attributed to an inhibition of CDK1-cyclin B1 complex, a master regulator required

for the initiation of mitosis [86]. Furthermore, increasing evidence demonstrated that *n*-3 PUFA can inhibit cell proliferation and increase cell differentiation by activating PPARγ, although this effect was only observed with DHA treatment [105].

Dysregulation of apoptosis is also a hallmark of cancer cells, and thus agents that activate apoptosis are highly desired. Treatment with EPA and DHA inhibited phosphorylation of Akt as well as the NF-κB DNA binding activity [96]. This represents a novel mechanism by which *n*-3 PUFA induce apoptosis in MDA-MB-231 cells [96]. Additionally, it has been shown that *n*-3 PUFA decreased expression of Bcl-2 and increased activity of pro-caspase-8, an apoptosis effector enzyme [71]. As a result, *n*-3 PUFA can inhibit BC development *in vitro* by both suppressing tumor cell proliferation and inducing tumor cell death.

5. The Effect of Individual *n*-3 PUFA on BC Development

5.1. ALA and BC

5.1.1. Inefficient Conversion from ALA to EPA and DHA

α-Linolenic acid (18:3*n*-3; ALA) is the major *n*-3 PUFA in the Western diet. Typical consumption of ALA in Europe, Australia and North America ranges between 0.6 and 1.7 g per day in men and 0.5–1.4 g per day in women [58]. This is about 10-fold lower than the consumption of *n*-6 PUFA. ALA, regarded as the precursor for long-chain PUFA, can be converted to EPA (20:5*n*-3), DPA (22:5*n*-3) and DHA (22:6*n*-3) by the pathway shown in Figure 1. Whether the essentiality of ALA in the diet primarily reflects the activity of ALA itself or of long-chain PUFA synthesized from ALA is a matter of debate. The concentration of ALA in plasma phospholipids, cells and tissues is found to less than 0.5% of total fatty acids [127]. Although the dietary intake of EPA and DHA are approximately 10-fold lower than those of ALA in North America, the concentrations of these long-chain PUFA in plasma, cell and tissue phospholipids are greater than those of ALA [127]. This apparent mismatch between dietary intakes and levels of incorporation further suggests that the primary biological role of ALA is for EPA and DHA synthesis. However, it is also possible that the low concentration of ALA may be due to negative selection in the incorporation of ALA into blood and cell membrane lipid pools [58]. Since consumption of EPA and DHA show a strong inverse association with the risk of BC, this raises the question of whether conversion of ALA to EPA and DHA in humans is a viable alternative to dietary sources of these long-chain PUFA. The majority of human studies estimate that ALA supplementation in human adults generally lead to an increase in EPA and DPA, but have little or no effect on DHA content [128–132]. Conservatively, Pawlosky *et al.* estimated the overall efficiency of conversion from ALA was 0.2% to EPA, 0.13% to DPA and 0.05% to DHA [129]. This inefficient conversion is due to the first rate limiting reaction catalyzed by Δ6-desaturase [58]. As a result, the extent of ALA conversion to EPA and DHA is inefficient and limited. Thus, ALA may not be considered as an effective alternative source to fish for providing EPA and DHA.

5.1.2. Individual Effect of ALA on Breast Cancer

Data derived from epidemiological and observational studies suggest that ALA present in the Western diet has protective effects in BC (Tables 7 and 8). Two case control studies compared the fatty acid composition in the adipose breast tissue from women with invasive non-metastatic breast carcinoma and women with benign breast disease [45,133]. Low ALA content in adipose tissue was found to be associated with an increased risk of BC. This observation was consistent with a previous cohort study on 121 BC patients, which demonstrated a link between a low level of ALA in adipose breast tissue and increased risk of metastatic development [134]. In particular, the ratio of *n*-6/*n*-3 PUFA was also shown to be positively correlated with BC in these patients, highlighting the role of *n*-3 and *n*-6 PUFA balance in BC.

Dietary supplementation of ALA reduced the growth of established mammary tumors in chemically-induced rats and xenograft rodent models. In OVX athymic mice with high circulating estrogen level, flaxseed oil and its high ALA content, attenuated (ER+) MCF-7 breast tumor growth by reducing cell proliferation and increasing apoptotic index [78]. This effect was probably due to the downregulation of tyrosine kinase receptors such as EGFR and HER2, with a subsequent reduction in pAkt. Compared to corn oil, consumption of ALA-rich flaxseed oil tends to modify the *n*-6/*n*-3 PUFA ratio by increasing serum ALA, EPA and DHA concentrations. This provides evidence that ALA absorption and conversion may contribute to the observed tumor reducing effect in the flaxseed oil diet [78]. Further, an *in vitro* study showed that pure ALA inhibited MCF-7 cell proliferation by 33%, which was in accordance with the *in vivo* reduction in palpable tumor growth (33%) [78]. This suggests that ALA itself, rather than the generated EPA and DHA, exerts this anti-tumorigenic effect [78]. Another *in vivo* study was designed to elucidate which component(s) of flaxseed (lignan or ALA) was responsible for enhancing tamoxifens effect on reducing the growth of established MCF-7 breast tumors at low circulating estrogen levels [105]. ALA-rich flaxseed oil had a stronger effect in reducing the palpable tumor size of tamoxifen-treated tumors compared with lignan-treated mice [105]. More importantly, ALA was found to downregulate HER2 expression, and subsequently modulate growth factor-mediated signaling pathways by repressing IGF-1R and Bcl-2 [105].

Table 7. Individual role of ALA, EPA and DHA on BC.

n-3 PUFA	Amount of Fatty Acid	Effect	Mechanism	Reference
ALA	NA	Moderate decrease BC risk	NA	[45]
	~22.8 g of ALA per kg diet	Reduced tumor cell proliferation	Inhibited HER2, EGFR expression	[78]
	~22.8 g of ALA per kg diet	Inhibited MCF-7 cell proliferation		[78]
	~11 g ALA per kg diet	Reduced tumor incidence and burden	Increased BAX/Bcl-2 ratio	[93]
	10.6 g ALA per kg diet	Decreased tumor growth rate	Inhibited HER2 expression	[105]
	72 μM ALA	Moderate inhibited ER-negative cell proliferation, not affect MCF-7	NA	[124]
	30 μM of ALA	Slightly inhibited MCF-7	NA	[122]
	NA	Inversely associated with BC risk	NA	[133]
	NA	Inversely correlated with metastasis development	NA	[134]
	55.9 g ALA per kg diet	Reduced tumor growth and metastasis	NA	[135]
	8 g ALA per kg diet	Decreased tumor growth rate	NA	[136]
	10 g ALA per kg diet	Reduced tumor burden and increased survival rate	NA	[137]
	2.5-40 μM of ALA	enhanced cytotoxic effects of Trastuzumab (at 10 μM of ALA)	Down-regulated HER2 (at 20 μM of ALA)	[138]
	10 μM of ALA	Diminished proteolytic cleavage of the extracellular domain of HER2	Inhibited HER-2 activity	[139]
	~21.2 g of ALA per kg diet	Minimal inhibited tumor growth w/wo Trastuzumab	NA	[140]
	52.8 g of ALA per kg diet	Inhibited mammary tumor development	NA	[141]
EPA	40–80 g of EPA per kg diet	Slowed down tumor growth, reduced tumor burden	Decreased AA derived-eicosanoid	[20]
	3–100 μM of EPA	Induced BC cell apoptosis (at 50 μM of EPA)	NA	[86]
	40–200 μM of EPA	Restored the growth inhibitory effect of Tamoxifen (at 40 μM of EPA)	Decreased pAkt (at 20 μM of EPA)	[97]
	20–80 g of EPA per kg diet	Inhibited the development of lung metastasis	NA	[126]
	100 μM of EPA	Inhibited MCF-7 cell growth	NA	[125]
	40 μM of EPA	Induced apoptosis, inhibited cell proliferation, arrested cell cycle at G0/G1	down-regulated Bcl-2 expression	[142]
	95 g of EPA per kg diet	Reduced KPL-1 cell proliferation rate and metastasis	NA	[143]
	42 g of EPA per kg diet	Suppressed cell proliferation in MCF-7 xenografts in rats	NA	[144]
	50 μM of EPA	Increased PPARγ at mRNA level	NA	[145]
	0–200 μM of EPA	Inhibited MCF-7 cell growth (at 60 μM of EPA)	NA	[146]

Table 7. *Cont.*

n-3 PUFA	Amount of Fatty Acid	Effect	Mechanism	Reference
DHA	120 μM of DHA	Decreased cancer cell viability, enhanced the cytotoxic activity of taxanes	Decreased the expression of Her-2/neu	[5]
	100 μM of DHA	Disrupted lipid rafts, induced apoptosis in HER-2 overexpressing cells	Decreased Akt activity and FAN	[6]
	100 μM of DHA	Decreased MDA-MB-231 cell proliferation, enhanced EGFR inhibitors	Altered EGFR phosphorylation and localization	[56]
	0–200 μM of DHA	Reduced MCF-7 cell viability and DNA synthesis (at 25 μM of DHA)	Increased lipid peroxidation, capase 8 activation	[146]
	20 or 100 μM of DHA	Inhibited MDA-MB-231 cell proliferation, promoted nuclear condensation	Increased caspase-3 activity (at 100 μM of DHA)	[147]
	10–160 μM of DHA	Inhibited MCF-7 cell growth and induced apoptosis (at 40 μM of DHA)	Downregulated Bcl-2, increased Bax/Bcl-2 ratio	[148]
	270 μM of DHA	50% inhibitory KPL-1 cell growth after 72 h treatment	Downregulated Bcl-2, increased Bax/Bcl-2 ratio	[149]
	40 g of DHA per kg diet	Decreased tumor growth rate and final tumor weight, increased apoptosis	Reduced tumor PGE2, decreased Ki-67	[150]
	32 g of DHA per kg diet	Reduced tumor incidence	Increased BRCA1 at protein level	[151]
	30 μM of DHA	50% inhibitory MCF7 cell growth after 96 h treatment	Increased BRCA1/2 at transcriptional level	[152]
	NA	Increased response of the tumor to chemotherapies, increased survival rate		[153]

NA: not available.

Table 8. Individual effect of ALA, EPA and DHA on different types of BC.

BC Cell Type	ALA	EPA	DHA
MDA-MB-231 (ER−)	✓	✓	✓
MDA-MB 435 (ER−)	NA	✓	✓
MCF-10A (ER−)	—	✓	✓
HBL-100 (ER−)	✓	✓	✓
MCF-7 (ER+)	—	✓	✓
ZR-75 (ER+)	—	✓	✓
T-47-D (ER+)	—	✓	✓
SK-Br3 and BT-474 (HER-2/neu positive)	✓	NA	✓

✓ have significant inhibitory effect on cell proliferation; — slightly inhibit the cell growth; NA: not available.

Remarkably, long-term changes in dietary ALA and LA ratio significantly affect mammary tumor outcomes. Feeding BALB/c mice with ALA-rich linseed oil inhibited the development of mammary tumors compared to mice on corn oil diets [135]. In a recent study, use of canola oil (10% ALA) instead of corn oil (1% ALA) in the diet of MDA-MB-231 implanted mice reduced tumor growth rate [136]. Progression to apoptosis is associated with a balance between pro-apoptotic Bax and anti-apoptotic Bcl-2. Mice exposed to canola oil had an increased ratio of Bax/Bcl-2, which was responsible for the apoptosis of defective epithelial cells [93]. Furthermore, the maternal diets have a life-long influence on development of BC in the daughter. Substitution of corn oil with canola oil in the maternal diet increased *n*-3 PUFA incorporation into mammary glands, which in turn delayed occurrence of mammary tumors and increased tumor cell apoptosis in offspring [93]. Elsewhere, maternal replacement of dietary soybean oil with canola oil significantly lowered the burden of MNU-induced mammary tumors, along with increased survival rate in the offspring [137]. As a result, maternal ALA supplementation brings about a stable epigenetic imprint of genes that are involved in the development and differentiation of the mammary gland, which are passed on to female offspring where they exert a protective effect against BC.

Notably, the protective role of ALA in BC was inconclusive in *in vitro* studies. In BT-474 and SkBr-3 cancer cells that naturally amplify the HER-2 oncogene, exogenous supplementation with ALA significantly suppressed HER-2 mRNA expression, thereby reducing the probability of activation that leads to tumor growth [138]. Moreover, ALA co-exposure was reported to synergistically enhance trastuzumab (an anti-cancer therapy) efficacy in HER2-overexpression BC cells [139]. However, in a separate study, ALA exerted minimal tumor-reducing effects in the presence of trastuzumab [140]. In addition, some studies had difficulties in characterizing the role of ALA in estrogen dependent and independent BC cell lines [122,124]. This suggests a variable effect of ALA on cell proliferation depending on the cell line assessed.

Overall, these findings suggest that diets rich in ALA can inhibit mammary tumor development in animals and *in vitro*, however, it cannot be ruled out that some of the effects are due in part to conversion of ALA to EPA and DHA, albeit limited.

5.2. Individual Effect of EPA on Breast Cancer

Fish oil is a mixture of EPA and DHA, which have been previously shown to have protective effects against BC. However, whether EPA and DHA differentially or similarly affect BC has not yet been determined. In support of the independent effect of EPA on BC, several *in vitro* studies consistently demonstrated the ability of EPA to induce apoptosis in human BC cells (Tables 7 and 8). Chiu *et al.* demonstrated that 40 μM EPA induced cell apoptosis through inhibition of anti-apoptotic regulator proteins, such as Bcl-2 [142]. Moreover, Akt was also shown to be susceptible to EPA [97]. Treatment with EPA lowered both total and phosphorylated Akt content in transfected MCF-7 cells that overexpressing constitutively active Akt [97]. Additionally, co-treatment with EPA enhanced the growth inhibitory response to tamoxifen in MCF-7 cells, thus suggesting that EPA may be useful as a nutritional adjuvant in the treatment of BC [97].

Diets rich in EPA have also been shown to inhibit the growth of spontaneous or transplanted mammary carcinomas in animal models and human clinical trials. EPA was observed to slow tumor growth and reduced metastasis in mice implanted with KPL-1 human BC cells [143]. In a separate study, when compared with LA diet, intake of EPA significantly inhibited tumor cell proliferation and development of lung metastasis in mice with induced mammary tumorigenesis [20,126]. These inhibitory effects were attributed to high incorporation of EPA into tumor phospholipids, and subsequently, disrupting inflammatory eicosanoid biosynthesis from AA [20]. More recently, EPA was found to regulate cell proliferation in MCF-7 xenografts via an inhibitory G protein-coupled receptor-mediated signal transduction pathway [144]. Furthermore, a human clinical study demonstrated a strong positive correlation between plasma EPA concentrations and PPARγ mRNA levels in adipose tissue of obese subjects [145]. Since activators of PPARγ are known to inhibit cell proliferation and tumorigensis, up-regulation of PPARγ gene expression by EPA might be a potential mechanism of action. These findings suggest that EPA can independently act to inhibit the development and progression of human BC.

5.3. Individual Effect of DHA on Breast Cancer

Several independent reports have shown that DHA can inhibit mammary carcinoma development and progression (Tables 7 and 8). However, the specific mechanisms underlying these protective effects of DHA remain to be determined.

HER-2 signaling is central to many processes involved in cellular proliferation and survival [6]. Treatment with DHA alone *in vitro* was shown to be effective in disrupting lipid rafts in HER-2 overexpressing cells, inhibiting HER-2 activity and its downstream signaling molecules (Akt and FAS, *etc.*), and consequently led to cell death [6]. Menendez *et al.* demonstrated that exogenous supplementation with DHA was able to downregulate HER-2/neu oncogene expression in SK-Br3 and BT-474 human BC cells [5]. This supports the therapeutic potential of DHA supplementation in the treatment of HER-2 positive BC. On the other hand, recent evidence suggests that DHA itself is capable

of decreasing EGFR localization in the lipid rafts of the MDA-MB-231 BC cell line [56]. Moreover, DHA supplementation was reported to significantly enhance the efficacy of EGFR inhibitors, which provides strong evidence for the potential development of combination therapies targeting EGFR [56]. The pre-exposure with DHA has been shown to synergistically enhance the cytotoxicity of antimitotic drugs including Taxane and Taxol against highly metastatic BC cells [5]. This was attributed to the incorporation of DHA into cellular lipids, and subsequently altered membrane fluidity and function, thereby increasing drug intake [5]. In agreement with this, a recent human clinical trial demonstrated that addition of DHA into chemotherapy increased survival in metastatic BC patients [154].

A number of earlier studies have suggested that the anti-cancer property of DHA is attributable to its ability to inhibit cell growth and induce apoptosis. Kang *et al.* demonstrated that DHA induced apoptosis in MCF-7 cells through a combination of pathways [146]. Mechanistically, it was largely due to increased lipid peroxidation, followed by accumulation of reactive oxygen species in cancer cells and higher oxidative stress, ultimately resulting in cell apoptosis [146]. It was also possible that the elevated intracellular levels of DHA stimulated activation of apoptosis effector enzyme, such as caspase-8 and caspase-3, and thus inducing apoptotic cell death [146,147]. Furthermore, Chiu *et al.* found that pure DHA inhibited growth of MCF-7 cells [148]. Although DHA did not affect pro-apoptotic Bax protein, it induced the downregulation of anti-apoptotic Bcl-2 gene expression time-dependently, and thus increasing the Bax/Bcl-2 ratio [148]. DHA has also shown to suppress KPL-1 cell growth *in vitro*, accompanied by downregulation of Bcl-2 [149]. Since the ratio of Bax/Bcl-2 is positively associated with apoptotic activity, the regulation of Bax and Bcl-2 can be considered an important step in the apoptotic actions of DHA.

Animal studies also support the anti-cancer role of DHA in BC. Dietary supplementation of pure DHA was showed to suppress tumor development in both chemically-induced carcinoma and xenograft rodent models [150,151]. Low level DHA administration markedly reduced tumor growth rates and tumor weight compared with rats fed LA [150]. These observed suppressive effects of DHA resulted from diminished tumor eicosanoid concentrations and decreased cell proliferation [150]. DHA has also been shown to decrease mammary tumor incidence coinciding with a 60% increase in BRCA1 protein, a major tumor suppressor [151]. In complement, DHA supplementation was shown to increase BRCA1 at the transcriptional level [152]. The correlation between the *in vitro* and *in vivo* observations adds weight to DHA's potential mechanism and supports a beneficial role for DHA against BC.

Altogether, ALA, EPA and DHA have various effects against mammary tumor development *in vivo*. In terms of *in vitro* studies, EPA and DHA individually exert an inhibitory effect on the proliferation of almost all types of tumor cells *in vitro*; whereas ALA only has effects on ER− and HER-2 positive BC cells (Table 8). As little as 50 μM of EPA or 30 μM of DHA can dramatically reduce tumor cell viability, while 72 μM of ALA only cause moderate inhibition on ER− cell proliferation. However, it is not appropriate to compare the effective dosage of individual *n*-3 PUFA from different type of studies, since the amount of tumor cells and the duration of treatment are different. Additional experimental studies are needed to compare the efficacy of each individual *n*-3 PUFA under the same conditions.

6. Plant-Derived *n*-3 (ALA) *vs.* Marine-Based *n*-3 (EPA, DHA)

The potential health benefits of *n*-3 PUFA have been examined in various types of studies. However, the relative potency of plant-based *n*-3 *versus* marine *n*-3 PUFA, as well as EPA *versus* DHA in inhibiting tumor growth remains unclear. There are only two studies that have compared the efficacy between EPA and DHA [120,155]. The first study compared the ability of dietary EPA or DHA to suppress MNU-induced mammary carcinogenesis in a rat model [120]. Although treatment with DHA or EPA alone was not as effective in combination, DHA was more potent in delaying tumor onset and reducing tumor multiplicity relative to EPA [120]. In accordance, several BC cell line studies indicated that DHA was more effective in inhibiting MDA-MB-231 and MCF-7 cell proliferation and invasion than EPA at the same concentration [86,96,121,125,155].

In terms of the efficacy of plant-based *n*-3 PUFA, there has not yet been a human clinical study directly comparing ALA *versus* EPA or DHA in BC. To the best of our knowledge, only two cell culture studies have examined the effect of different types of *n*-3 PUFA. ALA was less effective compared with EPA or DHA, but ALA had moderate inhibitory effects in some BC cell lines [122,124]. This may be due to the conversion of ALA to EPA and DHA which would lower the amount of ALA incubated with cancer cells. In addition, the lower incorporation of ALA into the cellular lipid pool may also be a confounding factor.

7. Conclusions

In summary, the present review has assessed the anticancer effects of *n*-3 PUFA when consumed or treated individually, as well as in *n*-3 PUFA mixtures. ALA, EPA and DHA can differentially inhibit mammary tumor development by changing the cell membrane fatty acid composition, suppressing AA-derived eicosanoid biosynthesis and influencing signaling transcriptional pathways to inhibit cell proliferation and induce apoptosis. This review also provided evidence for using *n*-3 PUFA as a nutritional intervention in the treatment of BC to enhance conventional therapeutics, or potentially lowering effective doses.

Overall, in order to provide definitive recommendations, additional human studies are required. Long term studies tracking fish or *n*-3 PUFA intake are needed to demonstrate a role for *n*-3 PUFA in prevention. Also, additional clinical trials are needed to evaluate the effect *n*-3 PUFA on BC outcomes. Nevertheless, evidence does not indicate harm and all forms of *n*-3 PUFA may be included in a healthy diet.

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References

1. DeSantis, C.; Ma, J.; Bryan, L.; Jemal, A. Breast cancer statistics, 2013. *CA Cancer J. Clin.* **2014**, *64*, 52–62. [CrossRef] [PubMed]
2. Canadian Cancer Statistics. *Canadian Cancer Society's Advisory*; Canadian Cancer statistics: Toronto, ON, Canada, 2013.
3. Gerber, M. Omega-3 fatty acids and cancers: A systematic update review of epidemiological studies. *Br. J. Nutr.* **2012**, *107* (Suppl. 2), S228–S239. [CrossRef] [PubMed]
4. Iwasaki, M.; Tsugane, S. Risk factors for breast cancer: Epidemiological evidence from Japanese studies. *Cancer Sci.* **2011**, *102*, 1607–1614. [CrossRef] [PubMed]
5. Menendez, J.A.; Lupu, R.; Colomer, R. Exogenous supplementation with omega-3 polyunsaturated fatty acid docosahexaenoic acid (DHA; 22:6*n*-3) synergistically enhances taxane cytotoxicity and downregulates Her-2/neu (c-erbB-2) oncogene expression in human breast cancer cells. *Eur. J. Cancer Prev.* **2005**, *14*, 263–270. [CrossRef] [PubMed]
6. Ravacci, G.R.; Brentani, M.M.; Tortelli, T., Jr.; Torrinhas, R.S.; Saldanha, T.; Torres, E.A.; Waitzberg, D.L. Lipid raft disruption by docosahexaenoic acid induces apoptosis in transformed human mammary luminal epithelial cells harboring HER-2 overexpression. *J. Nutr. Biochem.* **2013**, *24*, 505–515. [CrossRef] [PubMed]
7. Zheng, J.S.; Hu, X.J.; Zhao, Y.M.; Yang, J.; Li, D. Intake of fish and marine *n*-3 polyunsaturated fatty acids and risk of breast cancer: Meta-analysis of data from 21 independent prospective cohort studies. *BMJ* **2013**, *346*. [CrossRef] [PubMed]
8. Simopoulos, A.P. The importance of the ratio of omega-6/omega-3 essential fatty acids. *Biomed. Pharmacother.* **2002**, *56*, 365–379. [CrossRef] [PubMed]
9. Saadatian-Elahi, M.; Norat, T.; Goudable, J.; Riboli, E. Biomarkers of dietary fatty acid intake and the risk of breast cancer: A meta-analysis. *Int. J. Cancer* **2004**, *111*, 584–591. [CrossRef] [PubMed]
10. Boyd, N.F.; Stone, J.; Vogt, K.N.; Connelly, B.S.; Martin, L.J.; Minkin, S. Dietary fat and breast cancer risk revisited: A meta-analysis of the published literature. *Br. J. Cancer* **2003**, *89*, 1672–1685. [CrossRef] [PubMed]

11. Anderson, B.M.; Ma, D.W. Are all *n*-3 polyunsaturated fatty acids created equal? *Lipids Health Dis.* **2009**, *8*, 33. [CrossRef] [PubMed]
12. Zou, Z.; Bidu, C.; Bellenger, S.; Narce, M.; Bellenger, J. *n*-3 polyunsaturated fatty acids and HER2-positive breast cancer: Interest of the fat-1 transgenic mouse model over conventional dietary supplementation. *Biochimie* **2014**, *96*, 22–27. [CrossRef] [PubMed]
13. Murray, M. ω-3 Polyunsaturated fatty acids and their metabolites as inhibitors of mammalian tumorigenesis. *Phytochem. Rev.* **2014**, *13*, 139–156. [CrossRef]
14. Akhtar, S.; Ismail, T.; Riaz, M. Flaxseed—A miraculous defense against some critical maladies. *Pak. J. Pharm. Sci.* **2013**, *26*, 199–208. [PubMed]
15. Kris-Etherton, P.M.; Harris, W.S.; Appel, L.J. Fish consumption, fish oil, omega-3 fatty acids, and cardiovascular disease. *Circulation* **2002**, *106*, 2747–2757. [CrossRef] [PubMed]
16. Institute of Medicine of the National Academies. *Dietary Reference Intakes for Energy, Carbohydrate, Fiber, Fat, Fatty Acids, Cholesterol, Protein, and Amino Acids*; The National Academies Press: Washington, DC, USA, 2005.
17. Vannice, G.; Rasmussen, H. Position of the academy of nutrition and dietetics: Dietary fatty acids for healthy adults. *J. Acad. Nutr. Diet.* **2014**, *114*, 136–153. [CrossRef] [PubMed]
18. Bagga, D.; Anders, K.H.; Wang, H.J.; Glaspy, J.A. Long-chain *n*-3-to-*n*-6 polyunsaturated fatty acid ratios in breast adipose tissue from women with and without breast cancer. *Nutr. Cancer* **2002**, *42*, 180–185. [CrossRef] [PubMed]
19. Chajes, V.; Torres-Mejia, G.; Biessy, C.; Ortega-Olvera, C.; ngeles-Llerenas, A.; Ferrari, P.; Lazcano-Ponce, E.; Romieu, I. omega-3 and omega-6 Polyunsaturated fatty acid intakes and the risk of breast cancer in Mexican women: Impact of obesity status. *Cancer Epidemiol. Biomark. Prev.* **2012**, *21*, 319–326. [CrossRef]
20. Rose, D.P.; Connolly, J.M.; Rayburn, J.; Coleman, M. Influence of diets containing eicosapentaenoic or docosahexaenoic acid on growth and metastasis of breast cancer cells in nude mice. *J. Natl. Cancer Inst.* **1995**, *87*, 587–592. [CrossRef] [PubMed]
21. Leslie, M.A.; Abdelmagid, S.A.; Perez, K.; Muller, W.J.; Ma, D.W. Mammary tumour development is dose-dependently inhibited by *n*-3 polyunsaturated fatty acids in the MMTV-neu(ndl)-YD5 transgenic mouse model. *Lipids Health Dis.* **2014**, *13*, 96. [CrossRef] [PubMed]
22. Schley, P.D.; Brindley, D.N.; Field, C.J. (*n*-3) PUFA alter raft lipid composition and decrease epidermal growth factor receptor levels in lipid rafts of human breast cancer cells. *J. Nutr.* **2007**, *137*, 548–553. [PubMed]
23. Silva, V.; Barazzoni, R.; Singer, P. Biomarkers of fish oil omega-3 polyunsaturated fatty acids intake in humans. *Nutr. Clin. Pract.* **2014**, *29*, 63–72. [CrossRef] [PubMed]
24. Ziegler, R.G.; Hoover, R.N.; Pike, M.C.; Hildesheim, A.; Nomura, A.M.; West, D.W.; Wu-Williams, A.H.; Kolonel, L.N.; Horn-Ross, P.L.; Rosenthal, J.F.; *et al.* Migration patterns and breast cancer risk in Asian-American women. *J. Natl. Cancer Inst.* **1993**, *85*, 1819–1827. [CrossRef]
25. Singh, G.K.; Hiatt, R.A. Trends and disparities in socioeconomic and behavioural characteristics, life expectancy, and cause-specific mortality of native-born and foreign-born populations in the United States, 1979–2003. *Int. J. Epidemiol.* **2006**, *35*, 903–919. [CrossRef] [PubMed]
26. Manna, S.; Janarthan, M.; Ghosh, B.; Rana, B.; Rana, A.; Chatterjee, M. Fish oil regulates cell proliferation, protect DNA damages and decrease HER-2/neu and c-Myc protein expression in rat mammary carcinogenesis. *Clin. Nutr.* **2010**, *29*, 531–537. [CrossRef] [PubMed]
27. Manna, S.; Chakraborty, T.; Ghosh, B.; Chatterjee, M.; Panda, A.; Srivastava, S.; Rana, A.; Chatterjee, M. Dietary fish oil associated with increased apoptosis and modulated expression of Bax and Bcl-2 during 7,12-dimethylbenz(α)anthracene-induced mammary carcinogenesis in rats. *Prostaglandins Leukot. Essent. Fatty Acids* **2008**, *79*, 5–14. [CrossRef] [PubMed]
28. Manna, S.; Chakraborty, T.; Damodaran, S.; Samanta, K.; Rana, B.; Chatterjee, M. Protective role of fish oil (Maxepa) on early events of rat mammary carcinogenesis by modulation of DNA-protein crosslinks, cell proliferation and p53 expression. *Cancer Cell Int.* **2007**, *7*, 6. [CrossRef] [PubMed]
29. Su, H.M.; Hsieh, P.H.; Chen, H.F. A maternal high *n*-6 fat diet with fish oil supplementation during pregnancy and lactation in rats decreases breast cancer risk in the female offspring. *J. Nutr. Biochem.* **2010**, *21*, 1033–1037. [CrossRef] [PubMed]
30. Hardman, W.E.; Avula, C.P.; Fernandes, G.; Cameron, I.L. Three percent dietary fish oil concentrate increased efficacy of doxorubicin against MDA-MB 231 breast cancer xenografts. *Clin. Cancer Res.* **2001**, *7*, 2041–2049. [PubMed]

31. Larsson, S.C.; Kumlin, M.; Ingelman-Sundberg, M.; Wolk, A. Dietary long-chain *n*-3 fatty acids for the prevention of cancer: A review of potential mechanisms. *Am. J. Clin. Nutr.* **2004**, *79*, 935–945. [PubMed]
32. Terry, P.D.; Rohan, T.E.; Wolk, A. Intakes of fish and marine fatty acids and the risks of cancers of the breast and prostate and of other hormone-related cancers: A review of the epidemiologic evidence. *Am. J. Clin. Nutr.* **2003**, *77*, 532–543. [PubMed]
33. Stoll, B.A. *n*-3 fatty acids and lipid peroxidation in breast cancer inhibition. *Br. J. Nutr.* **2002**, *87*, 193–198. [CrossRef] [PubMed]
34. MacLennan, M.; Ma, D.W. Role of dietary fatty acids in mammary gland development and breast cancer. *Breast Cancer Res.* **2010**, *12*, 211. [CrossRef] [PubMed]
35. Wakai, K.; Tamakoshi, K.; Date, C.; Fukui, M.; Suzuki, S.; Lin, Y.; Niwa, Y.; Nishio, K.; Yatsuya, H.; Kondo, T.; *et al.* Dietary intakes of fat and fatty acids and risk of breast cancer: A prospective study in Japan. *Cancer Sci.* **2005**, *96*, 590–599. [CrossRef] [PubMed]
36. Gago-Dominguez, M.; Yuan, J.M.; Sun, C.L.; Lee, H.P.; Yu, M.C. Opposing effects of dietary *n*-3 and *n*-6 fatty acids on mammary carcinogenesis: The Singapore Chinese Health Study. *Br. J. Cancer* **2003**, *89*, 1686–1692. [CrossRef] [PubMed]
37. Murff, H.J.; Shu, X.O.; Li, H.; Yang, G.; Wu, X.; Cai, H.; Wen, W.; Gao, Y.T.; Zheng, W. Dietary polyunsaturated fatty acids and breast cancer risk in Chinese women: A prospective cohort study. *Int. J. Cancer* **2011**, *128*, 1434–1441. [CrossRef] [PubMed]
38. Shannon, J.; King, I.B.; Lampe, J.W.; Gao, D.L.; Ray, R.M.; Lin, M.G.; Stalsberg, H.; Thomas, D.B. Erythrocyte fatty acids and risk of proliferative and nonproliferative fibrocystic disease in women in Shanghai, China. *Am. J. Clin. Nutr.* **2009**, *89*, 265–276. [CrossRef]
39. Kim, J.; Lim, S.Y.; Shin, A.; Sung, M.K.; Ro, J.; Kang, H.S.; Lee, K.S.; Kim, S.W.; Lee, E.S. Fatty fish and fish omega-3 fatty acid intakes decrease the breast cancer risk: A case-control study. *BMC Cancer* **2009**, *9*, 216. [CrossRef] [PubMed]
40. Brasky, T.M.; Lampe, J.W.; Potter, J.D.; Patterson, R.E.; White, E. Specialty supplements and breast cancer risk in the VITamins And Lifestyle (VITAL) Cohort. *Cancer Epidemiol. Biomark. Prev.* **2010**, *19*, 1696–1708. [CrossRef]
41. Goodstine, S.L.; Zheng, T.; Holford, T.R.; Ward, B.A.; Carter, D.; Owens, P.H.; Mayne, S.T. Dietary (*n*-3)/(*n*-6) fatty acid ratio: Possible relationship to premenopausal but not postmenopausal breast cancer risk in U.S. women. *J. Nutr.* **2003**, *133*, 1409–1414. [PubMed]
42. Thiebaut, A.C.; Chajes, V.; Gerber, M.; Boutron-Ruault, M.C.; Joulin, V.; Lenoir, G.; Berrino, F.; Riboli, E.; Benichou, J.; Clavel-Chapelon, F. Dietary intakes of omega-6 and omega-3 polyunsaturated fatty acids and the risk of breast cancer. *Int. J. Cancer* **2009**, *124*, 924–931. [CrossRef] [PubMed]
43. Stripp, C.; Overvad, K.; Christensen, J.; Thomsen, B.L.; Olsen, A.; Moller, S.; Tjonneland, A. Fish intake is positively associated with breast cancer incidence rate. *J. Nutr.* **2003**, *133*, 3664–3669. [PubMed]
44. Witt, P.M.; Christensen, J.H.; Schmidt, E.B.; Dethlefsen, C.; Tjonneland, A.; Overvad, K.; Ewertz, M. Marine *n*-3 polyunsaturated fatty acids in adipose tissue and breast cancer risk: A case-cohort study from Denmark. *Cancer Causes Control* **2009**, *20*, 1715–1721. [CrossRef] [PubMed]
45. Maillard, V.; Bougnoux, P.; Ferrari, P.; Jourdan, M.L.; Pinault, M.; Lavillonniere, F.; Body, G.; Le, F.O.; Chajes, V. *n*-3 and *n*-6 fatty acids in breast adipose tissue and relative risk of breast cancer in a case-control study in Tours, France. *Int. J. Cancer* **2002**, *98*, 78–83. [CrossRef] [PubMed]
46. Kuriki, K.; Hirose, K.; Wakai, K.; Matsuo, K.; Ito, H.; Suzuki, T.; Hiraki, A.; Saito, T.; Iwata, H.; Tatematsu, M.; *et al.* Breast cancer risk and erythrocyte compositions of *n*-3 highly unsaturated fatty acids in Japanese. *Int. J. Cancer* **2007**, *121*, 377–385. [CrossRef] [PubMed]
47. Shannon, J.; King, I.B.; Moshofsky, R.; Lampe, J.W.; Gao, D.L.; Ray, R.M.; Thomas, D.B. Erythrocyte fatty acids and breast cancer risk: A case-control study in Shanghai, China. *Am. J. Clin. Nutr.* **2007**, *85*, 1090–1097. [PubMed]
48. Patterson, R.E.; Flatt, S.W.; Newman, V.A.; Natarajan, L.; Rock, C.L.; Thomson, C.A.; Caan, B.J.; Parker, B.A.; Pierce, J.P. Marine fatty acid intake is associated with breast cancer prognosis. *J. Nutr.* **2011**, *141*, 201–206. [CrossRef] [PubMed]
49. Signori, C.; DuBrock, C.; Richie, J.P.; Prokopczyk, B.; Demers, L.M.; Hamilton, C.; Hartman, T.J.; Liao, J.; El-Bayoumy, K.; Manni, A. Administration of omega-3 fatty acids and Raloxifene to women at high risk of

breast cancer: Interim feasibility and biomarkers analysis from a clinical trial. *Eur. J. Clin. Nutr.* **2012**, *66*, 878–884. [CrossRef] [PubMed]
50. Thompson, L.U.; Chen, J.M.; Li, T.; Strasser-Weippl, K.; Goss, P.E. Dietary flaxseed alters tumor biological markers in postmenopausal breast cancer. *Clin. Cancer Res.* **2005**, *11*, 3828–3835. [CrossRef] [PubMed]
51. Menendez, J.A.; Lupu, R. Fatty acid synthase and the lipogenic phenotype in cancer pathogenesis. *Nat. Rev. Cancer* **2007**, *7*, 763–777. [CrossRef] [PubMed]
52. Gu, Z.; Suburu, J.; Chen, H.; Chen, Y.Q. Mechanisms of omega-3 polyunsaturated fatty acids in prostate cancer prevention. *Biomed. Res. Int.* **2013**, *2013*. [CrossRef] [PubMed]
53. Wassall, S.R.; Stillwell, W. Docosahexaenoic acid domains: The ultimate non-raft membrane domain. *Chem. Phys. Lipids* **2008**, *153*, 57–63. [CrossRef] [PubMed]
54. Chenais, B.; Blanckaert, V. The Janus face of lipids in human breast cancer: How polyunsaturated Fatty acids affect tumor cell hallmarks. *Int. J. Breast Cancer* **2012**, *2012*. [CrossRef]
55. Murai, T. The role of lipid rafts in cancer cell adhesion and migration. *Int. J. Cell Biol.* **2012**, *2012*. [CrossRef]
56. Rogers, K.R.; Kikawa, K.D.; Mouradian, M.; Hernandez, K.; McKinnon, K.M.; Ahwah, S.M.; Pardini, R.S. Docosahexaenoic acid alters epidermal growth factor receptor-related signaling by disrupting its lipid raft association. *Carcinogenesis* **2010**, *31*, 1523–1530. [CrossRef] [PubMed]
57. Ma, D.W.; Seo, J.; Davidson, L.A.; Callaway, E.S.; Fan, Y.Y.; Lupton, J.R.; Chapkin, R.S. *n*-3 PUFA alter caveolae lipid composition and resident protein localization in mouse colon. *FASEB J.* **2004**, *18*, 1040–1042. [CrossRef] [PubMed]
58. Burdge, G.C.; Calder, P.C. Conversion of α-linolenic acid to longer-chain polyunsaturated fatty acids in human adults. *Reprod. Nutr. Dev.* **2005**, *45*, 581–597. [CrossRef] [PubMed]
59. Young, L.R.; Kurzer, M.S.; Thomas, W.; Redmon, J.B.; Raatz, S.K. Effect of dietary fat and omega-3 fatty acids on urinary eicosanoids and sex hormone concentrations in postmenopausal women: A randomized controlled feeding trial. *Nutr. Cancer* **2011**, *63*, 930–939. [CrossRef] [PubMed]
60. Healy, D.A.; Wallace, F.A.; Miles, E.A.; Calder, P.C.; Newsholm, P. Effect of low-to-moderate amounts of dietary fish oil on neutrophil lipid composition and function. *Lipids* **2000**, *35*, 763–768. [CrossRef] [PubMed]
61. Benoit, V.; Relic, B.; Leval, X.X.; Chariot, A.; Merville, M.P.; Bours, V. Regulation of HER-2 oncogene expression by cyclooxygenase-2 and prostaglandin E2. *Oncogene* **2004**, *23*, 1631–1635. [CrossRef] [PubMed]
62. Richards, J.A.; Brueggemeier, R.W. Prostaglandin E2 regulates aromatase activity and expression in human adipose stromal cells via two distinct receptor subtypes. *J. Clin. Endocrinol. Metab.* **2003**, *88*, 2810–2816. [CrossRef] [PubMed]
63. Kelley, D.S.; Taylor, P.C.; Nelson, G.J.; Schmidt, P.C.; Ferretti, A.; Erickson, K.L.; Yu, R.; Chandra, R.K.; Mackey, B.E. Docosahexaenoic acid ingestion inhibits natural killer cell activity and production of inflammatory mediators in young healthy men. *Lipids* **1999**, *34*, 317–324. [CrossRef] [PubMed]
64. Wei, N.; Wang, B.; Zhang, Q.Y.; Mi, M.T.; Zhu, J.D.; Yu, X.P.; Yuan, J.L.; Chen, K.; Wang, J.; Chang, H. Effects of different dietary fatty acids on the fatty acid compositions and the expression of lipid metabolic-related genes in mammary tumor tissues of rats. *Nutr. Cancer* **2008**, *60*, 810–825. [CrossRef] [PubMed]
65. Serhan, C.N. Novel eicosanoid and docosanoid mediators: Resolvins, docosatrienes, and neuroprotectins. *Curr. Opin. Clin. Nutr. Metab. Care* **2005**, *8*, 115–121. [CrossRef] [PubMed]
66. Serhan, C.N.; Arita, M.; Hong, S.; Gotlinger, K. Resolvins, docosatrienes, and neuroprotectins, novel omega-3-derived mediators, and their endogenous aspirin-triggered epimers. *Lipids* **2004**, *39*, 1125–1132. [CrossRef] [PubMed]
67. Weylandt, K.H.; Kang, J.X.; Wiedenmann, B.; Baumgart, D.C. Lipoxins and resolvins in inflammatory bowel disease. *Inflamm. Bowel. Dis.* **2007**, *13*, 797–799. [CrossRef] [PubMed]
68. Kang, J.X.; Weylandt, K.H. Modulation of inflammatory cytokines by omega-3 fatty acids. *Subcell. Biochem.* **2008**, *49*, 133–143. [PubMed]
69. Calder, P.C. *n*-3 polyunsaturated fatty acids, inflammation, and inflammatory diseases. *Am. J. Clin. Nutr.* **2006**, *83*, 1505S–1519S. [PubMed]
70. Zandi, R.; Larsen, A.B.; Andersen, P.; Stockhausen, M.T.; Poulsen, H.S. Mechanisms for oncogenic activation of the epidermal growth factor receptor. *Cell Signal.* **2007**, *19*, 2013–2023. [CrossRef] [PubMed]
71. Corsetto, P.A.; Montorfano, G.; Zava, S.; Jovenitti, I.E.; Cremona, A.; Berra, B.; Rizzo, A.M. Effects of *n*-3 PUFAs on breast cancer cells through their incorporation in plasma membrane. *Lipids Health Dis.* **2011**, *10*, 73. [CrossRef] [PubMed]

72. Bhargava, R.; Gerald, W.L.; Li, A.R.; Pan, Q.; Lal, P.; Ladanyi, M.; Chen, B. EGFR gene amplification in breast cancer: Correlation with epidermal growth factor receptor mRNA and protein expression and HER-2 status and absence of EGFR-activating mutations. *Mod. Pathol.* **2005**, *18*, 1027–1033. [CrossRef] [PubMed]
73. Hynes, N.E.; Stern, D.F. The biology of erbB-2/neu/HER-2 and its role in cancer. *Biochim. Biophys. Acta* **1994**, *1198*, 165–184. [PubMed]
74. Tovey, S.M.; Brown, S.; Doughty, J.C.; Mallon, E.A.; Cooke, T.G.; Edwards, J. Poor survival outcomes in HER2-positive breast cancer patients with low-grade, node-negative tumours. *Br. J. Cancer* **2009**, *100*, 680–683. [CrossRef] [PubMed]
75. Moasser, M.M. The oncogene HER2: Its signaling and transforming functions and its role in human cancer pathogenesis. *Oncogene* **2007**, *26*, 6469–6487. [CrossRef] [PubMed]
76. Bollig-Fischer, A.; Dziubinski, M.; Boyer, A.; Haddad, R.; Giroux, C.N.; Ethier, S.P. HER-2 signaling, acquisition of growth factor independence, and regulation of biological networks associated with cell transformation. *Cancer Res.* **2010**, *70*, 7862–7873. [CrossRef] [PubMed]
77. Yee, L.D.; Agarwal, D.; Rosol, T.J.; Lehman, A.; Tian, M.; Hatton, J.; Heestand, J.; Belury, M.A.; Clinton, S.K. The inhibition of early stages of HER-2/neu-mediated mammary carcinogenesis by dietary *n*-3 PUFAs. *Mol. Nutr. Food Res.* **2013**, *57*, 320–327. [CrossRef] [PubMed]
78. Truan, J.S.; Chen, J.M.; Thompson, L.U. Flaxseed oil reduces the growth of human breast tumors (MCF-7) at high levels of circulating estrogen. *Mol. Nutr. Food Res.* **2010**, *54*, 1414–1421. [CrossRef] [PubMed]
79. Desvergne, B.; Wahli, W. Peroxisome proliferator-activated receptors: Nuclear control of metabolism. *Endocr. Rev.* **1999**, *20*, 649–688. [PubMed]
80. Georgiadi, A.; Kersten, S. Mechanisms of gene regulation by fatty acids. *Adv. Nutr.* **2012**, *3*, 127–134. [CrossRef] [PubMed]
81. Gurnell, M.; Savage, D.B.; Chatterjee, V.K.; O'Rahilly, S. The metabolic syndrome: Peroxisome proliferator-activated receptor gamma and its therapeutic modulation. *J. Clin. Endocrinol. Metab.* **2003**, *88*, 2412–2421. [CrossRef] [PubMed]
82. Flachs, P.; Rossmeisl, M.; Bryhn, M.; Kopecky, J. Cellular and molecular effects of *n*-3 polyunsaturated fatty acids on adipose tissue biology and metabolism. *Clin. Sci.* **2009**, *116*, 1–16. [CrossRef] [PubMed]
83. Kliewer, S.A.; Sundseth, S.S.; Jones, S.A.; Brown, P.J.; Wisely, G.B.; Koble, C.S.; Devchand, P.; Wahli, W.; Willson, T.M.; Lenhard, J.M.; *et al.* Fatty acids and eicosanoids regulate gene expression through direct interactions with peroxisome proliferator-activated receptors alpha and gamma. *Proc. Natl. Acad. Sci. USA* **1997**, *94*, 4318–4323. [CrossRef] [PubMed]
84. Clay, C.E.; Namen, A.M.; Atsumi, G.; Willingham, M.C.; High, K.P.; Kute, T.E.; Trimboli, A.J.; Fonteh, A.N.; Dawson, P.A.; Chilton, F.H. Influence of J series prostaglandins on apoptosis and tumorigenesis of breast cancer cells. *Carcinogenesis* **1999**, *20*, 1905–1911. [CrossRef] [PubMed]
85. Mueller, E.; Sarraf, P.; Tontonoz, P.; Evans, R.M.; Martin, K.J.; Zhang, M.; Fletcher, C.; Singer, S.; Spiegelman, B.M. Terminal differentiation of human breast cancer through PPAR gamma. *Mol. Cell* **1998**, *1*, 465–470. [CrossRef] [PubMed]
86. Barascu, A.; Besson, P.; Le, F.O.; Bougnoux, P.; Jourdan, M.L. CDK1-cyclin B1 mediates the inhibition of proliferation induced by omega-3 fatty acids in MDA-MB-231 breast cancer cells. *Int. J. Biochem. Cell Biol.* **2006**, *38*, 196–208. [CrossRef] [PubMed]
87. Jiang, W.; Zhu, Z.; McGinley, J.N.; El, B.K.; Manni, A.; Thompson, H.J. Identification of a molecular signature underlying inhibition of mammary carcinoma growth by dietary *n*-3 fatty acids. *Cancer Res.* **2012**, *72*, 3795–3806. [CrossRef] [PubMed]
88. Elmore, S. Apoptosis: A review of programmed cell death. *Toxicol. Pathol.* **2007**, *35*, 495–516. [CrossRef] [PubMed]
89. Jacobson, M.D.; Burne, J.F.; King, M.P.; Miyashita, T.; Reed, J.C.; Raff, M.C. Bcl-2 blocks apoptosis in cells lacking mitochondrial DNA. *Nature* **1993**, *361*, 365–369. [CrossRef] [PubMed]
90. Van Slooten, H.J.; Clahsen, P.C.; van Dierendonck, J.H.; Duval, C.; Pallud, C.; Mandard, A.M.; obelle-Deroide, A.; van de Velde, C.J.; van de Vijver, M.J. Expression of Bcl-2 in node-negative breast cancer is associated with various prognostic factors, but does not predict response to one course of perioperative chemotherapy. *Br. J. Cancer* **1996**, *74*, 78–85. [CrossRef] [PubMed]
91. Oltvai, Z.N.; Milliman, C.L.; Korsmeyer, S.J. Bcl-2 heterodimerizes *in vivo* with a conserved homolog, Bax, that accelerates programmed cell death. *Cell* **1993**, *74*, 609–619. [CrossRef] [PubMed]

92. Raisova, M.; Hossini, A.M.; Eberle, J.; Riebeling, C.; Wieder, T.; Sturm, I.; Daniel, P.T.; Orfanos, C.E.; Geilen, C.C. The Bax/Bcl-2 ratio determines the susceptibility of human melanoma cells to CD95/Fas-mediated apoptosis. *J. Investig. Dermatol.* **2001**, *117*, 333–340. [CrossRef] [PubMed]
93. Ion, G.; Akinsete, J.A.; Hardman, W.E. Maternal consumption of canola oil suppressed mammary gland tumorigenesis in C3(1) TAg mice offspring. *BMC Cancer* **2010**, *10*, 81. [CrossRef] [PubMed]
94. Vivanco, I.; Sawyers, C.L. The phosphatidylinositol 3-Kinase AKT pathway in human cancer. *Nat. Rev. Cancer* **2002**, *2*, 489–501. [CrossRef] [PubMed]
95. Bai, D.; Ueno, L.; Vogt, P.K. Akt-mediated regulation of NFκB and the essentialness of NFκB for the oncogenicity of PI3K and Akt. *Int. J. Cancer* **2009**, *125*, 2863–2870. [CrossRef] [PubMed]
96. Schley, P.D.; Jijon, H.B.; Robinson, L.E.; Field, C.J. Mechanisms of omega-3 fatty acid-induced growth inhibition in MDA-MB-231 human breast cancer cells. *Breast Cancer Res. Treat.* **2005**, *92*, 187–195. [CrossRef] [PubMed]
97. DeGraffenried, L.A.; Friedrichs, W.E.; Fulcher, L.; Fernandes, G.; Silva, J.M.; Peralba, J.M.; Hidalgo, M. Eicosapentaenoic acid restores tamoxifen sensitivity in breast cancer cells with high Akt activity. *Ann. Oncol.* **2003**, *14*, 1051–1056. [CrossRef] [PubMed]
98. Nicholson, K.M.; Anderson, N.G. The protein kinase B/Akt signalling pathway in human malignancy. *Cell Signal.* **2002**, *14*, 381–395. [CrossRef] [PubMed]
99. Hussain, A.R.; Ahmed, S.O.; Ahmed, M.; Khan, O.S.; Al, A.S.; Platanias, L.C.; Al-Kuraya, K.S.; Uddin, S. Cross-talk between NFκB and the PI3-kinase/AKT pathway can be targeted in primary effusion lymphoma (PEL) cell lines for efficient apoptosis. *PLoS One* **2012**, *7*. [CrossRef] [PubMed]
100. Dolcet, X.; Llobet, D.; Pallares, J.; Matias-Guiu, X. NF-κB in development and progression of human cancer. *Virchows Arch.* **2005**, *446*, 475–482. [CrossRef] [PubMed]
101. Wu, J.T.; Kral, J.G. The NF-κB/IκB signaling system: A molecular target in breast cancer therapy. *J. Surg. Res.* **2005**, *123*, 158–169. [CrossRef] [PubMed]
102. Mayo, M.W.; Wang, C.Y.; Cogswell, P.C.; Rogers-Graham, K.S.; Lowe, S.W.; Der, C.J.; Baldwin, A.S., Jr. Requirement of NF-κB activation to suppress p53-independent apoptosis induced by oncogenic Ras. *Science* **1997**, *278*, 1812–1815. [CrossRef] [PubMed]
103. Yerushalmi, R.; Woods, R.; Ravdin, P.M.; Hayes, M.M.; Gelmon, K.A. Ki67 in breast cancer: Prognostic and predictive potential. *Lancet Oncol.* **2010**, *11*, 174–183. [CrossRef] [PubMed]
104. Scholzen, T.; Gerdes, J. The Ki-67 protein: From the known and the unknown. *J. Cell. Physiol.* **2000**, *182*, 311–322. [CrossRef] [PubMed]
105. Saggar, J.K.; Chen, J.; Corey, P.; Thompson, L.U. Dietary flaxseed lignan or oil combined with tamoxifen treatment affects MCF-7 tumor growth through estrogen receptor- and growth factor-signaling pathways. *Mol. Nutr. Food Res.* **2010**, *54*, 415–425. [CrossRef] [PubMed]
106. Taftachi, R.; Ayhan, A.; Ekici, S.; Ergen, A.; Ozen, H. Proliferating-cell nuclear antigen (PCNA) as an independent prognostic marker in patients after prostatectomy: A comparison of PCNA and Ki-67. *BJU Int.* **2005**, *95*, 650–654. [CrossRef] [PubMed]
107. Paunesku, T.; Mittal, S.; Protić, M.; Oryhon, J.; Korolev, S.V.; Joachimiak, A.; Woloschak, G.E. Proliferating cell nuclear antigen (PCNA): Ringmaster of the genome. *Int. J. Radiat. Biol.* **2001**, *77*, 1007–1021. [CrossRef] [PubMed]
108. Malkas, L.H.; Herbert, B.S.; bdel-Aziz, W.; Dobrolecki, L.E.; Liu, Y.; Agarwal, B.; Hoelz, D.; Badve, S.; Schnaper, L.; Arnold, R.J.; *et al.* A cancer-associated PCNA expressed in breast cancer has implications as a potential biomarker. *Proc. Natl. Acad. Sci. USA* **2006**, *103*, 19472–19477. [CrossRef] [PubMed]
109. Strzalka, W.; Ziemienowicz, A. Proliferating cell nuclear antigen (PCNA): A key factor in DNA replication and cell cycle regulation. *Ann. Bot.* **2011**, *107*, 1127–1140. [CrossRef] [PubMed]
110. Olivo, S.E.; Hilakivi-Clarke, L. Opposing effects of prepubertal low- and high-fat *n*-3 polyunsaturated fatty acid diets on rat mammary tumorigenesis. *Carcinogenesis* **2005**, *26*, 1563–1572. [CrossRef] [PubMed]
111. Kim, I.S.; Baek, S.H. Mouse models for breast cancer metastasis. *Biochem. Biophys. Res. Commun.* **2010**, *394*, 443–447. [CrossRef] [PubMed]
112. Karmali, R.A.; Marsh, J.; Fuchs, C. Effect of omega-3 fatty acids on growth of a rat mammary tumor. *J. Natl. Cancer Inst.* **1984**, *73*, 457–461. [PubMed]
113. Gonzalez, M.J.; Schemmel, R.A.; Gray, J.I.; Dugan, L., Jr.; Sheffield, L.G.; Welsch, C.W. Effect of dietary fat on growth of MCF-7 and MDA-MB231 human breast carcinomas in athymic nude mice: Relationship between

carcinoma growth and lipid peroxidation product levels. *Carcinogenesis* **1991**, *12*, 1231–1235. [CrossRef] [PubMed]
114. Hubbard, N.E.; Lim, D.; Erickson, K.L. Alteration of murine mammary tumorigenesis by dietary enrichment with *n*-3 fatty acids in fish oil. *Cancer Lett.* **1998**, *124*, 1–7. [CrossRef] [PubMed]
115. Yee, L.D.; Young, D.C.; Rosol, T.J.; Vanbuskirk, A.M.; Clinton, S.K. Dietary (*n*-3) polyunsaturated fatty acids inhibit HER-2/neu-induced breast cancer in mice independently of the PPARgamma ligand rosiglitazone. *J. Nutr.* **2005**, *135*, 983–988. [PubMed]
116. MacLennan, M.B.; Clarke, S.E.; Perez, K.; Wood, G.A.; Muller, W.J.; Kang, J.X.; Ma, D.W. Mammary tumor development is directly inhibited by lifelong *n*-3 polyunsaturated fatty acids. *J. Nutr. Biochem.* **2013**, *24*, 388–395. [CrossRef] [PubMed]
117. Dankort, D.; Maslikowski, B.; Warner, N.; Kanno, N.; Kim, H.; Wang, Z.; Moran, M.F.; Oshima, R.G.; Cardiff, R.D.; Muller, W.J. Grb2 and Shc adapter proteins play distinct roles in Neu (ErbB-2)-induced mammary tumorigenesis: Implications for human breast cancer. *Mol. Cell Biol.* **2001**, *21*, 1540–1551. [CrossRef] [PubMed]
118. Yee, L.D.; Lester, J.L.; Cole, R.M.; Richardson, J.R.; Hsu, J.C.; Li, Y.; Lehman, A.; Belury, M.A.; Clinton, S.K. Omega-3 fatty acid supplements in women at high risk of breast cancer have dose-dependent effects on breast adipose tissue fatty acid composition. *Am. J. Clin. Nutr.* **2010**, *91*, 1185–1194. [CrossRef] [PubMed]
119. Luijten, M.; Verhoef, A.; Dormans, J.A.; Beems, R.B.; Cremers, H.W.; Nagelkerke, N.J.; Adlercreutz, H.; Penalvo, J.L.; Piersma, A.H. Modulation of mammary tumor development in Tg.NK (MMTV/c-neu) mice by dietary fatty acids and life stage-specific exposure to phytoestrogens. *Reprod. Toxicol.* **2007**, *23*, 407–413. [CrossRef] [PubMed]
120. Yuri, T.; Danbara, N.; Tsujita-Kyutoku, M.; Fukunaga, K.; Takada, H.; Inoue, Y.; Hada, T.; Tsubura, A. Dietary docosahexaenoic acid suppresses *N*-methyl-*N*-nitrosourea-induced mammary carcinogenesis in rats more effectively than eicosapentaenoic acid. *Nutr. Cancer* **2003**, *45*, 211–217. [CrossRef] [PubMed]
121. Rose, D.P.; Connolly, J.M. Effects of fatty acids and inhibitors of eicosanoid synthesis on the growth of a human breast cancer cell line in culture. *Cancer Res.* **1990**, *50*, 7139–7144. [PubMed]
122. Grammatikos, S.I.; Subbaiah, P.V.; Victor, T.A.; Miller, W.M. *n*-3 and *n*-6 fatty acid processing and growth effects in neoplastic and non-cancerous human mammary epithelial cell lines. *Br. J. Cancer* **1994**, *70*, 219–227. [CrossRef] [PubMed]
123. Kachhap, S.K.; Dange, P.P.; Santani, R.H.; Sawant, S.S.; Ghosh, S.N. Effect of omega-3 fatty acid (docosahexanoic acid) on BRCA1 gene expression and growth in MCF-7 cell line. *Cancer Biother. Radiopharm.* **2001**, *16*, 257–263. [CrossRef] [PubMed]
124. Chajes, V.; Sattler, W.; Stranzl, A.; Kostner, G.M. Influence of *n*-3 fatty acids on the growth of human breast cancer cells *in vitro*: Relationship to peroxides and vitamin-E. *Breast Cancer Res. Treat.* **1995**, *34*, 199–212. [CrossRef] [PubMed]
125. Chamras, H.; Ardashian, A.; Heber, D.; Glaspy, J.A. Fatty acid modulation of MCF-7 human breast cancer cell proliferation, apoptosis and differentiation. *J. Nutr. Biochem.* **2002**, *13*, 711–716. [CrossRef] [PubMed]
126. Rose, D.P.; Connolly, J.M.; Coleman, M. Effect of ω-3 fatty acids on the progression of metastases after the surgical excision of human breast cancer cell solid tumors growing in nude mice. *Clin. Cancer Res.* **1996**, *2*, 1751–1756. [PubMed]
127. Arterburn, L.M.; Hall, E.B.; Oken, H. Distribution, interconversion, and dose response of *n*-3 fatty acids in humans. *Am. J. Clin. Nutr.* **2006**, *83*, 1467S–1476S. [PubMed]
128. Harper, C.R.; Edwards, M.J.; DeFilippis, A.P.; Jacobson, T.A. Flaxseed oil increases the plasma concentrations of cardioprotective (*n*-3) fatty acids in humans. *J. Nutr.* **2006**, *136*, 83–87. [PubMed]
129. Pawlosky, R.J.; Hibbeln, J.R.; Novotny, J.A.; Salem, N., Jr. Physiological compartmental analysis of α-linolenic acid metabolism in adult humans. *J. Lipid Res.* **2001**, *42*, 1257–1265. [PubMed]
130. Brenna, J.T. Efficiency of conversion of α-linolenic acid to long chain *n*-3 fatty acids in man. *Curr. Opin. Clin. Nutr. Metab Care* **2002**, *5*, 127–132. [CrossRef] [PubMed]
131. Ezaki, O.; Takahashi, M.; Shigematsu, T.; Shimamura, K.; Kimura, J.; Ezaki, H.; Gotoh, T. Long-term effects of dietary α-linolenic acid from perilla oil on serum fatty acids composition and on the risk factors of coronary heart disease in Japanese elderly subjects. *J. Nutr. Sci. Vitaminol.* **1999**, *45*, 759–772. [CrossRef] [PubMed]
132. Ghafoorunissa; Vani, A.; Laxmi, R.; Sesikeran, B. Effects of dietary α-linolenic acid from blended oils on biochemical indices of coronary heart disease in Indians. *Lipids* **2002**, *37*, 1077–1086. [CrossRef] [PubMed]

133. Klein, V.; Chajes, V.; Germain, E.; Schulgen, G.; Pinault, M.; Malvy, D.; Lefrancq, T.; Fignon, A.; Le, F.O.; Lhuillery, C.; Bougnoux, P. Low α-linolenic acid content of adipose breast tissue is associated with an increased risk of breast cancer. *Eur. J. Cancer* **2000**, *36*, 335–340. [CrossRef] [PubMed]
134. Bougnoux, P.; Koscielny, S.; Chajes, V.; Descamps, P.; Couet, C.; Calais, G. α-Linolenic acid content of adipose breast tissue: A host determinant of the risk of early metastasis in breast cancer. *Br. J. Cancer* **1994**, *70*, 330–334. [CrossRef] [PubMed]
135. Fritsche, K.L.; Johnston, P.V. Effect of dietary α-linolenic acid on growth, metastasis, fatty acid profile and prostaglandin production of two murine mammary adenocarcinomas. *J. Nutr.* **1990**, *120*, 1601–1609. [PubMed]
136. Hardman, W.E. Dietary canola oil suppressed growth of implanted MDA-MB 231 human breast tumors in nude mice. *Nutr. Cancer* **2007**, *57*, 177–183. [CrossRef]
137. Mabasa, L.; Cho, K.; Walters, M.W.; Bae, S.; Park, C.S. Maternal dietary canola oil suppresses growth of mammary carcinogenesis in female rat offspring. *Nutr. Cancer* **2013**, *65*, 695–701. [CrossRef] [PubMed]
138. Menendez, J.A.; Vazquez-Martin, A.; Ropero, S.; Colomer, R.; Lupu, R. HER2 (erbB-2)-targeted effects of the omega-3 polyunsaturated fatty acid, α-linolenic acid (ALA; 18:3*n*-3), in breast cancer cells: The "fat features" of the "Mediterranean diet" as an "anti-HER2 cocktail". *Clin. Transl. Oncol.* **2006**, *8*, 812–820. [CrossRef] [PubMed]
139. Menendez, J.A.; Ropero, S.; Lupu, R.; Colomer, R. Dietary fatty acids regulate the activation status of Her-2/neu (c-erbB-2) oncogene in breast cancer cells. *Ann. Oncol.* **2004**, *15*, 1719–1721. [CrossRef] [PubMed]
140. Mason, J.K.; Fu, M.H.; Chen, J.; Yu, Z.; Thompson, L.U. Dietary flaxseed-trastuzumab interactive effects on the growth of HER2-overexpressing human breast tumors (BT-474). *Nutr. Cancer* **2013**, *65*, 451–459. [CrossRef] [PubMed]
141. Hirose, M.; Masuda, A.; Ito, N.; Kamano, K.; Okuyama, H. Effects of dietary perilla oil, soybean oil and safflower oil on 7,12-dimethylbenz (*a*) anthracene (DMBA) and 1,2-dimethyl-hydrazine (DMH)-induced mammary gland and colon carcinogenesis in female SD rats. *Carcinogenesis* **1990**, *11*, 731–735. [CrossRef] [PubMed]
142. Chiu, L.C.; Wan, J.M. Induction of apoptosis in HL-60 cells by eicosapentaenoic acid (EPA) is associated with downregulation of bcl-2 expression. *Cancer Lett.* **1999**, *145*, 17–27. [CrossRef] [PubMed]
143. Senzaki, H.; Iwamoto, S.; Ogura, E.; Kiyozuka, Y.; Arita, S.; Kurebayashi, J.; Takada, H.; Hioki, K.; Tsubura, A. Dietary effects of fatty acids on growth and metastasis of KPL-1 human breast cancer cells in vivo and in vitro. *Anticancer Res.* **1998**, *18*, 1621–1627. [PubMed]
144. Sauer, L.A.; Dauchy, R.T.; Blask, D.E.; Krause, J.A.; Davidson, L.K.; Dauchy, E.M. Eicosapentaenoic acid suppresses cell proliferation in MCF-7 human breast cancer xenografts in nude rats via a pertussis toxin-sensitive signal transduction pathway. *J. Nutr.* **2005**, *135*, 2124–2129. [PubMed]
145. Chambrier, C.; Bastard, J.P.; Rieusset, J.; Chevillotte, E.; Bonnefont-Rousselot, D.; Therond, P.; Hainque, B.; Riou, J.P.; Laville, M.; Vidal, H. Eicosapentaenoic acid induces mRNA expression of peroxisome proliferator-activated receptor gamma. *Obes. Res.* **2002**, *10*, 518–525. [CrossRef] [PubMed]
146. Kang, K.S.; Wang, P.; Yamabe, N.; Fukui, M.; Jay, T.; Zhu, B.T. Docosahexaenoic acid induces apoptosis in MCF-7 cells *in vitro* and *in vivo* via reactive oxygen species formation and caspase 8 activation. *PLoS One* **2010**, *5*. [CrossRef] [PubMed]
147. Blanckaert, V.; Ulmann, L.; Mimouni, V.; Antol, J.; Brancquart, L.; Chenais, B. Docosahexaenoic acid intake decreases proliferation, increases apoptosis and decreases the invasive potential of the human breast carcinoma cell line MDA-MB-231. *Int. J. Oncol.* **2010**, *36*, 737–742. [CrossRef] [PubMed]
148. Chiu, L.C.; Wong, E.Y.; Ooi, V.E. Docosahexaenoic acid from a cultured microalga inhibits cell growth and induces apoptosis by upregulating Bax/Bcl-2 ratio in human breast carcinoma MCF-7 cells. *Ann. N. Y. Acad. Sci.* **2004**, *1030*, 361–368. [CrossRef] [PubMed]
149. Tsujita-Kyutoku, M.; Yuri, T.; Danbara, N.; Senzaki, H.; Kiyozuka, Y.; Uehara, N.; Takada, H.; Hada, T.; Miyazawa, T.; Ogawa, Y.; *et al.* Conjugated docosahexaenoic acid suppresses KPL-1 human breast cancer cell growth *in vitro* and *in vivo*: Potential mechanisms of action. *Breast Cancer Res.* **2004**, *6*, R291–R299. [CrossRef] [PubMed]
150. Connolly, J.M.; Gilhooly, E.M.; Rose, D.P. Effects of reduced dietary linoleic acid intake, alone or combined with an algal source of docosahexaenoic acid, on MDA-MB-231 breast cancer cell growth and apoptosis in nude mice. *Nutr. Cancer* **1999**, *35*, 44–49. [CrossRef] [PubMed]

151. Jourdan, M.L.; Maheo, K.; Barascu, A.; Goupille, C.; de Latour, M.P.; Bougnoux, P.; Rio, P.G. Increased BRCA1 protein in mammary tumours of rats fed marine omega-3 fatty acids. *Oncol. Rep.* **2007**, *17*, 713–719. [PubMed]
152. Bernard-Gallon, D.J.; Vissac-Sabatier, C.; ntoine-Vincent, D.; Rio, P.G.; Maurizis, J.C.; Fustier, P.; Bignon, Y.J. Differential effects of *n*-3 and *n*-6 polyunsaturated fatty acids on BRCA1 and BRCA2 gene expression in breast cell lines. *Br. J. Nutr.* **2002**, *87*, 281–289. [CrossRef] [PubMed]
153. Bougnoux, P.; Chajes, V.; Germain, E.; Hubert, B.; Lhuillery, C.; Le, F.O.; Body, G.; Calais, G. Cytotoxic drug efficacy correlates with adipose tissue docosahexaenoic acid level in locally advanced breast carcinoma. *Lipids* **1999**, *34*, S109. [CrossRef] [PubMed]
154. Bougnoux, P.; Hajjaji, N.; Ferrasson, M.N.; Giraudeau, B.; Couet, C.; Le, F.O. Improving outcome of chemotherapy of metastatic breast cancer by docosahexaenoic acid: A phase II trial. *Br. J. Cancer* **2009**, *101*, 1978–1985. [CrossRef] [PubMed]
155. Rahman, M.M.; Veigas, J.M.; Williams, P.J.; Fernandes, G. DHA is a more potent inhibitor of breast cancer metastasis to bone and related osteolysis than EPA. *Breast Cancer Res. Treat.* **2013**, *141*, 341–352. [CrossRef] [PubMed]

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Review

Evidence and Mechanisms of Fat Depletion in Cancer

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Abstract: The majority of cancer patients experience wasting characterized by muscle loss with or without fat loss. In human and animal models of cancer, body composition assessment and morphological analysis reveals adipose atrophy and presence of smaller adipocytes. Fat loss is associated with reduced quality of life in cancer patients and shorter survival independent of body mass index. Fat loss occurs in both visceral and subcutaneous depots; however, the pattern of loss has been incompletely characterized. Increased lipolysis and fat oxidation, decreased lipogenesis, impaired lipid depositionand adipogenesis, as well as browning of white adipose tissue may underlie adipose atrophy in cancer. Inflammatory cytokines such as interleukin-6 (IL-6), tumor necrosis factor alpha (TNF-α), and interleukin-1 beta (IL-1β) produced by the tumor or adipose tissue may also contribute to adipose depletion. Identifying the mechanisms and time course of fat mass changes in cancer may help identify individuals at risk of adipose depletion and define interventions to circumvent wasting. This review outlines current knowledge of fat mass in cancer and illustrates the need for further studies to assess alterations in visceral and subcutaneous adipose depots and possible mechanisms for loss of fat during cancer progression.

Keywords: adipose tissue; cancer; computed tomography; fat mobilization

1. Introduction

Adipose tissue (AT) is an active secretory organ that regulates energy balance, homeostasis, appetite, inflammation, insulin sensitivity, angiogenesis, and fat metabolism [1]. Adipose tissue metabolism and whole body fat mass are regulated through two major pathways: lipolysis (fat breakdown) and lipogenesis (fat synthesis) [2]. Adipose tissue dysfunction, fat mass changes and concurrent alterations in the production of adipokines, inflammatory cytokines, and lipid metabolites are common in metabolic disorders, such as insulin resistance, type 2 diabetes, cardiovascular disease, and obesity-related cancers such as colorectal and breast cancer [3,4].

The link between obesity and increased cancer incidence is well established [5,6], but the relationship between fat mass and cancer progression is much less clear. Studies indicate that the majority of cancer patients experience some degree of cancer-related wasting of both muscle and/or fat during the disease trajectory [7]. However, little is known about the importance of fat loss in cancer because the majority of studies of cancer-associated wasting typically focus on muscle. Potential links between fat loss and poor outcomes have been identified that indicate fat loss to be a poor prognostic factor in advanced cancer regardless of a patient' body weight [8,9]. This article reviews current knowledge of adipose tissue depletion in cancer, focusing on both assessment of fat tissue and morphological determination of adipose tissue in cancer populations. Possible mechanisms of fat loss are also discussed. Biological alterations in adipose tissue metabolism precede the physical manifestation of adipose tissue loss. Thus, understanding mechanisms and potential markers of fat

loss in cancer are important for early detection which facilitates prevention of further loss to preserve fat and improve survival in cancer patients.

2. Adipose Atrophy in Cancer

Fat loss has been reported to be associated with shorter survival time [8,9]. Analysis of adipose tissue morphology and body composition has revealed body fat depletion in human and animal models of cancer cachexia. In the majority of human studies discussed in this review, cachexia is defined as $\geq$5% weight loss (WL) over 3 months or $\geq$10% within the previous 6 months. Weight loss does not necessarily reflect the severity of cachexia and fat loss but is the first outcome measurement typically used in studies of cancer. Validated data for classification of cachexia based on recent consensus are emerging [10].

The murine adenocarcinoma (MAC16) causes diminished adipocyte size with increased mitochondrial density, and elevated adipose tissue fibrosis in cachectic mice, compared to pair-fed and control animals [11]. The Walker 256 carcinoma, a well-established cancer cachexia model, affects adipose tissue in a time and depot-dependent manner [12–14]. Seven days after Walker 256 tumor injection, no significant changes were observed in adipocyte size. However, after 14 days, adipocyte size of retroperitoneal, and epididymal adipose tissue was decreased. On the other hand, mesenteric adipose tissue was not lost and size of mesenteric adipocytes increased after 14 days [13,14]. In support of these experimental studies, reduction in fat cell volume has been reported in weight-losing gastrointestinal (GI) cancer patients [15–17]. Cachectic patients exhibited smaller adipocytes compared to weight-stable controls [15–18] and non-cancer patients [16] but total body fat cell number was not altered [16,17]. Collectively, these studies suggest altered adipocyte size and reduced lipid storage capacity in the presence of a tumor.

3. Assessment of Fat Tissue over the Cancer Trajectory

The body mass index (BMI) has been used frequently as a clinically accessible measure of human body composition. However, the BMI does not differentiate between fat and fat-free mass or fat depots. Accumulation or loss of specific fat tissues are differentially associated with health outcomes. For example, there is a relationship between visceral adipose tissue (VAT) accumulation and insulin resistance [19]. Insulin resistant adipocytes that reside in VAT are more sensitive to catecholamine-induced lipolysis than subcutaneous adipose tissue (SAT) [1,20]. Lipolysis of fat from VAT enables direct delivery of free fatty acids to liver, which may lead to elevated hepatic triglyceride (TG) production, increased very low density lipoprotein secretion, and higher plasma TGs which exacerbates an already dysregulated metabolic state [20]. Therefore, an understanding of the intensity of loss and the type of fat being lost (VAT *vs.* SAT) is required. Potential differences in fat loss between depots has not been consistently demonstrated, partly due to the use of analytical techniques with limited applicability, as well as the variability among studies with regards to tumor type, stage and the time-point in the cancer trajectory that patients are studied. Discrepant methods of assessing fat and reporting values as cross sectional area (cm^2) or volume (cm^3), total fat mass (kg or %), change in area or the rate of changes also limit the ability to interpret and compare studies.

Body composition is assessed in cancer patients using a variety of methods including bioelectrical impedance analysis (BIA), dual-energy X-ray absorptiometry (DEXA), magnetic resonance imaging (MRI) and computed tomography (CT) scan analysis [21]. Body composition analysis using BIA has demonstrated lower body fat (% or kg) in cachectic patients compared to weight-stable cancer controls [15–17,22], healthy controls [23], or non-malignant controls [16,22]. When DEXA was applied to malnourished palliative cancer patients, no differences were observed in absolute fat mass (kg) during follow-up (4–62 months) [9]. However, the relative change (percentage of change from initial values) revealed a loss of fat concurrent with a marginal increase in lean mass during cancer progression [9]. As DEXA quantifies regional lean body mass, this study raised the possibility that patients may not have been gaining skeletal muscle per se but rather lean mass in internal organs

such as the liver and spleen which has been reported as patients approached death in a subsequent study [24].

In an oncologic population, CT images are a routine part of treatment and are available from patient records as a chart review. CT image analysis has emerged as the gold standard for body composition assessment in cancer patients due to its ability to discriminate and quantify muscle, adipose tissue and organs. Shen *et al.* established that single slice tissue areas can be used to estimate whole body muscle and adipose tissue volumes [25]. Cachectic cancer patients exhibit lower adipose tissue mass compared to weight stable and/or controls [15–17,26]. Volumes of total adipose tissue, VAT and SAT were calculated in newly diagnosed GI cancer patients receiving no anticancer treatment [26]. Cachectic groups were separated into two groups, those with and without gastrointestinal obstruction that interfered with their food intake. Cachectic groups were compared to weight-stable cancer patients. Deterioration in nutritional status was confirmed by a higher Patient-Generated Subjective Global Assessment (PG-SGA) score in the cachectic patients with GI obstruction. Both BIA and CT analysis indicated that total fat mass (kg), and visceral and abdominal subcutaneous volumes were lower in cachectic patients compared to the weight-stable group. The cachectic patients with GI obstruction lost approximately two times more weight but VAT volume was greater compared to cachectic group without GI obstruction [26]. This study applied CT scans taken at one point in time; therefore, intensity of loss over time can not be determined. A lower amount of VAT, not the loss per se, was observed in cachectic group who did not have altered food intake.

Approaching death, the intensity of tissue loss increases and patients experience the greatest and most accelerated rate of loss [8,9,24,27]. Analysis of sequential CT images in 34 advanced colorectal cancer patients revealed that the greatest changes in body composition occur starting at 4.2 months from death [24]. One month from death, liver and spleen mass increase, whereas skeletal muscle and fat mass decrease [24]. A study by Murphy *et al.* quantified fat mass in 108 colorectal and lung cancer patients with at least two abdominal CT images in the last 500 days of life. Beginning seven months prior to death, both VAT and SAT mass decreased in cancer patients, reaching intensities of 10 kg of fat loss/100 days [8]. A recent study in pancreatic cancer patients suggested that the rate of visceral adipose tissue loss, rather than the absolute amount, may be an important indicator of survival [28]. Patients with at least two abdominal CT scans between diagnosis and death, receiving surgery (62%) or chemotherapy (88%) during cancer progression, were selected for this study. The rate of change (% change/100 days) for SAT was similar to VAT but a change in VAT was significantly correlated with survival in cancer patients. The presence of co-morbidities such as diabetes and anemia may have accelerated loss of VAT [28]. In another study, cachectic gastrointestinal cancer patients had significantly lower VAT and SAT three months prior to death compared to the benign controls. However, there was a tendency for cachectic patients to have smaller visceral and subcutaneous area compared to cancer patients without cachexia [27]. At present, no other studies exist regarding the pattern of fat loss in cancer and further studies are needed to establish the timeline and pattern of fat mass alterations in different adipose tissue depots during cancer progression. Further, the majority of studies assessing fat mass focus on gastrointestinal cancer patients so there is a gap in knowledge related to other malignant tumors.

While the majority of human studies focus on cachectic *vs.* non-cachectic patients, less is known about the effect of cancer treatments, which may also induce alterations in fat mass. For example, cancer surgery contributes to weight loss. Six months after surgery, weight is reduced from the baseline due to the catabolic response to the operation [29–31] and stabilizes after 12 months [31]. Adams reported that weight loss occurs rapidly in the three months following surgery [29]. Body composition assessment before, and 6 and 12 months after gastrectomy, measured from total body potassium and water, indicated 40% of fat mass was lost during the six months after surgery [30]. In a study by our group, two to six months after surgery, patients with colorectal liver metastasis were losing VAT at a greater rate than SAT, measured using consecutive CT scans (Figure 1). This supports the work of others in pancreatic cancer patients during early stages of disease progression. Intra-abdominal and

subcutaneous adipose tissue mass were assessed before and after surgery using CT scans. Fat loss from intra-abdominal depot was greater than abdominal subcutaneous following surgery [32]. Therefore, surgical procedures may contribute to weight and fat loss due to the catabolic and inflammatory response to the surgery.

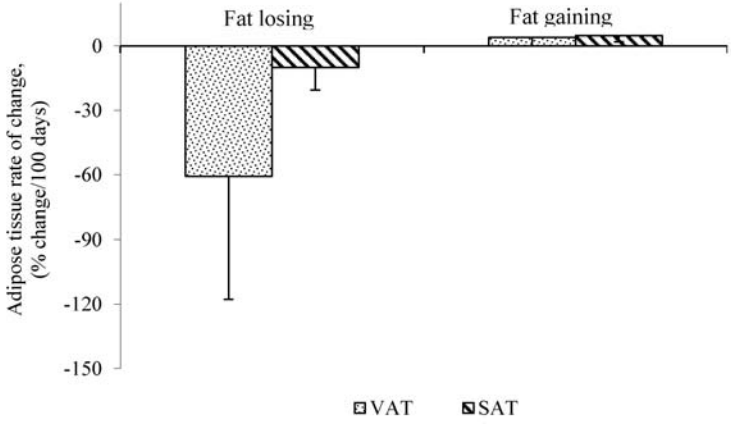

Figure 1. Mean rate of visceral adipose tissue (VAT) and subcutaneous adipose tissue (SAT) change in fat losing cancer patients assessed by consecutive computed tomography (CT) scans. Data are represented as Mean ± SD, $n = 5$ (Fat Losing) and $n = 2$ (Fat Stable), $p < 0.05$ VAT, visceral adipose tissue; SAT, subcutaneous adipose tissue.

Fat loss or gain after chemotherapy will depend on the tumor type, drug type, dose and overall response to chemotherapy. Following at least one cycle of chemotherapy treatment (cisplatin, 5-fluorouracil and/or epirubicin), patients with locally advanced oesophagogastric cancers lost an average of 1.3 ± 3.2 kg (6%) fat mass [33]. In advanced pancreatic cancer patients, a multivariate survival analysis revealed that VAT loss (determined from CT pre and post chemoradiation) but not muscle loss was significantly related to shorter survival [34].

Three months after chemotherapy initiation, testicular cancer patients who received 3 or 4 cycles of cisplatin-based chemotherapy had significantly higher VAT volume without changes in SAT [35]. However, nine months later, both VAT and SAT increased significantly, suggesting a capacity to rebuild lost adipose tissue [35]. A recent study using CT imaging to understand the loss and gain of muscle and adipose tissue during the year preceding death revealed that anabolic potential does exist, as some patients gained muscle and adipose tissue, but were only capable of doing this >3 months prior to death [36]. These results will initiate further research aiming to define the appropriate time to initiate nutritional intervention to preserve both muscle and fat tissue.

Fat loss may precede the loss of lean tissues (Table 1). The only patient group in which this question has been addressed is patients with newly diagnosed GI cancers. However, in all studies that have addressed this question to date, changes in adipose tissue were observed in absence of changes in lean tissues. The majority of these studies use BIA and DEXA for body composition assessments which are limited in ability to provide a direct estimate of muscle mass; further studies are needed to confirm that these findings are attributable to muscle loss or other lean body mass loss. Only one study used CT scans to assess body composition in GI cancer patients and that study showed no difference in abdominal muscle volume between cachectic and weight-stable cancer patients. However, that study assessed CT images at only one time point [26]. Adipose depletion may occur more rapidly than muscle during disease progress. Advanced pancreatic cancer patients lost both VAT and SAT over time, and the rate of change (%change/100 days) in total adipose tissue (−40.4 ± 25.4%/100 days)

was much greater than muscle tissue ($-3.1 \pm 12.0\%/100$ days). No significant differences in adipose tissue mass were observed between patients who were or were not receiving chemotherapy [37]. These observations are supported by an experimental study in which lung carcinoma or melanoma cells were injected subcutaneously to induce cachexia in mice. Fat loss occurred prior to muscle loss, at early stages of tumor growth, at an intensity that was greater than muscle loss [38]. White adipose tissue browning, which contributes to fat loss in cancer, occurred before skeletal muscle wasting in mouse models of cancer cachexia [39].

Table 1. Articles reporting fat and lean tissue loss in newly diagnosed cancer patients.

Authors	Subjects [1]	Cancer Type	Body Composition Assessment	General Comments
Fouladiun *et al.*, [9]	Malnourished patients (n = 132; 66 ± 3 years) advanced cancer with malnutrition (T4N1M1)	GI (n = 123) Breast (n = 1) Melanomas (n = 2) Other (n = 6), followed for 6–42 months	DEXA	Whole body fat loss was related to shorter survival Body fat loss more intense and pronounced compared to lean tissue
Agusstson *et al.*, [15]	Weight stable cancer patients (n = 11), Weight-losing cachectic cancer patients with (n = 8) and without (n = 7) malnutrition	GI cancer with no treatment before surgery	BIA	No differences in lean body mass between groups Increased lipolysis in cancer cachectic patients
Dahlman *et al.*, [17]	Cachectic patients (n = 13) Weight-stable cancer (n = 14)	GI cancer with no treatment before surgery	BIA	Decreased body fat mass but similar lean body mass between cachectic and control patients
Ryden *et al.*, [16]	Cachectic patients (n = 13) Weight stable cancer patients (n = 10), Without cancer (n = 5)	GI cancer with no treatment before surgery	BIA	No difference in lean body mass between groups Elevated lipolysis with no changes in lipogenesis No local inflammation
Agustsson *et al.*, [26]	Cancer cachectic without (n = 13) and with gastrointestinal obstruction (n = 10), Weight losing-cancer (n = 17)	GI cancer with no treatment before surgery	BIA, CT	No changes were observed in lean mass Visceral fat volume was lower in cachectic group compared to weight stable

[1] No patients received chemotherapy or radiotherapy.

4. Mechanisms for Adipose Depletion in Cancer

Elevated energy expenditure, decreased food intake and alterations in circulating levels of hormones including insulin, leptin, catecholamines, as well as elevated catabolism due to the tumor presence (high energy demands of tumor, inflammatory mediators produced by tumor) and tumor-host interactions are factors contributing to wasting in cancer [40]. These factors can cause abnormalities in lipid metabolism which may also lead to fat loss. Increased lipolytic activity, evidenced by elevated fasting plasma glycerol and free fatty acids is a driver of fat loss in advanced cancer patients [15,23,41] but the underlying causes of elevated lipolysis are not known. Other mechanisms including decreased lipogenesis [42,43], impairment in adipogenesis [11,14], elevated fat oxidation [17,23,44], and decreased lipid deposition [45–49] have also been attributed to fat loss in cancer (Figure 2).

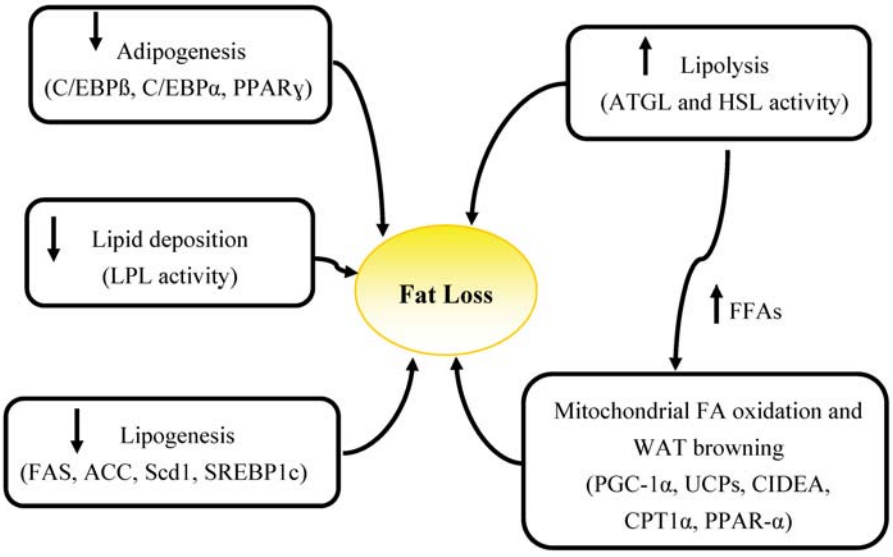

Figure 2. Summary of mechanisms and specific genes involved in adipose atrophy in cancer. WAT, white adipose tissue; FFAs, free fatty acids; ATGL, adipose triglyceride lipase, HSL, hormone sensitive lipase; PGC-1α, peroxisome proliferator-activated receptor-gamma coactivator 1 alpha; UCPs, un-coupling proteins; CIDEA, Cell death-inducing DFFA (DNA fragmentation factor-alpha)-like effector A; CPT1α, carnitine palmitoyltransferase 1 alpha; PPAR-γ, Peroxisome proliferator-activatedreceptor gamma; C/EBPα, CCAAT-enhancer-binding proteinα; LPL, lipoprotein lipase; FAS, fatty acid synthase; ACC, Acetyl-CoA carboxylase; Scd1, Stearoyl-CoA desaturase; SREBP1c, sterol regulatory element binding protein-1c.

Human and experimental models have been used to study the mechanisms of fat loss in cancer. Animal models are necessary to elevate our understanding of cancer associated weight-loss. However, each model may represent only some aspects of human cancer cachexia and choice of animal model is based on research objectives. For example, the MAC16 adenocarcinoma induces cachexia in the absence of anorexia and is suitable to study wasting related to the tumor produced factors rather than food intake. Yoshida ascites hepatoma AH130 (YAH-130), on the other hand, induces cachexia and anorexia accompanied by inflammation [50]. Therefore, the result of studies investigating the mechanisms underlying fat loss in cancer should be interpreted with caution as each specific tumor type, in various stages of growth, can affect various adipose tissue depots in a different manner.

4.1. Decreased Food Intake and Hypermetabolism

Anorexia alone does not explain reduced body weight or/and fat mass in cancer patients and cachexia-associated wasting can not be completely reversed by elevated nutritional intake [10]. Compared to pair-fed controls, the MAC-16 tumor leads to greater fat loss in mice indicating that tumors with high energy demands, rather than calorie restriction, may be responsible for adipose depletion [11]. Likewise, human studies have shown that in the absence of changes in food intake, hyper metabolism, characterized by elevated resting energy expenditure (REE), may be a contributing factor to the weight loss in cancer. Weight-losing and weight-stable cancer patients with various solid tumors had similar dietary intakes but weight losing patients had a higher REE determined by indirect calorimetry [51]. REE was also higher in weight-stable cancer patients compared to non-cancer controls which indicate that the tumor contributes to an elevated REE [52]. Johnson *et al.* reported no difference in measured REE between weight-losing and stable cancer patients but rather attributed higher REE in

weight-losing cancer patients to elevated C-reactive protein (CRP) [53]. Although REE can contribute to weight loss in cancer, factors like tumor type, stage and duration of the disease also affect the REE in cancer [52,54].

In palliative cancer patients, during 4–62 months follow-up, body weight and fat mass (% change from baseline) decreased in the absence of changes in REE. Despite providing nutritional support to patients who had baseline calorie intake less than 90% of their energy requirement, body weight and fat mass did not increase [9]. Therefore, factors other than nutrient intake and hypermetabolism may contribute to fat loss in cancer.

4.2. Lipolysis and Elevated Fat Oxidation

It is well accepted that elevated lipolysis is the main cause of fat loss in cancer [15–17,22,23,55,56], however the specific mechanisms contributing to lipolysis have not been clearly defined. Hormone sensitive lipase (HSL) and adipose triglyceride lipase (ATGL) are major enzymes that contribute to TG breakdown in adipose tissue. Adipose triglyceride lipase catalyzes the first step in TG hydrolysis. During adipose tissue lipolysis, free fatty acids (FFA) and glycerol molecules are produced by the hydrolysis of triglyceride. HSL activity is regulated by hormones, *i.e.*, catecholamines, insulin and glucagon, through a cAMP-mediated process [57,58]. Catecholamines stimulate lipolysis, whereas insulin has anti-lipolytic functions [59]. Binding of hormones to G-protein-coupled receptors results in up-regulation of adenylate cyclase which leads to an increase in intracellular cyclic adenosine monophosphate (cAMP) concentrations. cAMP stimulates a protein kinase which in turn phosphorylates and activates HSL [57,58]. Phosphorylated HSL translocates from the cytosol to the surface of lipid droplets to induce lipolysis.

Elevated expression of HSL mRNA [15,22,60] and protein [15,22] has been reported in cancer cachectic patients compared to weight-stable cancer. Higher mRNA expression of HSL in SAT was associated with higher serum FFAs in cancer patients, however, no significant differences were observed in mRNA expression of lipoprotein lipase (LPL), fatty acid synthase (FAS), insulin and tumor necrosis factor alpha (TNF-α) in adipose tissue of cancer patents compared to controls [60]. These results are supported by a study that reported HSL mRNA and protein over-expression, as well as increased hormone-stimulated lipolysis in cachectic cancer patients compared to malnourished weight-losing and weight stable cancer patients, explained the elevated adipose atrophy in cancer cachexia [15]. The ratio of plasma glycerol/body fat (index of *in vivo* lipolysis) was two times higher in cachectic patients. *Ex vivo* culture of adipocytes from the same patients revealed no difference in basal lipolysis (glycerol release to the media) between groups. However, incubation of adipocytes with catecholamines and natriuretic peptides elevated glycerol release to the media in the cachectic group suggesting that the adipocytes were more sensitive to the same amount of stimuli, and therefore more catabolic. There was no significant difference in plasma levels of catecholamines and natriuretic peptides between groups [15]. An explanation of the lack of difference in plasma hormone levels could be that lipolytic effects of hormones are elevated at the receptor level, evidenced by elevated β1-adrenoceptor (ADRB1) expression on adipocyte membranes in cachectic GI cancer patients [22]. Consequently, higher HSL expression and activity, which positively correlated with ADRB1 expression, were associated with higher plasma glycerol/fat mass and FFA/fat mass [22]. Therefore, lipolysis can be elevated in cancer cachectic patients due to increased expression of receptors on adipocytes membrane and their response to lipolytic effects of hormones, rather than elevated levels of mediators.

In study by Agustsson *et al.* [15], elevated HSL mRNA and protein expression contributed to the increased lipolysis. No significant difference in mRNA expression of ATGL was observed between cachectic cancer patients and controls; however, protein expression was not measured in this study [15]. Das and Hoefler [61] reported that ATGL mRNA expression may not translate to enzyme activity as its function is regulated via post-translational modifications. In another study, Das *et al.* reported higher ATGL and HSL activity in VAT of cachectic patients compared to non-cancer and cancer patients without cachexia, which has been previously reported [38]. Animal studies suggest that ATGL plays a

more important role in adipose tissue lipolysis than HSL [38,62]. In mice bearing cachexia-inducing lung carcinoma or melanoma cells, lower body weight, decreased fat and muscle mass and elevated lipolysis were observed in tumor group compared to the controls. In HSL deficient mice, the tumor could reduce body weight and fat mass due to elevated ATGL activity. However, in ATGL deficient mice, the tumor did not induce elevated lipolysis and there was no significant difference in weight and fat mass between control and tumor group. Fat preservation in ATGL deficient mice, prevented muscle loss in tumor bearing animals [38]. Consistent with these findings, a recent study in mice bearing cachectic Colon-26 carcinoma revealed lower fat mass and increased lipolysis in cachectic mice compared to control mice. Increased lipolysis was induced by ATGL rather than the PKA/HSL pathway during late stages of cancer cachexia. ATGL protein levels increased in cachectic mice, however, no changes were observed in ATGL mRNA expression [62]. Therefore, not only mRNA expression but also protein expression and/or activity of these enzymes need to be determined in adipose tissue to investigate mechanisms that underlie elevated lipolysis.

The majority of studies indicated elevated lipolysis as a reason for fat loss in cancer; and consequently, increasing fatty acid oxidation could be a tentative approach to utilize surplus fatty acids (FAs). By increasing fatty acid oxidation within adipose tissue, liberated FAs are oxidized and can not be re-esterified into TG. Zuijdgeest-van Leeuwen *et al.* [23] reported higher lipolysis and reduced food intake in weight-losing cancer patients compared to healthy weight-stable adults. Whole body lipolysis and fatty acid oxidation were higher in cancer patients compared to healthy subjects, even after adjusting for food intake. However, this heterogeneous population of cancer patients had varying degrees of weight loss (5.3%–25%/6 months) and were at different stages in the disease trajectory (1–180 months since diagnosis) [23]. Up-regulation of genes involved in mitochondrial fat oxidation such as peroxisome proliferator-activated receptor-gamma coactivator 1 alpha (PGC-1α), and uncoupling protein 2 (UCP-2) have been demonstrated in animal models of cancer cachexia [11]. Enhanced fat oxidation reflected by a decreased respiratory quotient (RQ) [17,44], higher expression of genes related to energy and FA metabolism pathways such as Krebs TCA cycle, oxidative phosphorylation, and FA degradation have been reported in patients with cachexia [17]. No differences were observed in expression of genes involved in fatty acid oxidation including PPARα, PGC-1α, carnitine palmitoyltransferase 1 alpha (CPT1α) in mice bearing Colon-26 carcinoma compared to controls. However, mRNA expression of peroxisomal bifunctional enzyme (Pbe), specific for peroxisomal fatty acid oxidation, was higher in cachectic mice [62]. Another study found that mRNA levels of Cell death-inducing DFFA (DNA fragmentation factor-alpha)-like effector A (Cidea), which mediates oxidation of excess FAs rather than glucose, is increased in SAT of cachectic patients. In addition, cachectic patients had lower plasma TGs, increased FAs and glycerol, and lower RQ indicating elevated FA oxidation [44]. In pancreatic cancer patients, Cidea expression was higher in intra-abdominal adipose tissue compared to subcutaneous in early stages of tumor progression. CT image analysis of these same patients revealed that patients were losing intra-abdominal fat more than subcutaneous fat [32].

Excess fatty acids from enhanced lipolysis are oxidized by mitochondria to produce energy. However, the appearance of brown adipocytes within the white adipose tissue can dissipate energy of substrate oxidation as heat through uncoupling fatty acid oxidation from ATP production by uncoupling protein-1 (UCP1) [63]. Recently, studies found that white adipose tissue browning can contribute to adipose atrophy in cancer by enhancing white fat thermogenesis [39,64]. Small adipocytes with large nuclei were observed during early stages of cachexia in SAT of mouse models of lung and pancreatic cancer. Multi-locular cells interspersed in the white adipose tissue, resembling brown adipocytes, positively stained for UCP-1 [39]. White adipose tissue browning was associated with increased expression of brown fat markers including UCP-1, PGC-1α, PPARγ and Cidea in cachectic mice compared to the controls [39,64]. β-adrenergic signaling, inflammatory cytokines like interleukin-6 (IL-6) [39] and tumor-derived parathyroid hormone-related protein (PTHrP) [64] can mediate white adipose browning by inducing expression of thermogenic genes. Blocking of these

mediators might help to prevent adipose atrophy in cancer cachexia [39,64]. Collectively, lipolysis, increase fatty acid oxidation and elevated white adipose tissue thermogenesis play an important role in AT depletion in cancer.

4.3. Lipogenesis and Lipid Deposition

Despite the importance of lipolysis in fat loss in cancer, fat depletion can also occur when lipogenesis is limited in white adipose tissue. In murine cachectic models (Yoshida AH-130 ascites hepatoma), decreased AT lipogenesis was accompanied by an increase in liver lipogenesis and hypertriglyceridemia [42]. Decreased lipogenesis was accompanied by lower activities of FAS, citrate cleavage enzyme, and malic enzyme in rats bearing a mammary adenocarcinoma during late phases of tumor progression [43]. Deterioration in lipid synthesis capacity of epididymal adipose tissue was observed in MAC16 bearing rats, evidenced by decreased mRNA levels of important lipogenic enzymes such as acetyl-CoA carboxylase, FAS, stearoyl-CoA desaturase-1 and glycerol-3-phosphate acyltransferase [11].

Increased lipolysis and decreased lipogenesis has been reported in male Japanese white rabbits bearing the VX2 tumor cells compared to food-restricted animals. Body weight reduction and fat loss occurred before any decrease in food intake [65]. Adipocyte apoptosis (20–30 days after tumor implantation) was also observed in tumor groups, however no changes in total body fat cell numbers has been reported in previous human studies [15–17]. Discrepancies may be caused by the fact that patients in previous studies were at early stages of disease and also the fat cell numbers were extrapolated based on the total body fat and mean fat cell volume. In contrast to those human studies, animals were followed during cancer progression and also biological differences and limitations of extrapolating results between different species may contribute to the discrepancies.

LPL mediate FAs uptake in adipose tissue by hydrolysis of very-low-density lipoproteins and chylomicrons. Numerous animal studies suggest reduced LPL activity in cancer [42,43,45,46]. Reduction in AT LPL activity in tumor bearing mice to the levels of starved animals was associated with impaired lipid deposition, fat loss, reduced breakdown of plasma lipoproteins and increased circulating lipid concentrations [47]. Decreased adipose tissue LPL activity was associated with hypertriglyceridemia during early stages of tumor growth in Lewis rats bearing a mammary adenocarcinoma [43]. Decreased fat content and LPL activity in WAT was accompanied by increasing circulating triglycerides, and body weight loss induced by the Yoshida AH-130 ascites hepatoma in rats [42,45,46]. In mice bearing MAC16, plasma TGs decreased during cancer progression, regardless of the amount of weight loss. At early stages, plasma FFA decreased and LPL activity increased; however, at advanced stages of tumor, LPL activity decreased [49].

While the majority of studies have utilized animal models to investigate lipogenesis and LPL activity during cancer progression, the human studies have reported decreased mRNA expression and activity of LPL and FAS in VAT in proximity to a tumor compared to distal adipose tissue in colorectal cancer patients [48]. Decreased FAS activity in adipose tissue and elevated activity in tumor cells might be important for tumor cell growth [48]. No changes were observed in lipogenesis in adipocytes isolated from cancer patients' SAT, compared to controls [16]. Lower plasma TG and higher glycerol and FFAs have been observed in cachectic patients [16,17,27] but the activity or expression of LPL was not determined in these studies. Further studies are required to determine the lipogenesis capacity and fatty acid uptake by adipose tissue in various groups of cancer patients at different stages during disease trajectory.

4.4. Adipogenesis

Fat loss may arise from impairment in the adipose tissue development and ability for fat synthesis and storage capacity. Adipogenesis is a highly regulated process which encompasses preadipocyte proliferation and differentiation into mature adipocytes. Adipogenesis is then followed by lipogenesis to store lipid in fat cells. TNF-α, a proinflammatory cytokine produced by both tumor

and adipose tissue regulates adipocyte differentiation [66]. Therefore, higher production of TNF-α in cancer-associated cachexia may lead to the altered differentiation status of adipocytes. A reduction in mRNA levels of adipogenic transcription factors including CCAAT-enhancer-binding proteins (c/EBPβ), PPARγ, c/EBPα, sterol regulatory element binding protein-1c (SREBP-1c) in epididymal adipose tissue of mice bearing MAC16 tumor was associated with diminished adipocytes size [11]. Expression of adipogenic factors including C/EBPα, SREBP1C and PPARγ decreased in rats bearing Walker 256 during early stages of cachexia. Morphological changes evident by smaller adipocytes occurred during late stages of cachexia which supports a reduction in expression of adipogenic genes [14]. Lower expression of adipogenic genes such as C/EBPα, Reverba, Per2 and PPARγ has been reported in cachectic mice bearing the Colon-26 carcinoma [62]. More research in both animal and human models is required to demonstrate the possible alterations in adipogenesis during cancer progression.

5. Local Adipose Tissue Inflammation

Proinflammatory cytokines, *i.e.*, IL-1β, TNF-α, IL-6 produced by tumor or host tissue due to tumor presence leads to both systemic and local inflammation in cancer [67,68]. Visceral adipose tissue is a more active producer of inflammatory cytokines IL-6, TNF-α [69,70]. However, data on local adipose tissue inflammation in cancer are inconsistent, being reported as either increased [14,62,71] or unchanged [11,16,17,31,32]. Walker 256 carcinoma caused elevated expression of macrophage markers (f4/80, CD68) especially during late stages of tumor progression [14]. Mesenteric and epididymal adipose tissue were the most and least commonly affected fat depots by macrophages, respectively [14]. In mice bearing Colon-26 carcinoma, depleted fat mass was associated with enhanced inflammatory IL-6/STAT3 cytokine signaling pathway [62]. IL-6 induces signal transducer and activator of transcription-3 (STAT3) activation through phosphorylation. Elevated levels of phosphorylated STAT3 in cachectic mice compared to the control group were observed [62]. Higher mRNA expression of TNF-α in SAT, not VAT, of cachectic GI cancer patients compared to weight-stable cancer patients was reported in newly diagnosed cancer patients. [71]. Contrary to those studies, no change in mRNA expression of inflammatory markers including IL-6 and TNF-α were observed in SAT from cancer patients [16,17] or in an animal model [11]. This paralleled the observation that macrophages or lymphocytes did not infiltrate SAT, as there were no changes in mRNA levels of CD68 (macrophages infiltration marker), CD3 (T-lymphocytes marker) [16] in humans, and MAC1 and F4/80 (macrophage markers) expression in animals [11]. Monocyte chemoattractant protein-1 (MCP-1) and TNF-α mRNA levels in both intra-abdominal and subcutaneous depots did not differ between pancreatic cancer patient and non-cancer controls. However, mRNA expression of MCP-1 and TNF-α in intra-abdominal adipose tissue negatively correlated with post-operative change in intra-abdominal mass assessed by CT scans [32]. This paradox may be due to differences in tumor stages between studies, involvement of other cytokines such as transforming growth factor-b (TGF-b), IL-1 or interferon gamma in cancer associated cachexia or the balance between anti- and pro-inflammatory cytokines of might be important for cachexia-associated inflammation [61,72]. Another explanation is that a cytokine like TNF-α is involved in early stages of cachexia but is transient in nature. Therefore, due to its short half-life and different assay sensitivities, results should be interpreted with caution (reviewed by Das and Hoefler) [61]. Alternate markers such as TNF-R1 and TNF-R2 (Soluble TNF-α membrane receptors) may be more accurate markers than TNF-α due to their longer half-life and stability [73]. Overall, a major gap remains related to comparison of local inflammatory markers in both visceral and subcutaneous depots. Inflammatory cytokines can mediate fat loss in cancer (reviewed by Bing [74]), therefore, assessing whether depot-specific differences in inflammatory cytokines transcription may contribute to inflammatory factors production and subsequent alterations in fat mass would be of great value.

6. Conclusions

Alterations in adipose tissue fat metabolism including changes in expression of genes involved in fat synthesis, storage, mobilization or oxidation, browning of white adipose tissue, adipocytes development, and elevated inflammatory signaling may have a role in fat loss in cancer patients. Fat accumulation at the time of diagnosis may contribute to cancer progression but the accelerated rate of adipose tissue loss would be expected to be associated with shorter survival time during cancer progression. Alterations in fat mass and composition between visceral and subcutaneous depots are equivocal in cancer trajectory and little is known regarding these alterations. The prognostic significance of these depots needs to be investigated in large populations throughout the cancer progression. Due to various roles of adipose tissue in controlling human metabolism, further identification of mechanisms and mediators of fat loss in cancer would help in the identifying fat-losing cancer patients that would benefit from early therapeutic interventions which could improve survival and prevent muscle atrophy in these patients. Finally, results need to be interpreted carefully as factors including tumor type, cancer stage, response to treatment and metabolic capacity of patients may influence findings.

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References

1. Ibrahim, M.M. Subcutaneous and visceral adipose tissue: Structural and functional differences. *Obes. Rev.* **2010**, *11*, 11–18.
2. Ntambi, J.M.; Young-Cheul, K. Adipocyte differentiation and gene expression. *J. Nutr.* **2000**, *130*, 3122–3126.
3. Park, J.; Euhus, D.M.; Scherer, P.E. Paracrine and endocrine effects of adipose tissue on cancer development and progression. *Endocr. Rev.* **2011**, *32*, 550–570. [PubMed]
4. Guilherme, A.; Virbasius, J.V.; Puri, V.; Czech, M.P. Adipocyte dysfunctions linking obesity to insulin resistance and type 2 diabetes. *Nat. Rev. Mol. Cell Biol.* **2008**, *9*, 367–377. [CrossRef] [PubMed]
5. Calle, E.E.; Kaaks, R. Overweight, obesity and cancer: Epidemiological evidence and proposed mechanisms. *Nat. Rev. Cancer* **2004**, *4*, 579–591. [CrossRef] [PubMed]
6. Prieto-Hontoria, P.L.; Perez-Matute, P.; Fernandez-Galilea, M.; Bustos, M.; Martinez, J.A.; Moreno-Aliaga, M.J. Role of obesity-associated dysfunctional adipose tissue in cancer: A molecular nutrition approach. *Biochim. Biophys. Acta* **2011**, *1807*, 664–678. [CrossRef] [PubMed]
7. Fearon, K.C. Cancer cachexia: Developing multimodal therapy for a multidimensional problem. *Eur. J. Cancer* **2008**, *44*, 1124–1132. [CrossRef] [PubMed]
8. Murphy, R.A.; Wilke, M.S.; Perrine, M.; Pawlowicz, M.; Mourtzakis, M.; Lieffers, J.R.; Maneshgar, M.; Bruera, E.; Clandinin, M.T.; Baracos, V.E.; *et al.* Loss of adipose tissue and plasma phospholipids: Relationship to survival in advanced cancer patients. *Clin. Nutr.* **2010**, *29*, 482–487. [CrossRef] [PubMed]
9. Fouladiun, M.; Korner, U.; Bosaeus, I.; Daneryd, P.; Hyltander, A.; Lundholm, K.G. Body composition and time course changes in regional distribution of fat and lean tissue in unselected cancer patients on palliative care—Correlations with food intake, metabolism, exercise capacity, and hormones. *Cancer* **2005**, *103*, 2189–2198. [CrossRef] [PubMed]
10. Fearon, K.; Strasser, F.; Anker, S.D.; Bosaeus, I.; Bruera, E.; Fainsinger, R.L.; Jatoi, A.; Loprinzi, C.; MacDonald, N.; Mantovani, G.; *et al.* Definition and classification of cancer cachexia: An international consensus. *Lancet Oncol.* **2011**, *12*, 489–495. [CrossRef] [PubMed]
11. Bing, C.; Russell, S.; Becket, E.; Pope, M.; Tisdale, M.J.; Trayhurn, P.; Jenkins, J.R. Adipose atrophy in cancer cachexia: Morphologic and molecular analysis of adipose tissue in tumour-bearing mice. *Br. J. Cancer* **2006**, *95*, 1028–1037. [CrossRef]
12. Machado, A.P.; Costa Rosa, L.F.; Seelaender, M.C. Adipose tissue in walker 256 tumour-induced cachexia: Possible association between decreased leptin concentration and mononuclear cell infiltration. *Cell Tissue Res.* **2004**, *318*, 503–514. [CrossRef] [PubMed]

13. Bertevello, P.S.; Seelaender, M.C. Heterogeneous response of adipose tissue to cancer cachexia. *Braz. J. Med. Biol. Res.* **2001**, *34*, 1161–1167. [CrossRef] [PubMed]
14. Batista, M.L., Jr.; Neves, R.X.; Peres, S.B.; Yamashita, A.S.; Shida, C.S.; Farmer, S.R.; Seelaender, M. Heterogeneous time-dependent response of adipose tissue during the development of cancer cachexia. *J. Endocrinol.* **2012**, *215*, 363–373. [CrossRef] [PubMed]
15. Agustsson, T.; Ryden, M.; Hoffstedt, J.; van Harmelen, V.; Dicker, A.; Laurencikiene, J.; Isaksson, B.; Permert, J.; Arner, P. Mechanism of increased lipolysis in cancer cachexia. *Cancer Res.* **2007**, *67*, 5531–5537. [CrossRef] [PubMed]
16. Ryden, M.; Agustsson, T.; Laurencikiene, J.; Britton, T.; Sjolin, E.; Isaksson, B.; Permert, J.; Arner, P. Lipolysis—Not inflammation, cell death, or lipogenesis—Is involved in adipose tissue loss in cancer cachexia. *Cancer* **2008**, *113*, 1695–1704. [CrossRef] [PubMed]
17. Dahlman, I.; Mejhert, N.; Linder, K.; Agustsson, T.; Mutch, D.M.; Kulyte, A.; Isaksson, B.; Permert, J.; Petrovic, N.; Nedergaard, J.; *et al.* Adipose tissue pathways involved in weight loss of cancer cachexia. *Br. J. Cancer* **2010**, *102*, 1541–1548. [CrossRef] [PubMed]
18. Mracek, T.; Stephens, N.A.; Gao, D.; Bao, Y.; Ross, J.A.; Ryden, M.; Arner, P.; Trayhurn, P.; Fearon, K.C.; Bing, C.; *et al.* Enhanced ZAG production by subcutaneous adipose tissue is linked to weight loss in gastrointestinal cancer patients. *Br. J. Cancer* **2011**, *104*, 441–447. [CrossRef] [PubMed]
19. Otake, S.; Takeda, H.; Suzuki, Y.; Fukui, T.; Watanabe, S.; Ishihama, K.; Saito, T.; Togashi, H.; Nakamura, T.; Matsuzawa, Y.; *et al.* Association of visceral fat accumulation and plasma adiponectin with colorectal adenoma: Evidence for participation of insulin resistance. *Clin. Cancer Res.* **2005**, *11*, 3642–3646. [CrossRef] [PubMed]
20. Hellmer, J.; Marcus, C.; Sonnenfeld, T.; Arner, P. Mechanisms for differences in lipolysis between human subcutaneous and omental fat cells. *J. Clin. Endocrinol. Metab.* **1992**, *75*, 15–20. [PubMed]
21. Fabbro, E.D.; Bruera, E.; Demark-Wahnefried, W.; Bowling, T.; Hopkinson, J.B.; Baracos, V.E. *Nutrition and the Cancer Patient*; Oxford University Press: New York, NY, USA, 2010; pp. 24–28.
22. Cao, D.X.; Wu, G.H.; Yang, Z.A.; Zhang, B.; Jiang, Y.; Han, Y.S.; He, G.D.; Zhuang, Q.L.; Wang, Y.F.; Huang, Z.L.; *et al.* Role of beta1-adrenoceptor in increased lipolysis in cancer cachexia. *Cancer Sci.* **2010**, *101*, 1639–1645. [CrossRef] [PubMed]
23. Zuijdgeest-van Leeuwen, S.D.; van den Berg, J.W.; Wattimena, J.L.; van der Gaast, A.; Swart, G.R.; Wilson, J.H.; Dagnelie, P.C. Lipolysis and lipid oxidation in weight-losing cancer patients and healthy subjects. *Metabolism* **2000**, *49*, 931–936. [CrossRef] [PubMed]
24. Lieffers, J.R.; Mourtzakis, M.; Hall, K.D.; McCargar, L.J.; Prado, C.M.; Baracos, V.E. A viscerally driven cachexia syndrome in patients with advanced colorectal cancer: Contributions of organ and tumor mass to whole-body energy demands. *Am. J. Clin. Nutr.* **2009**, *89*, 1173–1179. [CrossRef] [PubMed]
25. Shen, W.; Punyanitya, M.; Wang, Z.; Gallagher, D.; St-Onge, M.P.; Albu, J.; Heymsfield, S.B.; Heshka, S. Total body skeletal muscle and adipose tissue volumes: Estimation from a single abdominal cross-sectional image. *J. Appl. Physiol.* **2004**, *97*, 2333–2338. [CrossRef] [PubMed]
26. Agustsson, T.; Wikrantz, P.; Ryden, M.; Brismar, T.; Isaksson, B. Adipose tissue volume is decreased in recently diagnosed cancer patients with cachexia. *Nutrition* **2012**, *28*, 851–855. [CrossRef] [PubMed]
27. Ogiwara, H.; Takahashi, S.; Kato, Y.; Uyama, I.; Takahara, T.; Kikuchi, K.; Iida, S. Diminished visceral adipose tissue in cancer cachexia. *J. Surg. Oncol.* **1994**, *57*, 129–133. [CrossRef] [PubMed]
28. Di Sebastiano, K.M.; Yang, L.; Zbuk, K.; Wong, R.K.; Chow, T.; Koff, D.; Moran, G.R.; Mourtzakis, M. Accelerated muscle and adipose tissue loss may predict survival in pancreatic cancer patients: The relationship with diabetes and anaemia. *Br. J. Nutr.* **2013**, *109*, 302–312. [CrossRef] [PubMed]
29. Adams, J.F. The clinical and metabolic consequences of total gastrectomy. I. Morbidity, weight, and nutrition. *Scand. J. Gastroenterol.* **1967**, *2*, 137–149. [CrossRef] [PubMed]
30. Liedman, B.; Andersson, H.; Bosaeus, I.; Hugosson, I.; Lundell, L. Changes in body composition after gastrectomy: Results of a controlled, prospective clinical trial. *World J. Surg.* **1997**, *21*, 416–421. [CrossRef] [PubMed]
31. Bachmann, J.; Heiligensetzer, M.; Krakowski-Roosen, H.; Buchler, M.W.; Friess, H.; Martignoni, M.E. Cachexia worsens prognosis in patients with resectable pancreatic cancer. *J. Gastrointest. Surg.* **2008**, *12*, 1193–1201. [CrossRef] [PubMed]

32. Haugen, F.; Labori, K.J.; Noreng, H.J.; Buanes, T.; Iversen, P.O.; Drevon, C.A. Altered expression of genes in adipose tissues associated with reduced fat mass in patients with pancreatic cancer. *Arch. Physiol. Biochem.* **2011**, *117*, 78–87. [CrossRef] [PubMed]
33. Awad, S.; Tan, B.H.; Cui, H.; Bhalla, A.; Fearon, K.C.; Parsons, S.L.; Catton, J.A.; Lobo, D.N. Marked changes in body composition following neoadjuvant chemotherapy for oesophagogastric cancer. *Clin. Nutr.* **2012**, *31*, 74–77. [CrossRef] [PubMed]
34. Dalal, S.; Hui, D.; Bidaut, L.; Lem, K.; del Fabbro, E.; Crane, C.; Reyes-Gibby, C.C.; Bedi, D.; Bruera, E. Relationships among body mass index, longitudinal body composition alterations, and survival in patients with locally advanced pancreatic cancer receiving chemoradiation: A pilot study. *J. Pain Symptom Manag.* **2012**, *44*, 181–191. [CrossRef]
35. Willemse, P.P.; van der Meer, R.W.; Burggraaf, J.; van Elderen, S.G.; de Kam, M.L.; de Roos, A.; Lamb, H.J.; Osanto, S. Abdominal visceral and subcutaneous fat increase, insulin resistance and hyperlipidemia in testicular cancer patients treated with cisplatin-based chemotherapy. *Acta Oncol.* **2014**, *53*, 351–360. [CrossRef]
36. Prado, C.M.; Sawyer, M.B.; Ghosh, S.; Lieffers, J.R.; Esfandiari, N.; Antoun, S.; Baracos, V.E. Central tenet of cancer cachexia therapy: Do patients with advanced cancer have exploitable anabolic potential? *Am. J. Clin. Nutr.* **2013**, *98*, 1012–1019. [CrossRef]
37. Tan, B.H.; Birdsell, L.A.; Martin, L.; Baracos, V.E.; Fearon, K.C. Sarcopenia in an overweight or obese patient is an adverse prognostic factor in pancreatic cancer. *Clin. Cancer Res.* **2009**, *15*, 6973–6979. [CrossRef] [PubMed]
38. Das, S.K.; Eder, S.; Schauer, S.; Diwoky, C.; Temmel, H.; Guertl, B.; Gorkiewicz, G.; Tamilarasan, K.P.; Kumari, P.; Trauner, M.; *et al.* Adipose triglyceride lipase contributes to cancer-associated cachexia. *Science* **2011**, *333*, 233–238. [CrossRef] [PubMed]
39. Petruzzelli, M.; Schweiger, M.; Schreiber, R.; Campos-Olivas, R.; Tsoli, M.; Allen, J.; Swarbrick, M.; Rose-John, S.; Rincon, M.; Robertson, G.; *et al.* A switch from white to brown fat increases energy expenditure in cancer-associated cachexia. *Cell Metab.* **2014**, *20*, 433–447. [CrossRef] [PubMed]
40. Tisdale, M.J. Cachexia in cancer patients. *Nat. Rev. Cancer* **2002**, *2*, 862–871. [CrossRef] [PubMed]
41. Ryden, M.; Arner, P. Fat loss in cachexia—Is there a role for adipocyte lipolysis? *Clin. Nutr.* **2007**, *26*, 1–6. [CrossRef] [PubMed]
42. Lopez-Soriano, J.; Argiles, J.M.; Lopez-Soriano, F.J. Lipid metabolism in rats bearing the Yoshida AH-130 ascites hepatoma. *Mol. Cell. Biochem.* **1996**, *165*, 17–23. [CrossRef] [PubMed]
43. Lanza-Jacoby, S.; Lansey, S.C.; Miller, E.E.; Cleary, M.P. Sequential changes in the activities of lipoprotein lipase and lipogenic enzymes during tumor growth in rats. *Cancer Res.* **1984**, *44*, 5062–5067. [PubMed]
44. Laurencikiene, J.; Stenson, B.M.; Arvidsson Nordstrom, E.; Agustsson, T.; Langin, D.; Isaksson, B.; Permert, J.; Ryden, M.; Arner, P. Evidence for an important role of CIDEA in human cancer cachexia. *Cancer Res.* **2008**, *68*, 9247–9254. [CrossRef] [PubMed]
45. Lopez-Soriano, J.; Argiles, J.M.; Lopez-Soriano, F.J. Sequential changes in lipoprotein lipase activity and lipaemia induced by the Yoshida AH-130 ascites hepatoma in rats. *Cancer Lett.* **1997**, *116*, 159–165. [CrossRef] [PubMed]
46. Lopez-Soriano, J.; Argiles, J.M.; Lopez-Soriano, F.J. Marked hyperlipidaemia in rats bearing the Yoshida AH-130 ascites hepatoma. *Biochem. Soc. Trans.* **1995**, *23*, 492.
47. Thompson, M.P.; Koons, J.E.; Tan, E.T.; Grigor, M.R. Modified lipoprotein lipase activities, rates of lipogenesis, and lipolysis as factors leading to lipid depletion in C57BL mice bearing the preputial gland tumor, ESR-586. *Cancer Res.* **1981**, *41*, 3228–3232. [PubMed]
48. Notarnicola, M.; Miccolis, A.; Tutino, V.; Lorusso, D.; Caruso, M.G. Low levels of lipogenic enzymes in peritumoral adipose tissue of colorectal cancer patients. *Lipids* **2012**, *47*, 59–63. [CrossRef] [PubMed]
49. Briddon, S.; Beck, S.A.; Tisdale, M.J. Changes in activity of lipoprotein lipase, plasma free fatty acids and triglycerides with weight loss in a cachexia model. *Cancer Lett.* **1991**, *57*, 49–53. [CrossRef] [PubMed]
50. Bennani-Baiti, N.; Walsh, D. Animal models of the cancer anorexia-cachexia syndrome. *Support. Care Cancer* **2011**, *19*, 1451–1463. [CrossRef] [PubMed]
51. Bosaeus, I.; Daneryd, P.; Lundholm, K. Dietary intake, resting energy expenditure, weight loss and survival in cancer patients. *J. Nutr.* **2002**, *132*, 3465–3466.

52. Cao, D.X.; Wu, G.H.; Zhang, B.; Quan, Y.J.; Wei, J.; Jin, H.; Jiang, Y.; Yang, Z.A. Resting energy expenditure and body composition in patients with newly detected cancer. *Clin. Nutr.* **2010**, *29*, 72–77. [CrossRef] [PubMed]
53. Johnson, G.; Salle, A.; Lorimier, G.; Laccourreye, L.; Enon, B.; Blin, V.; Jousset, Y.; Arnaud, J.P.; Malthiery, Y.; Simard, G.; *et al.* Cancer cachexia: Measured and predicted resting energy expenditures for nutritional needs evaluation. *Nutrition* **2008**, *24*, 443–450. [CrossRef] [PubMed]
54. Fredrix, E.W.; Soeters, P.B.; Wouters, E.F.; Deerenberg, I.M.; von Meyenfeldt, M.F.; Saris, W.H. Effect of different tumor types on resting energy expenditure. *Cancer Res.* **1991**, *51*, 6138–6141. [PubMed]
55. Klein, S.; Wolfe, R.R. Whole-body lipolysis and triglyceride-fatty acid cycling in cachectic patients with esophageal cancer. *J. Clin. Investig.* **1990**, *86*, 1403–1408. [CrossRef]
56. Jeevanandam, M.; Horowitz, G.D.; Lowry, S.F.; Brennan, M.F. Cancer cachexia and the rate of whole body lipolysis in man. *Metabolism* **1986**, *35*, 304–310. [CrossRef] [PubMed]
57. Jaworski, K.; Sarkadi-Nagy, E.; Duncan, R.E.; Ahmadian, M.; Sul, H.S. Regulation of triglyceride metabolism. IV. Hormonal regulation of lipolysis in adipose tissue. *Am. J. Physiol. Gastrointest. Liver Physiol.* **2007**, *293*, 1–4. [CrossRef]
58. Jocken, J.W.; Blaak, E.E. Catecholamine-induced lipolysis in adipose tissue and skeletal muscle in obesity. *Physiol. Behav.* **2008**, *94*, 219–230. [CrossRef] [PubMed]
59. Holm, C. Molecular mechanisms regulating hormone-sensitive lipase and lipolysis. *Biochem. Soc. Trans.* **2003**, *31*, 1120–1124. [CrossRef] [PubMed]
60. Thompson, M.P.; Cooper, S.T.; Parry, B.R.; Tuckey, J.A. Increased expression of the mRNA for hormone-sensitive lipase in adipose tissue of cancer patients. *Biochim. Biophys. Acta* **1993**, *1180*, 236–242. [CrossRef] [PubMed]
61. Das, S.K.; Hoefler, G. The role of triglyceride lipases in cancer associated cachexia. *Trends Mol. Med.* **2013**, *19*, 292–301. [CrossRef] [PubMed]
62. Tsoli, M.; Schweiger, M.; Vanniasinghe, A.S.; Painter, A.; Zechner, R.; Clarke, S.; Robertson, G. Depletion of white adipose tissue in cancer cachexia syndrome is associated with inflammatory signaling and disrupted circadian regulation. *PLoS One* **2014**, *9*. [CrossRef] [PubMed]
63. Harms, M.; Seale, P. Brown and beige fat: Development, function and therapeutic potential. *Nat. Med.* **2013**, *19*, 1252–1263. [CrossRef] [PubMed]
64. Kir, S.; White, J.P.; Kleiner, S.; Kazak, L.; Cohen, P.; Baracos, V.E.; Spiegelman, B.M. Tumour-derived PTH-related protein triggers adipose tissue browning and cancer cachexia. *Nature* **2014**, *513*, 100–104. [CrossRef] [PubMed]
65. Ishiko, O.; Nishimura, S.; Yasui, T.; Sumi, T.; Hirai, K.; Honda, K.; Ogita, S. Metabolic and morphologic characteristics of adipose tissue associated with the growth of malignant tumors. *Jpn. J. Cancer Res.* **1999**, *90*, 655–659. [CrossRef] [PubMed]
66. Cawthorn, W.P.; Heyd, F.; Hegyi, K.; Sethi, J.K. Tumour necrosis factor-alpha inhibits adipogenesis *via* a beta-catenin/TCF4 (TCF7L2)-dependent pathway. *Cell Death Differ.* **2007**, *14*, 1361–1373. [CrossRef] [PubMed]
67. Bing, C.; Trayhurn, P. New insights into adipose tissue atrophy in cancer cachexia. *Proc. Nutr. Soc.* **2009**, *68*, 385–392. [CrossRef] [PubMed]
68. Lin, W.W.; Karin, M. A cytokine-mediated link between innate immunity, inflammation, and cancer. *J. Clin. Investig.* **2007**, *117*, 1175–1183. [CrossRef] [PubMed]
69. Harman-Boehm, I.; Bluher, M.; Redel, H.; Sion-Vardy, N.; Ovadia, S.; Avinoach, E.; Shai, I.; Kloting, N.; Stumvoll, M.; Bashan, N.; *et al.* Macrophage infiltration into omental *versus* subcutaneous fat across different populations: Effect of regional adiposity and the comorbidities of obesity. *J. Clin. Endocrinol. Metab.* **2007**, *92*, 2240–2247. [CrossRef] [PubMed]
70. Fain, J.N.; Madan, A.K.; Hiler, M.L.; Cheema, P.; Bahouth, S.W. Comparison of the release of adipokines by adipose tissue, adipose tissue matrix, and adipocytes from visceral and subcutaneous abdominal adipose tissues of obese humans. *Endocrinology* **2004**, *145*, 2273–2282. [CrossRef] [PubMed]
71. Batista, M.L., Jr.; Olivan, M.; Alcantara, P.S.; Sandoval, R.; Peres, S.B.; Neves, R.X.; Silverio, R.; Maximiano, L.F.; Otoch, J.P.; Seelaender, M.; *et al.* Adipose tissue-derived factors as potential biomarkers in cachectic cancer patients. *Cytokine* **2013**, *61*, 532–539. [CrossRef] [PubMed]

72. Argiles, J.M.; Alvarez, B.; Lopez-Soriano, F.J. The metabolic basis of cancer cachexia. *Med. Res. Rev.* **1997**, *17*, 477–498. [CrossRef] [PubMed]
73. Hosono, K.; Yamada, E.; Endo, H.; Takahashi, H.; Inamori, M.; Hippo, Y.; Nakagama, H.; Nakajima, A. Increased tumor necrosis factor receptor 1 expression in human colorectal adenomas. *World J. Gastroenterol.* **2012**, *18*, 5360–5368. [CrossRef] [PubMed]
74. Bing, C. Lipid mobilization in cachexia: Mechanisms and mediators. *Curr. Opin. Support. Palliat. Care* **2011**, *5*, 356–360. [CrossRef] [PubMed]

nutrients

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Review

The Role of Dietary Fat throughout the Prostate Cancer Trajectory

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Abstract: Prostate cancer is the second most common cancer diagnosed world-wide; however, patients demonstrate exceptionally high survival rates. Many lifestyle factors, including obesity and diet, are considered risk factors for advanced prostate cancer. Dietary fat is a fundamental contributor to obesity and may be specifically important for prostate cancer patients. Prostate cancer treatment can result in changes in body composition, affecting quality of life for survivors by increasing the risk of co-morbidities, like cardiovascular disease and diabetes. We aim to examine dietary fat throughout the prostate cancer treatment trajectory, including risk, cancer development and survivorship. Focusing on one specific nutrient throughout the prostate cancer trajectory provides a unique perspective of dietary fat in prostate cancer and the mechanisms that may exacerbate prostate cancer risk, progression and recurrence. Through this approach, we noted that high intake of dietary fat, especially, high intake of animal and saturated fats, may be associated with increased prostate cancer risk. In contrast, a low-fat diet, specifically low in saturated fat, may be beneficial for prostate cancer survivors by reducing tumor angiogenesis and cancer recurrence. The insulin-like growth factor (IGF)/Akt signaling pathway appears to be the key pathway moderating dietary fat intake and prostate cancer development and progression.

Keywords: risk; progression; survivorship; IGF signaling; saturated fatty acids; monounsaturated fatty acids; polyunsaturated fatty acids; trans fatty acids

1. Introduction

Prostate cancer is the second most commonly diagnosed malignancy in men worldwide. Prostate cancer diagnoses accounts for 15% of all male cancer diagnoses, second to only lung cancer [1,2]. In 2012, more than 1.1 million prostate cancer cases were diagnosed worldwide [1,2]; however, prostate cancer mortality rates are exceptionally low, with only 307,000 prostate cancer deaths estimated in 2012, accounting for only 6.6% of male deaths. [1]. This remarkably low mortality rate is attributed to the wide-spread use of prostate-specific antigen (PSA) screening in most developed countries where the incidence of prostate cancer is higher. PSA screening allows the detection of smaller and earlier stage tumors that may or may not progress to more advanced cancer. Because of the growing incidence of prostate cancer and the low death rates, prevention of prostate cancer and, specifically, aggressive and advanced prostate cancer is of the utmost importance. Many lifestyle factors, such as obesity, diet, physical activity levels and smoking, are considered risk factors in the development of prostate cancer. Consequently, a large body of literature endeavours to elucidate the role of lifestyle factors, including obesity, in prostate cancer risk, development of the tumour and successful survivorship.

Obesity and prostate cancer endure a complex relationship. Obesity is associated with increased incidence of high-risk or aggressive prostate cancer [3]. Additionally, it is associated with increased

incidence of prostate cancer recurrence [4], which may be attributed to increased adiposity and reduced muscularity in prostate cancer patients who undergo androgen deprivation therapy (ADT) [5]. One of the potential factors thought to link obesity and prostate cancer is dietary fat intake. Dietary fat is a fundamental contributor to obesity [6] and may help explain the complicated relationship between obesity and prostate cancer. For prostate cancer patients, obesity is not only a potential risk factor, but changes in body composition may affect the quality of life of prostate cancer survivors by increasing the risk of co-morbidities, like cardiovascular disease [7], and by decreasing functional outcomes [8]. Changes in dietary fat intake may help ameliorate some of the negative outcomes associated with changes in body composition in the prostate cancer survivor. Thus, many studies have looked at diet and exercise manipulation to counter the expected increase in adiposity and the loss of muscle. Further consideration into the role of obesity, dietary fat and the prostate cancer trajectory is warranted to help clarify this complex relationship.

The aim of this manuscript is to review the literature examining dietary fat throughout the prostate cancer trajectory, including risk, development and survivorship. The majority of reviews that examine the role of dietary fat in prostate cancer consider many nutrients at one particular time point during the prostate cancer trajectory (*i.e.*, the role of diet in prostate cancer risk). This narrative review will examine and summarize the role of dietary fat in prostate cancer throughout the trajectory to provide a unique and comparative perspective across the time-course of the disease. We also aim to investigate the mechanisms that exacerbate prostate cancer risk, progression and recurrence and how they may be related. Saturated fat, *n*-3 fatty acids and trans-fatty acids will be given special considerations, as these types of dietary fat may be of particular importance for the prostate cancer patient. The perspectives described in this review will facilitate the identification of gaps in the literature and will aid future studies to advance this discipline.

2. Dietary Fat and Prostate Cancer Risk

There are numerous nutritional factors associated with obesity and prostate cancer risk, including positive energy balance [9], red meat and dairy intake [10], saturated fat [11], trans fatty acid intake [12] and total dietary fat intake [11]. Conversely, *n*-3 fatty acids have been identified as having a potentially protective effect against prostate cancer [11]. The earliest identification of dietary fat as a potential risk factor for prostate cancer stems from correlations and case-controls studies; however, the results were mixed, with studies demonstrating positive, negative and no associations between dietary fat and the risk of prostate cancer. These studies are based on the principle that participants moving from countries where the risk of prostate cancer is low, such as Japan, had a significant increase in the risk of developing prostate cancer upon moving to North America [13,14]. These studies hypothesized that increased dietary fat consumption may be driving this relationship [15]; however, they did not quantify dietary fat, nor did they control for confounders, which may affect the association with prostate cancer. Early case-control studies examining dietary fat as a risk factor for prostate cancer also demonstrated mixed results [16–23]. Some of these case-control studies demonstrated positive associations between both dietary and saturated fat intake [16–19], while others found no association between dietary fat intake and prostate cancer diagnosis [20–23]. Early prospective cohort studies also demonstrated mixed results, with some demonstrating no association between high meat and dairy products [24–26] and prostate cancer risk, while animal product intake was positively associated with the risk of prostate cancer, though these associations were weak (relative risk: 1.3–1.5; $p > 0.1$ [27]; relative risk: 1.38, 95% CI: 0.89–2.16) [27,28]. These studies were also limited by the methods used to assess dietary intake. It was not until Giovannucci and colleagues [29] published their prospective analysis of dietary fat and prostate cancer risk using the Health Professionals follow-up study that the relationship between dietary fat and prostate cancer risk began to become clearer. Giovannucci *et al.* [29] used a semi-quantitative food frequency questionnaire to assess dietary fat in these men. They concluded that total fat consumption was related to the risk of advanced cancer and that this relationship was primarily related to animal fat consumption (relative risk: 1.79; 95% CI: 1.04–3.07).

Fat from dairy products, with the exception of butter, appeared to be unrelated to advanced prostate cancer risk.

These early studies formed the basis for much of the current work that has evaluated dietary fat intake as a potential risk factor for prostate cancer. A systematic review by Ma and Chapman [30] indicated that based on the Oxford Centre for Evidenced-Based Medicine Levels of Evidence, there is Level 2b evidence to suggest that dietary fat, and in particular, high intake of animal and saturated fats (~40% total fat intake; [31]), is associated with the increased risk of prostate cancer. Most of the studies evaluated in the systematic review were primarily Level 4 studies (Case series), but also including some Level 2 (prospective comparative) and 3 (retrospective cohort and case-control) studies. Ma and Chapman [30] examined numerous types of fat and identified that there is suggestive evidence to support total fat intake, animal and saturated fat intake, monounsaturated fatty acids and α-linoleic acid as being associated with the increased risk of prostate cancer, while there was not enough evidence to draw conclusions about polyunsaturated fatty acid intake, as well as eicosapentaenoic acid (EPA).

Gathirua-Mwangi and Zhang [32] evaluated the relationship between dietary fat intake and the risk of advanced cancer. Despite the limited number of studies, their conclusions were similar to Ma and Chapman [30], indicating that total fat intake (odds ratios: 1.25–1.80; 95% CI: 0.75–2.91) and, specifically, saturated fat intake are significantly associated with the increased risk of advanced prostate cancer (odds ratios: 1.44–1.80; 95% CI: 0.82–5.20) [32]. When they evaluated the classification of fatty acids, they concluded that monounsaturated and polyunsaturated fatty acids were not associated with the risk of advanced prostate cancer, nor were linoleic and linolenic acids. They also noted that higher animal fat intake was also associated with advanced prostate cancer, and a borderline inverse relationship was noted for total vegetable fat intake and advanced prostate cancer risk. The most recent evidence for this relationship comes from Pelser and colleagues [11]. They suggested that a dichotomous relationship between dietary fat consumption and prostate cancer risk exists. Using data from the NIH-AARP Diet and Health Study, they demonstrated that total fat intake and mono- and poly-unsaturated fats were not associated with the incidence of prostate cancer, but saturated fat increased the risk of advanced prostate cancer (hazards ratio: 1.21; 95% CI: 1.00–1.46) and prostate cancer death (hazards ratio: 1.47; 95% CI: 1.01–2.15). They also noted that EPA was associated with decreased risk of fatal prostate cancer (hazards ratio: 0.82; 95% CI: 0.64–1.04).

Despite the limited number of studies, the systematic reviews cited here indicated some evidence for the increased risk of prostate cancer and, specifically, advanced or fatal prostate cancer with increased dietary fat intake. When evaluating specific categories of fats, saturated and animal fats appear to pose the greatest risk for prostate cancer development, while EPA may have a protective effect. However, more evidence is needed to understand the potential mechanisms that drive the relationship between fat intake and prostate cancer development.

3. Effects of Dietary Fat on Prostate Cancer Development

A number of potential mechanisms have been identified that may mediate the effects of dietary fat on prostate cancer development and progression. These include changes in the insulin-like growth factor (IGF)-Akt pathway, androgen signaling and alterations in cell proliferation and angiogenesis.

3.1. The IGF Signaling Pathway

The IGF signaling pathway is one of the main regulating pathways in which dietary fat can promote prostate cancer development and progression. Dietary fat intake is positively correlated with circulating levels of IGF-1, which may ultimately result in increased signaling through the IGF signaling pathway [33,34]. Fat intake is also negatively correlated with insulin-like growth factor binding protein-3 (IGFBP-3), the major binding protein of IGF-1 in plasma [33,34], which is also independently associated with the risk of prostate cancer [35]. Thus, increases in circulating levels of IGF-1 and decreases in IFGBP-3 can increase the stimulation of the IGF signaling cascade, which becomes disrupted in malignant cells. Given that perturbations in the IGF system play a critical role

in cell proliferation, differentiation, apoptosis and transformation, understanding the function of IGF signaling is key to determining the mechanisms of dietary fat in prostate cancer development and proliferation.

In non-malignant cells, IGF-1 binds to one of two receptors, IGF-1R and IGF-2R, with a preference for IGF-1R [36]. IGF-1R is a type 2 tyrosine kinase receptor that, under normal conditions, is involved in proliferation and differentiation; however, in transformed malignant cells, IGF-1R plays a key role in the establishment and progression of cancerous cells [36]. Cells lines without IGF-1R have an impaired ability to transform into malignant cells, though the exact mechanism is unknown [37]. In contrast, the presence of IGF-1R may also contribute to the ability of malignant cells to metastasize [36]. The primary role of IGF-1R cell growth is mediated through the IGF-1R/insulin receptor substrate (IRS)-1 axis. Numerous reviews on the role of the IGF-1R/IRS-1 axis and its role in cancer are available [36–41]. Briefly, when a ligand binds to IGF-1R, it activates the tyrosine kinase of the cytoplasmic domain of the receptor [36]. This results in the phosphorylation of the numerous IGF-1R substrates—IRS-1 and -2 Src- and collagen-homology (SHC) and growth factor receptor protein 2 (Grb2) [38–41]. The phosphorylated IRS and SHC in combination with Grb2 activate the mitogen-activated protein kinase (MAPK) cascade, resulting in cell growth and proliferation [38–41]. IRS-1 also phosphorylates phosphatidylinositol 3′-kinase (PI3K) and the Akt complex, which blocks Bad and Caspase 9, which are key pro-apoptotic proteins [38–41]. These pathways help regulate the metabolic and anti-apoptotic signal of IGF-1 [38–41]. Akt can also activate the transcription of nuclear factor-κB (NFκB) and mammalian target of rapamycin (mTOR). When NFκB is active in healthy cells, it functions as a regulator for many genes that control proliferation and cell survival; in cancerous cells, it is dysregulated, resulting in decreased cell death [38–41]. Akt activation promotes signaling in the IGF-1R/IRS-1 axis, which contributes to the dysregulation of NFκB and, ultimately, cancer cell growth. The IGF-1R/IRS-1 axis also indirectly increases mTOR activity [38–41]. mTOR then promotes cell growth through the S6kinase, Protein Kinase C (PKC), p21 and Glycogen Synthase Kinase 3β (GSK3β) activation [38]. These pathways change the cell cycle, ultimately promoting cell growth. Thus, increased dietary fat intake may potentially promote malignant cell growth through increased IGF-1 and decreased IGFBP-3, resulting in increased IGF-1 signaling through the IGF-1R, a receptor implicated in the transformation of healthy cells to cancerous cells and a mediator of cell growth through the IGF-1R/IRS-1 axis.

3.2. Increases in Androgen Signaling Due to Dietary Fat Intake

Along with IGF-1 signaling, androgen signaling is another pathway in which dietary fat intake can influence prostate cancer development. Some have demonstrated that decreased dietary fat intake is associated with decreased androgen [42,43] and testosterone levels [44–46], which subsequently improves signaling mediated through the IGF-1 signaling pathway. Androgens play a key role in the development of normal healthy prostate tissue; however, androgen signaling and, specifically, the androgen receptor, also known as nuclear receptor subfamily 3, group C, member 4, (NR3C4), is the principle stimulant of prostate cancer progression. In the early stages of development, malignant prostate cancer cells require androgen stimulation for growth [47]. However, increased androgen receptor growth is associated with the progression or switch of hormone sensitive cancers to hormone-resistant cancers, the more aggressive form of prostate cancer [47,48]. Androgens stimulate prostate cancer cell growth via the Erk-2 pathway, where Erk-2 activation increases the androgen receptor complex content in the prostate cells [49]. Androgens also increase IGF-1R expression [50], which is associated with prostate cancer development, as previously described, but IGF-1 can also have a more direct effect on the androgen receptor. Stimulation of the IGF-1 signaling cascade activates MAPK, which decreases the acetylation of heat shock protein (HSP) 90. HSP90 is a chaperone protein of the androgen receptor [51]. This decreased acetylation increases the association of the HSP90 with the androgen receptor, which further stimulates the signaling through the androgen receptor pathway. Ultimately, stimulation of this pathway results in upregulation of the androgen receptors, their associated proteins and androgen receptor regulated genes. High-fat diets have been shown

to increase stimulation through the IGF-1 axis [33,34], as well as being associated with increased androgen and testosterone levels [42–46]. Diets low in total and saturated fat and high in *n*-3 fatty acids counter this pathway by inhibiting IGF-1 binding and decreasing HSP90 association with the androgen receptor [52]. Consequently, there is increased acetylation of androgen receptors resulting in their degradation, ultimately reducing androgen receptor proteins, as well as the number of androgen receptor-regulated genes.

3.3. Dietary Fat Mediation of Cell Proliferation and Angiogenesis

While total dietary fat and saturated fat intake have not been shown to have a direct effect in the cell cycle and angiogenesis, *n*-3 fatty acid intake has been shown to inhibit malignant cell proliferation and angiogenesis [53]. *n*-3 fatty acids work both intrinsically (mitochondrial pathway) and extrinsically (death receptor pathway) to induce apoptosis. Specifically, they can inhibit PI3K activity, which phosphorylates the Akt complex. Phosphorylated Akt regulates a number of downstream factors that can directly affect apoptosis and the cell cycle. Briefly, phosphorylated Akt inhibits caspase 9 and pro-apoptotic proteins Bad and BAK, leading to decreased apoptosis; inhabitation of these pathways via *n*-3 fatty acids ultimately results in increased cell death. Phosphorylated Akt also increases p27 and inactivates NFκB signaling, which can independently and directly halt the cell cycle. Thus, *n*-3 fatty acids may have an important role in inhibiting malignant cell proliferation and angiogenesis.

Total dietary fat and saturated fat may work indirectly to enhance cell proliferation and angiogenesis through the creation of reactive oxygen species (ROS) [54]. ROS generated endogenously and externally are associated with cancer progression by inducing a number of neoplastic transformations. ROS alter the conformational structure of the p53 protein, resulting in changes in protein behaviour and causing a mutated phenotype. These types of p53 mutations are specifically important in prostate cancer progression. Total dietary fat, especially *n*-6 fatty acids, as well as androgens can all serve as oxidants directly increasing oxidative stress and altering a number of transcription factors. Oxidative stress has been shown to be higher in the benign epithelium of men with prostate cancer when compared to men without prostate cancer [54], while Lee *et al.* [55] demonstrated that inactivation of glutathione-s-transferase pi, a pro-oxidant scavenging enzyme, is critical in the development of prostate cancer carcinogenesis. Specifically, dietary fat consumption may contribute to the carcinogenesis of prostate tissue via lipid peroxidation [56], thus resulting in increased oxidative stress.

The relationship between dietary fat, cell proliferation and angiogenesis is less direct than some of the other relationships previously described in this review. There is little evidence to suggest that total and saturated fat intake play any part in malignant cell proliferation and angiogenesis. Dietary fat may be working though secondary pathways, such as ROS generation, to stimulate proliferation and angiogenesis. More research in these areas is needed to elucidate this complex relationship.

4. Fatty Acid Type and Its Relationship with Prostate Cancer

While there are many proposed mechanisms through which dietary fat may affect prostate cancer development and progression, much of the literature examining prostate cancer and dietary fat intake lacks a definite conclusion as to the negative impact of dietary fat in prostate cancer. This discrepancy may be, in part, attributed to the diverse physiological effects of different types and distributions of dietary fats; thus, an evaluation of total dietary fat intake may miss important relationships between specific types of dietary fat intake and prostate cancer development. The direct and indirect roles, as well as the interrelationships between saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), polyunsaturated fatty acids (PUFA) and trans fatty acids (TFA) on prostate cancer development and progression need to be elucidated in future work.

There is diverse epidemiological evidence suggesting that SFA intake is a risk factor for prostate cancer. Some studies show increased risk of prostate cancer risk and progression, while others are inconclusive. Animal models suggest that the quality of dietary fat, and, specifically, the PUFA content

of dietary fat intake, may be an important prognosticator [57,58]. Long-chain SFA may negatively affect prostate cancer, while short-chain fatty acids may be beneficial. Escobar *et al.* [59] fed rats two isocaloric low-fat diets, in which only 7% of total calorie content was derived from fat. In one diet, fat was derived from lard and the other from linseed oil, which Vereshagin and Novitskaya [60] identified as: 52%–55% α-linolenic acid, 18:3*n*-3; ~7% palmitic acid, 16:0; ~4% stearic acid, 18:0; ~18%–23% oleic acid, 18:1*n*-9; and ~14%–17% linoleic acid, 18:2*n*-6. The lard-derived diet, which was high in palmitic acid and oleic acid, increased prostate weight, testosterone, cell proliferation and androgen receptor expression, compared to the diet rich in α-linolenic acid. These data support the notion that long-chain SFA may have negative effects on various physiological factors in prostate cancer; however, this has yet to be investigated in humans.

The role of MUFA is less clear than the role of SFA in prostate cancer development and progression. The Mediterranean diet, which is rich in oleic acid (18:1*n*-9), was originally thought to reduce the risk of prostate cancer [61]; however, this early evidence remains inconclusive, as studies have demonstrated protective [62], no association [63,64] and negative effects of MUFA on prostate cancer [65,66]. As the Mediterranean diet contains a variety of potential protective agents, such as lycopene-rich tomatoes, fish that are high in *n*-3 fatty acids and low quantities of red meat, it is challenging to pinpoint the role of MUFA.

There is extensive research examining the role of PUFA in prostate cancer. It is reported that *n*-6 fatty acids increase prostate cancer risk, while *n*-3 fatty acids decrease prostate cancer risk [57,58]. Specifically, the anti-cancer benefits of a diet with low *n*-6-to-*n*-3 fatty acid ratios are supported in the literature [67]. This type of diet is in specific contrast to the Western style diet with a 30:1 ratio of *n*-6 to *n*-3 fatty acids [68–71]. The main mechanisms of action seem to converge on the IGF-1 signaling pathway, leading to prevention or inhibition of malignant growth in prostate cancer cells.

Conversely, a limited number of studies have examined the role of TFA in prostate cancer risk and development. Overall, they appear to suggest an increased risk of prostate cancer with increased serum TFA. Previously, Smith *et al.* [72] reviewed the role of TFA in a number of cancers and identified six studies that examined prostate cancer. Bakker *et al.* [73] conducted an ecological study examining the fatty acid component of adipose tissue in 690 participants across eight European countries and Israel and found no significant relationship between TFA levels and risk of prostate cancer. King *et al.* [74] and Chavarro *et al.* [75] used case-control and nested case-control methodologies, respectively, and demonstrated increased risk in prostate cancer with increased levels of the number of different TFA in both serum phospholipids [74] and whole blood [75]. Food frequency questionnaires have also been used to examine TFA intake and prostate cancer risk and have demonstrated the increased risk of advanced cancer [76] and no relationship [77,78]. More recently, Brasky *et al.* [79] demonstrated an inverse risk between serum TFA and high risk prostate cancer and suggested that the relationship between TFA and prostate cancer risk may be more complicated than earlier hypotheses. Interestingly, Laake *et al.* [80] demonstrated that the source of the TFAs may play a significant role in determining risk, as they demonstrated no association between dietary intake levels of TFA from vegetable sources, but increased risk of prostate cancer when the TFA source is a fish source. This evidence is in the very early stages, where data is associative; thus, more research is warranted to identify potential mechanisms in which prostate cancer is affected by TFA intake, as this relationship appears to be more complex than previously thought.

5. Dietary Fat in the Prostate Cancer Survivor

As it is the second most common malignancy diagnosed in men worldwide and because the survival rates are so high [1,2], the number of prostate cancer survivors is constantly increasing. Thus, understanding how manipulating dietary fat may positively influence quality of life in prostate cancer survivorship is important. Another important consideration for prostate cancer survivors is the use of aADT, a common treatment for aggressive prostate cancer, which causes significant loss of skeletal muscle and increases in adipose tissue and has been related to increased risk of cardiovascular disease

and diabetes in prostate cancer survivors [7]. However, there is limited data examining dietary fat throughout this stage of the prostate cancer trajectory.

Early reports demonstrate that fat intake, specifically saturated fat intake, may decrease disease-specific survival. Fradet and colleagues [81] followed a group of men diagnosed with prostate cancer for an average of 5.2 years. After controlling for cancer grade, clinical stage, treatment age and total energy intake, men in the lowest tertile of saturated fat intake had a decreased risk of dying from prostate cancer as compared to those in the highest tertile of saturated fat intake (hazards ratio: 3.13; 95% CI: 1.28–7.67). These findings align with the idea that fat intake, specifically saturated fat intake, promotes an environment conducive to prostate cancer growth. While this observational evidence supports the hypothesis that high levels of dietary fat have negative effects on prostate cancer survival, well-designed intervention studies are needed to identify if manipulating dietary fat can have positive effects on survivorship. These interventions should also investigate different sources of dietary fat and their ability to improve quality of life for the prostate cancer survivor by mitigating cancer recurrence, as some literature suggests that plant-based fats may be less harmful than animal-based fats [31,32,59].

Davies and colleagues [82] reviewed the evidence of the effects of low-fat diets on prostate cancer progression and identified five studies that manipulated dietary fat intake in some way to examine the potentially protective effects of these interventions. Ornish *et al.* [83] used a randomized control trial (RCT) design to examine the effect of an entire lifestyle intervention, including low-fat vegan diet supplemented with fish oil and a number of other vitamins and minerals combined with physical activity and stress management techniques, in a group of prostate cancer survivors and examined the effects on the prostate specific antigen (PSA) levels and LNCaP (human prostatic adenocarcinoma) cell growth *in vitro*. This comprehensive lifestyle intervention demonstrated significant improvements for prostate cancer patients *versus* the control group; however, the combined nature of this intervention makes it difficult to discern the individual effects of the lifestyle components (*i.e.*, exercise *vs.* low-fat diet *vs.* stress management techniques). Perhaps the synergistic interactions between these components improve patient outcomes. Two studies have examined the effects of low-fat diets supplemented with flaxseed on prostate cancer outcomes. Demark-Wahnefried *et al.* [84] demonstrated decreased proliferation rates in the men supplemented with flaxseed and that the low-fat diet group had significantly reduced serum cholesterol levels following ~30 days of supplementation. Heymach *et al.* [85] demonstrated that, as compared to the control arm, a low-fat diet, a flaxseed-supplemented diet and a low-fat diet with a flaxseed supplementation for 30 days had each decreased a number of angiogenic factors, though the results were greatest in the low-fat diet alone group. They speculate that the NFκB pathway may be regulating this response. A review by Hori *et al.* [86] supports the hypothesis suggested by the flax-supplemented diet that *n*-3 fatty acid may be beneficial for prostate cancer patients. Like Ornish *et al.* [83], Aronson *et al.* [87] used an RCT design to look at the effects of a four-week low-fat diet intervention as compared to a traditional Western diet on the effects of LNCaP cell growth. Serum for the low-fat diet group decreased the growth of the LNCaP cells as compared to the serum from the men on the Westernized diet. While there appears to be some evidence as to the protective effect of a low-fat diet for prostate cancer survivors, more research is warranted to better elucidate the specific components of these lifestyle interventions that will be most effective for prostate cancer patients.

Androgen Deprivation Therapy and Dietary Fat

ADT is a common treatment for prostate cancer patients; however, its use can have negative consequences for prostate cancer survivors. Specifically, patients undergoing ADT lose skeletal muscle mass and gain fat mass [88]. These changes are associated with increased risk of cardiovascular disease and diabetes [7]. Because of the associated changes in body composition and risk of comorbidities in survivorship, dietary intervention may be useful for prostate cancer patients receiving ADT. However, these considerations are beyond the scope of this review. Saylor and Smith [89] suggest lifestyle intervention, including low-fat diet and increased physical activity and weight control, may be

beneficial in the prevention of these comorbidities in men receiving ADT and that future investigations are justified.

6. Conclusions

The aim of this paper was to examine dietary fat throughout the prostate cancer trajectory, including risk, development and survivorship. In most cases, there is limited evidence linking dietary fat and prostate cancer; however, some trends do emerge. Dietary fat, and, in particular, high intake of animal and saturated fats, may be associated with prostate cancer risk. The IGF/Akt signaling pathway appears to be the key signaling pathway moderating malignant cell growth and changes in androgen receptor signaling. The type of fat consumed may mediate the relationship between dietary fat and prostate cancer. Saturated fat and TFA have been negatively associated with prostate cancer development, while PUFA may have a protective effect, though these relationships remain tenuous. For prostate cancer survivors, a diet low in fat and particularly low in saturated fat may be beneficial, as it may reduce tumor angiogenesis and cancer recurrence. Integrating research throughout the prostate cancer trajectory may provide new insights into the relationship between dietary fat intake and prostate cancer, as many of the same pathways are implicated throughout the trajectory. In conclusion, preliminary evidence suggests diets low in fat may be beneficial at any point in the prostate cancer trajectory; however, much more research is needed to elucidate the complex relationships that exist between dietary fat and prostate cancer biology.

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References

1. Ferlay, J.; Soerjomataram, I.; Ervik, M.; Dikshit, R.; Eser, S.; Mathers, C.; Rebelo, M.; Parkin, D.M.; Forman, D.; Bray, F. GLOBOVAN 2012 v1.0, Cancer Incidence and Mortality Worldwide: IARC CancerBase No. 11. Available online: http://globocan.iarc.fr/Pages/fact_sheets_cancer.aspx (accessed on 29 September 2014).
2. Bray, F.; Ren, J.S.; Masuyer, E.; Ferlay, J. Estimates of global cancer prevalence for 27 sites in the adult population in 2008. *Int. J. Cancer* **2013**, *132*, 1133–1145. [CrossRef] [PubMed]
3. Discacciati, A.; Orsini, N.; Wolk, A. Body mass index and incidence of localized and advanced prostate cancer—A dose-response meta-analysis of prospective studies. *Ann. Oncol.* **2012**, *23*, 1665–1671. [CrossRef] [PubMed]
4. Cao, Y.; Ma, J. Body mass index, prostate-cancer specific mortality, and biochemical recurrence: A systematic review and meta-analysis. *Cancer Prev. Res. (Phila)* **2011**, *4*, 486–501. [CrossRef]
5. Nguyen, P.L.; Alibhai, S.M.; Basaria, S.; D'Amico, A.V.; Kantoff, P.W.; Keating, N.L.; Penson, D.F.; Rosario, D.J.; Tombal, B.; Smith, M.R. Adverse effects of androgen deprivation therapy and strategies to mitigate them. *Eur. Urol.* **2014**, in press.
6. Bray, G.A.; Popkin, B.M. Dietary fat intake does affect obesity! *Am. J. Clin. Nutr.* **1998**, *68*, 1157–1173. [PubMed]
7. Keating, N.L.; O'Malley, A.J.; Freedland, S.J.; Smith, M.R. Diabetes and cardiovascular disease during androgen deprivation therapy: Observational study of veterans with prostate cancer. *J. Natl. Cancer Inst.* **2010**, *102*, 39–46. [CrossRef] [PubMed]
8. Resnick, M.J.; Koyama, T.; Fan, K.H.; Albertsen, P.C.; Goodman, M.; Hamilton, A.S.; Hoffman, R.M.; Potosky, A.L.; Stanford, J.L.; Stroup, A.M.; *et al.* Long-term functional outcomes after treatment for localized prostate cancer. *N. Engl. J. Med.* **2013**, *368*, 436–445. [CrossRef] [PubMed]

9. Andersson, S.O.; Wolk, A.; Bergstrom, R.; Adami, H.O.; Englholm, G.; Englund, A.; Nyrén, O. Body size and prostate cancer: A 20 year follow-up study among 135006 Swedish construction worker. *J. Natl. Cancer Inst.* **1997**, *5*, 385–389. [CrossRef]
10. Mandair, D.; Rossi, R.E.; Pericleous, M.; Whyand, T.; Caplin, M.E. Prostate cancer and the influence of dietary factors and supplements: A systematic review. *Nutr. Metab. (Lond.)* **2014**, *11*. [CrossRef]
11. Pelser, C.; Mondul, A.M.; Hollenbeck, A.R.; Park, Y. Dietary fatty acids and risk of prostate cancer in the NIH-AARP diet and health study. *Cancer Epidemiol. Biomark. Prev.* **2013**, *22*, 697–707. [CrossRef]
12. Hu, J.; la Vecchia, C.; de Groh, M.; Negri, E.; Morrison, H.; Mery, L. Dietary transfatty acids and cancer risk. *Eur. J. Cancer Prev.* **2011**, *20*, 530–538. [CrossRef] [PubMed]
13. Haenszel, W.; Kurihara, M. Studies of Japanese migrants. I. Mortality from cancer and other disease among Japanese in the United States. *J. Natl. Cancer Inst.* **1968**, *40*, 43–68. [PubMed]
14. Staszewski, W.; Haenszel, W. Cancer mortality among the Polish-born in the United States. *J. Natl. Cancer Inst.* **1965**, *35*, 291–297. [PubMed]
15. Armstrong, B.; Doll, R. Environmental factors and cancer incidence and mortality in different countries, with special reference to dietary practice. *Int. J. Cancer* **1975**, *15*, 617–631. [CrossRef] [PubMed]
16. Graham, S.; Haughey, B.; Marshall, J.; Priore, R.; Byers, T.; Rzepka, T.; Mettlin, C.; Pontes, J.E. Diet in the epidemiology of the carcinoma of the prostate gland. *J. Natl. Cancer Inst.* **1983**, *70*, 687–692. [PubMed]
17. Heshmat, M.Y.; Kaul, L.; Kovi, J.; Jackson, M.A.; Jackson, A.G.; Jones, G.W.; Edson, M.; Enterline, J.P.; Worrell, R.G.; Perry, S.L. Nutrition and prostate cancer: A case-control study. *Prostate* **1985**, *6*, 7–17. [CrossRef] [PubMed]
18. Kolonel, L.N.; Yoshizawa, C.N.; Hankin, J.H. Diet and prostatic cancer: A case-control study in Hawaii. *Am. J. Epidemiol.* **1988**, *127*, 999–1012. [PubMed]
19. West, D.W.; Slattery, M.L.; Robinson, L.M.; French, T.K.; Mahoney, A.W. Adult dietary intake and prostate cancer risk in Utah: A case-control study with special emphasis on aggressive tumors. *Cancer Causes Control* **1991**, *2*, 85–94. [CrossRef]
20. Ross, R.K.; Paganini-Hill, A.; Henderson, B.E. The etiology of prostate cancer: What does the epidemiology suggest? *Prostate* **1983**, *4*, 333–344. [CrossRef] [PubMed]
21. Kaul, L.; Heshmat, M.Y.; Kovi, J.; Jackson, M.A.; Jackson, A.G.; Jones, G.W.; Edson, M.; Enterline, J.P.; Worrell, R.G.; Perry, S.L. The role of diet in prostate cancer. *Nutr. Cancer* **1987**, *9*, 123–128. [CrossRef] [PubMed]
22. Mettlin, C.; Selenskas, S.; Natarajan, N.; Huben, R. Beta-carotene and animal fats an their relationship to prostate cancer risk. A case-control study. *Cancer* **1989**, *64*, 605–612. [CrossRef] [PubMed]
23. Ohno, Y.; Yoshida, O.; Oishi, K.; Okada, K.; Yamabe, H.; Schroeder, F.H. Dietary beta-carotene and cancer of the prostate: A case-control study is Kyoto, Japan. *Cancer Res.* **1988**, *48*, 1331–1336. [PubMed]
24. Hirayama, T. Epidemiology or prostate cancer with special reference to the role of diet. *Natl. Cancer Inst. Monogr.* **1979**, *53*, 149–155. [PubMed]
25. Hsing, A.W.; McLaughlin, J.K.; Schuman, L.M.; Bjelke, E.; Gridley, G.; Wacholder, S.; Chien, H.T.; Blot, W.J. Diet, tobacco use, and fatal prostate cancer: Results from the Lutheran Brotherhood Cohort Study. *Cancer Res.* **1990**, *50*, 6836–6840. [PubMed]
26. Severson, R.K.; Nomura, A.M.; Grove, J.S.; Stemmermann, G.N. A prospective study of demographics, diet, and prostate cancer among men of Japanese ancestry in Hawaii. *Cancer Res.* **1989**, *49*, 1857–1860. [PubMed]
27. Mills, P.K.; Beeson, W.L.; Phillips, R.L.; Fraser, G.E. Cohort study of diet, lifestyle, and prostate cancer in Adventist men. *Cancer* **1989**, *64*, 598–604. [CrossRef] [PubMed]
28. Snowdon, D.A.; Phillips, R.L.; Choi, W. Diet, obesity, and risk of fatal prostate cancer. *Am. J. Epidemiol.* **1984**, *120*, 244–250. [PubMed]
29. Giovannucci, E.; Rimm, E.B.; Colditz, G.A.; Stampfer, M.J.; Ascherio, A.; Chute, C.G.; Willett, W.C. A prospective study of dietary fat and risk of prostate cancer. *J. Natl. Cancer Inst.* **1993**, *85*, 1571–1579. [CrossRef] [PubMed]
30. Ma, R.W.L.; Chapman, K. A systematic review of the effect of diet in prostate cancer prevention and treatment. *J. Hum. Nutr. Diet* **2009**, *22*, 187–199. [CrossRef] [PubMed]
31. Bairati, I.; Meyer, F.; Fradet, Y.; Moore, L. Dietary fat and advanced prostate cancer. *J. Urol.* **1998**, *159*, 1271–1275. [CrossRef] [PubMed]

32. Gathirua-Mwangi, W.G.; Zhang, J. Dietary factors and risk for advanced prostate cancer. *Eur. J. Cancer Prev.* **2014**, *23*, 96–109. [CrossRef] [PubMed]
33. Kaklamani, V.G.; Linos, A.; Kaklamani, E.; Markaki, I.; Koumantaki, Y.; Mantzoros, C.S. Dietary fat and carbohydrates are independently associated with circulating insulin-like growth factor 1 and insulin-like growth factor-binging protein 3 concentrations in healthy adults. *J. Clin. Oncol.* **1999**, *17*, 3291–3298. [PubMed]
34. Holmes, M.D.; Pollack, M.N.; Willett, W.C.; Hankinson, S.E. Dietary correlates of plasma insulin-like growth factor 1 and insulin-like growth factor binding protein 3 concentrations. *Cancer Epidemiol. Biomark. Prev.* **2002**, *11*, 852–861.
35. Rowlands, M.A.; Gunnell, D.; Harris, R.; Vatten, L.J.; Holly, J.M.; Martin, R.M. Circulating insulin-like growth factor peptides and prostate cancer risk: A systematic review and meta-analysis. *Int. J. Cancer* **2009**, *124*, 2416–2429. [CrossRef] [PubMed]
36. Singh, P.; Alex, J.M.; Bast, F. Insulin receptor (IR) and insulin-like-growth factor receptor 1 (IGF-1R) signaling systems: Novel treatment strategies for cancer. *Med. Oncol.* **2014**, *31*, 1–14. [CrossRef]
37. Baserga, R.; Peruzzi, F.; Reiss, K. The IGF-1 receptor in cancer biology. *Int. J. Cancer* **2003**, *107*, 873–877. [CrossRef] [PubMed]
38. Vankateswaran, V.; Klotz, L.H. Diet and prostate cancer: Mechanisms of action and implications for chemoprevention. *Nat. Rev. Urol.* **2010**, *7*, 442–453. [CrossRef] [PubMed]
39. LeRoith, D.; Roberts, C.T., Jr. The insulin-like growth factor system and cancer. *Cancer Lett.* **2003**, *195*, 127–137. [CrossRef] [PubMed]
40. Rubin, R.; Baserga, R. Insulin-like growth factor-1 receptor. Its role in cell proliferation, apoptosis, and tumorigenicity. *Lab. Investig.* **1995**, *73*, 311–331. [PubMed]
41. Pollack, M. Insulin and insulin-like growth factor signaling in neoplasia. *Nat. Rev. Cancer* **2008**, *8*, 915–928. [CrossRef] [PubMed]
42. Hill, P.; Wynder, E.L.; Garbaczewski, L.; Garnes, H.; Walker, A.R. Diet and urinary steroids in black and white North American men and black South African men. *Cancer Res.* **1979**, *39*, 5101–5105. [PubMed]
43. Fleshner, N.; Zlotta, A.R. Prostate cancer prevention: Past present and future. *Cancer* **2007**, *110*, 1889–1899. [CrossRef] [PubMed]
44. Hamalainen, E.; Adlercreutz, H.; Puska, P.; Pietinen, P. Diet and serum sex hormones in healthy men. *J. Steroid Biochem.* **1984**, *20*, 459–464. [CrossRef] [PubMed]
45. Hamalainen, E.; Adlercreutz, H.; Puska, P.; Pietinen, P. Decrease of serum total and free testosterone during a low-fat high-fibre diet. *J. Steroid Biochem.* **1983**, *18*, 369–370. [CrossRef] [PubMed]
46. Rosenthal, M.B.; Barnard, R.J.; Rose, D.P.; Inkeles, S.; Hall, J.; Pritikin, N. Effects of a high-complex-carbohydrate, low-fat, low-cholesterol diet on levels of serum lipids and estradiol. *Am. J. Med.* **1985**, *78*, 23–27. [CrossRef] [PubMed]
47. Grossmann, M.; Cheung, A.S.; Zajac, J.D. Androgens and prostate cancer pathogenesis and deprivation therapy. *Best Pract. Res. Clin. Endocrinol. Metab.* **2013**, *27*, 603–616. [CrossRef] [PubMed]
48. Knudsen, K.E.; Scher, H.I. Starving the addiction: New opportunities for durable suppression of AR signaling in prostate cancer. *Clin. Cancer Res.* **2009**, *15*, 4792–4798. [CrossRef] [PubMed]
49. Chatterjee, B. The role of the androgen receptor in the development of prostatic hyperplasia and prostate cancer. *Mol. Cell. Biochem.* **2003**, *253*, 89–101. [CrossRef] [PubMed]
50. Wu, J.D.; Haugk, K.; Woodke, L.; Nelson, P.; Coleman, I.; Plymate, S.R. Interaction of IGF signaling and the androgen receptor in prostate cancer progression. *J. Cell. Biochem.* **2006**, *99*, 392–401. [CrossRef] [PubMed]
51. Chen, Y.; Sawyers, C.L.; Scher, H.I. Targeting the androgen receptor pathway in prostate cancer. *Curr. Opin. Pharmacol.* **2008**, *8*, 440–448. [CrossRef] [PubMed]
52. McCarty, M.F.; Hejazi, J.; Rastmanesh, R. Beyond androgen deprivation: Ancillary integrative strategies for targeting the androgen receptor addiction of prostate cancer. *Integr. Cancer Ther.* **2014**, *13*, 389–395. [CrossRef]
53. Spencer, L.; Mann, C.; Metcalfe, M.; Webb, M.; Pollard, C.; Spencer, D.; Berry, D.; Steward, W.; Dennison, A. The effects of omega-3 FAs on tumour angiogenesis and their therapeutic potential. *Eur. J. Cancer* **2009**, *45*, 2077–2086. [CrossRef] [PubMed]
54. Fleshner, N.E.; Klotz, L.H. Diet, androgens, oxidative stress and prostate cancer susceptibility. *Cancer Metastasis Rev.* **1999**, *17*, 325–330. [CrossRef]

55. Lee, W.H.; Morton, R.A.; Epstein, J.I.; Brooks, J.D.; Campbell, P.A.; Bova, G.S.; Hsieh, W.S.; Isaacs, W.B.; Nelson, W.G. Cytidine methylation of regulatory sequences near the pi-class glutathione *S*-transferase gene accompanies human prostatic carcinogenesis. *Proc. Natl. Acad. Sci. USA* **1994**, *91*, 11733–11737. [CrossRef] [PubMed]
56. Vaco, C.E.; Wilhelm, J.; Harms-Rigdahl, M. Interaction of lipid peroxidation products with DNA. A review. *Mutat. Res.* **1988**, *195*, 137–149. [CrossRef] [PubMed]
57. Berquin, I.M.; Edwards, I.J.; Kridel, S.J.; Chen, Y.Q. Polyunsaturated fatty acid metabolism in prostate cancer. *Cancer Metastasis Rev.* **2011**, *30*, 295–309. [CrossRef] [PubMed]
58. Chen, Y.Q.; Edwards, I.J.; Kridel, S.J.; Thornburg, T.; Berquin, I.M. Dietary fat-gene interactions in cancer. *Cancer Metastasis Rev.* **2007**, *26*, 535–551. [CrossRef] [PubMed]
59. Escobar, E.L.; Gomes-Marcondes, M.C.; Carvalho, H.F. Dietary fatty acid quality affects AR and PPARgamma levels and prostate growth. *Prostate* **2009**, *69*, 548–558. [CrossRef] [PubMed]
60. Vereshagin, A.G.; Novitskaya, G.V. The triglyceride composition of linseed oil. *J. Am. Oil Chem. Soc.* **1965**, *42*, 970–974. [CrossRef] [PubMed]
61. Trichopoulou, A.; Lagiou, P.; Kuper, H.; Trichopoulos, D. Cancer and Mediterranean dietary traditions. *Cancer Epidemiol. Biomark. Prev.* **2000**, *9*, 869–873.
62. Lopez-Miranda, J.; Perez-Jimenez, F.; Ros, E.; de Caterina, R.; Badimon, L.; Covas, M.I.; Escrich, E.; Ordovás, J.M.; Soriguer, F.; Abiá, R.; *et al.* Olive oil and health: Summary of the II international conference on olive oil and health consensus report, Jaen and Cordoba (Spain) 2008. *Nutr. Metab. Cardiovasc. Dis.* **2010**, *20*, 284–294. [CrossRef] [PubMed]
63. Park, S.Y.; Wilkens, L.R.; Henning, S.M.; le Marchand, L.; Gao, K.; Goodman, M.T.; Murphy, S.P.; Henderson, B.E.; Kolonel, L.N. Circulating fatty acids and prostate cancer risk in a nested case-control study: The Multiethnic Cohort. *Cancer Causes Control* **2009**, *20*, 211–223. [CrossRef] [PubMed]
64. Crowe, F.L.; Key, T.J.; Appleby, P.N.; Travis, R.C.; Overvad, K.; Jakobsen, M.U.; Johnsen, N.F.; Tjønneland, A.; Linseisen, J.; Rohrmann, S.; *et al.* Dietary fat intake and risk of prostate cancer in the European Prospective Investigation into Cancer and Nutrition. *Am. J. Clin. Nutr.* **2008**, *87*, 1405–1413. [PubMed]
65. Narita, S.; Tsuchiya, N.; Saito, M.; Inoue, T.; Kumazawa, T.; Yuasa, T.; Nakamura, A.; Habuchi, T. Candidate genes involved in enhanced growth of human prostate cancer under high fat feeding identified by microarray analysis. *Prostate* **2008**, *68*, 321–335. [CrossRef] [PubMed]
66. Bidoli, E.; Talamini, R.; Bosetti, C.; Negri, E.; Maruzzi, D.; Montella, M.; Franceschi, S.; la Vecchia, C. Macronutrients, fatty acids, cholesterol and prostate cancer risk. *Ann. Oncol.* **2005**, *16*, 152–157. [CrossRef] [PubMed]
67. Williams, C.D.; Whitley, B.M.; Hoyo, C.; Grant, D.J.; Iraggi, J.D.; Newman, K.A.; Gerber, L.; Taylor, L.A.; McKeever, M.G.; Freedland, S.J. A high ratio of dietary *n*-6/*n*-3 polyunsaturated fatty acids is associated with increased risk of prostate cancer. *Nutr. Res.* **2011**, *31*, 1–8. [CrossRef] [PubMed]
68. Kobayashi, N.; Barnard, R.J.; Said, J.; Hong-Gonzalez, J.; Corman, D.M.; Ku, M.; Doan, N.B.; Gui, D.; Elashoff, D.; Cohen, P.; *et al.* Effect of low-fat diet on development of prostate cancer and Akt phosphorylation in the Hi-Myc transgenic mouse model. *Cancer Res* **2008**, *68*, 3066–3073. [CrossRef] [PubMed]
69. Berquin, I.M.; Min, Y.; Wu, R.; Wu, J.; Perry, D.; Cline, J.M.; Thomas, M.J.; Thornburg, T.; Kulik, G.; Smith, A.; *et al.* Modulation of prostate cancer genetic risk by omega-3 and omega-6 fatty acids. *J. Clin. Investig.* **2007**, *117*, 1866–1875. [CrossRef] [PubMed]
70. Kobayashi, N.; Barnard, R.J.; Henning, S.M.; Elashoff, D.; Reddy, S.T.; Cohen, P.; Leung, P.; Hong-Gonzalez, J.; Freedland, S.J.; Said, J.; *et al.* Effect of altering dietary omega-6/omega-3 fatty acid ratios on prostate cancer membrane composition, cyclooxygenase-2, and prostaglandin E2. *Clin. Cancer Res.* **2006**, *12*, 4662–4670. [CrossRef] [PubMed]
71. Akinsete, J.A.; Ion, G.; Witte, T.R.; Hardman, W.E. Consumption of high omega-3 fatty acid diet suppressed prostate tumorigenesis in C3(1) Tag mice. *Carcinogenesis* **2012**, *33*, 140–148. [CrossRef] [PubMed]
72. Smith, B.K.; Robinson, L.E.; Nam, R.; Ma, D.W.L. Trans-fatty acids and cancer: A mini-review. *Br. J. Nutr.* **2009**, *102*, 1254–1266. [CrossRef] [PubMed]
73. Bakker, N.; Van't, V.P.; Zock, P.L. Adipose fatty acids and cancers of the breast, prostate and colon: An ecological study. *Int. J. Cancer* **1997**, *72*, 587–591. [CrossRef] [PubMed]

74. King, I.B.; Kristal, A.R.; Schaffer, S.; Thronquist, M.; Goodman, G.E. Serum trans-fatty acids are associated with risk of prostate cancer in b-Carotene and Retinol Efficacy Trial. *Cancer Epidemiol. Biomark. Prev.* **2005**, *14*, 988–992. [CrossRef]
75. Chavarro, J.E.; Stampfer, M.J.; Campos, H.; Kurth, T.; Willett, W.C.; Ma, J. A prospective study of trans-fatty acid levels in blood and risk of prostate cancer. *Cancer Epidemiol. Biomark. Prev.* **2008**, *17*, 95–101. [CrossRef]
76. Liu, X.; Schumacher, F.R.; Plummer, S.J.; Jorgenson, E.; Casey, G.; Witte, J.S. Trans-fatty acid intake and increased risk of advanced prostate cancer: Modification by RNASEL R462Q variant. *Carcinogenesis* **2007**, *28*, 1232–1236. [CrossRef] [PubMed]
77. Schuurman, A.G.; van den Brandt, P.A.; Dorant, E.; Brants, H.A.; Goldbohm, R.A. Association of energy and fat intake with prostate carcinoma risk: Results from The Netherlands Cohort Study. *Cancer* **1999**, *86*, 1019–1027. [CrossRef] [PubMed]
78. Hodge, A.M.; English, D.R.; McCredie, M.R.; Severi, G.; Boyle, P.; Hopper, J.L.; Giles, G.G. Foods, nutrients and prostate cancer. *Cancer Causes Control* **2004**, *15*, 11–20. [CrossRef] [PubMed]
79. Brasky, T.M.; Till, C.; White, E.; Neuhouser, M.L.; Song, X.; Goodman, P.; Thompson, I.M.; King, I.B.; Albanes, D.; Kristal, A.R. Serum phospholipid fatty acids and prostate cancer risk: Results from the prostate cancer prevention trial. *Am. J. Epidemiol.* **2011**, *173*, 1429–1439.
80. Laake, I.; Carlsen, M.H.; Pedersen, J.I.; Weiderpass, E.; Selmer, R.; Kirkhus, B.; Thune, I.; Veierød, M.B. Intake of trans fatty acids from partially hydrogenated vegetable and fish oil and ruminant fat in relation to cancer risk. *Int. J. Cancer* **2013**, *132*, 1389–1403. [CrossRef] [PubMed]
81. Fradet, Y.; Meyer, F.; Bairati, I.; Shadmani, R.; Moore, L. Dietary fat and prostate cancer progression and survival. *Eur. Urol.* **1999**, *35*, 388–391. [CrossRef] [PubMed]
82. Davies, N.J.; Batehup, L.; Thomas, R. The role of diet and physical Activity in breast, colorectal, and prostate cancer survivorship: A review of the literature. *Br. J. Cancer* **2011**, *105*, 52–73. [CrossRef]
83. Ornish, D.; Weidner, G.; Fair, W.R.; Marlin, R.; Pettengill, E.B.; Raisin, C.J.; Dunn-Emke, S.; Crutchfield, L.; Jacobs, F.N.; Barnard, R.J.; *et al.* Intensive lifestyle changes may affect the progression of prostate cancer. *J. Urol.* **2005**, *174*, 1065–1070.
84. Demark-Wahnefried, W.; Polascik, T.J.; George, S.L.; Switzer, B.R.; Madden, J.F.; Ruffin, M.T.; Snyder, D.C.; Owzar, K.; Hars, V.; Albala, D.M.; *et al.* Flaxseed supplementation (not dietary fat restriction) reduces prostate cancer proliferation rates in men pre surgery. *Cancer Epidemiol. Biomarkers Prev.* **2008**, *17*, 3577–3587. [CrossRef] [PubMed]
85. Heymach, J.V.; Schackleford, T.J.; Tran, H.T.; Yoo, S.Y.; Do, K.A.; Wergin, M.; Saintigny, P.; Vollmer, R.T.; Polascik, T.J.; Snyder, D.; *et al.* Effects of low-fat diets on plasma levels of NF-kB-regulated inflammatory cytokine and angiogenic factors in men with prostate cancer. *Cancer Prev. Res. (Phila)* **2011**, *4*, 1590–1598. [CrossRef]
86. Hori, S.; Butler, E.; McLoughlin, J. Prostate cancer and diet: Food for thought? *BJUI Int.* **2011**, *107*, 1348–1359. [CrossRef]
87. Aronson, W.J.; Barnard, R.J.; Freedland, S.J.; Henning, S.; Elashoff, D.; Jardack, P.M.; Cohen, P.; Heber, D.; Kobayashi, N. Growth inhibitory effect of low fat diet on prostate cancer cells: Results of a prospective, randomized dietary intervention trial in men with prostate cancer. *J. Urol.* **2010**, *183*, 345–350. [CrossRef] [PubMed]
88. Haseen, F.; Murray, L.J.; Cardwell, C.R.; O'Sullivan, J.M.; Cantwell, M.M. The effects of androgen deprivation therapy on body composition in men with prostate cancer: Systematic review and meta-analysis. *J. Cancer Surviv.* **2010**, *4*, 128–139. [CrossRef] [PubMed]
89. Saylor, P.J.; Smith, M.R. Prostate cancer: How can we improve the health of men who receive ADT? *Nat. Rev. Urol.* **2009**, *6*, 529–531. [CrossRef] [PubMed]

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An Investigation into the Association between DNA Damage and Dietary Fatty Acid in Men with Prostate Cancer

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Abstract: Prostate cancer is a growing problem in New Zealand and worldwide, as populations adopt a Western style dietary pattern. In particular, dietary fat is believed to be associated with oxidative stress, which in turn may be associated with cancer risk and development. In addition, DNA damage is associated with the risk of various cancers, and is regarded as an ideal biomarker for the assessment of the influence of foods on cancer. In the study presented here, 20 men with prostate cancer adhered to a modified Mediterranean style diet for three months. Dietary records, blood fatty acid levels, prostate specific antigen, C-reactive protein and DNA damage were assessed pre- and post-intervention. DNA damage was inversely correlated with dietary adherence ($p = 0.013$) and whole blood monounsaturated fatty acids ($p = 0.009$) and oleic acid ($p = 0.020$). DNA damage was positively correlated with the intake of dairy products ($p = 0.043$), red meat ($p = 0.007$) and whole blood omega-6 polyunsaturated fatty acids ($p = 0.015$). Both the source and type of dietary fat changed significantly over the course of the dietary intervention. Levels of DNA damage were correlated with various dietary fat sources and types of dietary fat.

Keywords: DNA damage; Mediterranean style diet; fatty acids; prostate cancer

1. Introduction

Prostate cancer in New Zealand and worldwide is an increasing problem with respect to prevalence and receipt of appropriate, and in some countries, timely treatment. Prostate cancer is the most common cancer amongst men in New Zealand, accounting for 27% of all new male cancer cases [1]. In addition to older age, ethnicity and family history being risk factors for prostate cancer, lifestyle is also believed to play a role [2]. This belief is supported by evidence obtained from migrants who adopted the lifestyle of their new country to varying degrees [3]. Such migrants also adopted the risk levels associated with that country, rather than their country of origin, depending on the extent to which they changed their lifestyle [3]. It is widely accepted that diet plays an important role in the development of cancers and that a Mediterranean style diet, as opposed to a Western style diet, may ameliorate the risk and progression of prostate cancer due to the effect of various Mediterranean style dietary components on inflammation and oxidative stress, amongst other factors [4]. The source and components of dietary fat vary enormously between Mediterranean and Western dietary patterns. The

former is higher in monounsaturated fatty acid (MUFA) rich-plant foods including oleic acid-rich olive oils, as well as the long chain omega 3 polyunsaturated fatty acids (PUFA) that are largely sourced from oily fish (which are high in the omega 3 fatty acids (*n*3PUFA), eicosapentanoiec acid (EPA) and docosahexaneoic acid (DHA)) [5]. A Western style dietary pattern on the other hand is higher in omega 6 fatty acids (*n*6PUFA) sourced largely from seed oils and animal fats [5].

Exogenous and endogenous factors can influence oxidative stress [6], which is caused by an imbalance between antioxidants and reactive oxygen species. Lifestyle and diet can be a source of antioxidants and can also promote oxidative stress. Examples of foods that promote oxidative stress include meat cooked at high temperature, as well as some processed and smoked meats [7,8]. Meat cooked at high heat can generate heterocyclic amines (HCA) and polycyclic aromatic hydrocarbons and these can induce DNA instability [7–10]. The susceptibility to prostate cancer risk as a result of consumption of such compounds may be modified by genotype [11]. The consumption of processed meats may also promote the formation of cancers as they contain potentially harmful nitrates and nitrites [9]. Other dietary sources of fat, such as dairy, contain calcium and angiotensin-converting enzyme inhibitors that may decrease oxidative stress, at least in people who are obese [12]. Despite such evidence, dairy intake has received mixed reviews with respect to association with prostate cancer risk [13–15].

There is some controversy regarding dietary fat intake and prostate cancer prevalence and progression [16–18]. Total and saturated fat intake has been positively associated with prostate specific antigen (PSA) levels [19], increased risk of prostate cancer, and aggressive prostate cancer [16,18], whilst saturated fat intake has been associated with fatal prostate cancer [18].

The dietary fatty acids that are discussed herein are shown in relation to one another in Figure 1. Both animal and plants consist of different types of fats in varying proportions. Animal fats consist predominantly of saturated fats (single carbon bonds in the hydrocarbon chains), and plant fats consist predominantly of unsaturated fats (with a varying number of double bonds). There are some exceptions, for example coconut oil contains predominantly saturated fat, and fish consists primarily of PUFA. Unsaturated trans fats are only found in trace amounts in meat and dairy, but they are often produced during the hydrogenation of vegetable oils to produce saturated fats, and therefore are common in processed foods [20].

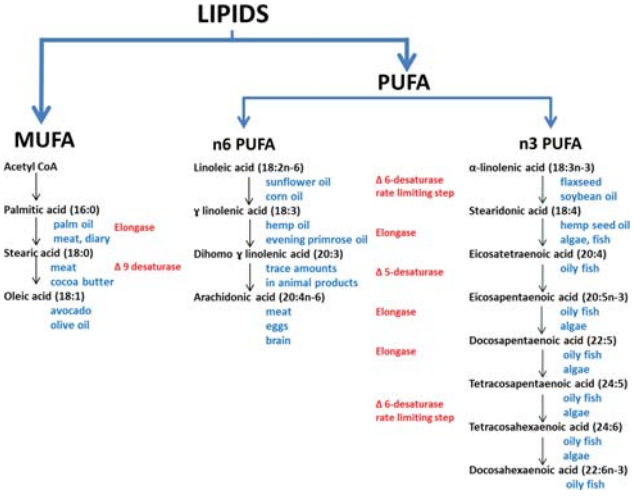

Figure 1. The biosynthesis pathways of the omega 3, 6 and 9 family of poly- and mono-unsaturated fatty acids (adapted from [21–23]).The main dietary sources are shown in blue text, and the enzymes in red text.

Linoleic and α-linolenic acid are essential fatty acids, whilst other fatty acids, to some degree, can be synthesised from precursors [22] (Figure 1). In the *n*3 PUFA and *n*6PUFA pathways there is competition for the Δ6-desaturase and Δ5-desaturase enzymes, although both enzymes preferentially catalyse the *n*3PUFA pathway [21]. In a Review by Plourde and Cunnane [24] the authors discuss the acceptance of the view that there is an "extremely limited efficiency" of the desaturase conversion of ALA to DHA. The controversy of the conversion of LA and ALA to the long chain PUFAs arose in part due to the early use of rat models and also due to using animals that were deficient in essential fatty acids [25]. These two approaches were misleading as rats have a more efficient conversion of LA and ALA to longer chain PUFA, and fatty acid deficiency stimulates the conversion of LA and ALA to longer chain PUFA [25]. Although EPA and DHA biosynthesis is generally regarded as being inefficient [22], the extent of this inefficiency is controversial as measurements of longer chain PUFA may be quite different in plasma *versus* levels measured in other tissues, and it is plasma levels that are more commonly measured and reported [24,26]. However, it seems that the most predictable means of achieving adequate levels of EPA and DHA in plasma and tissues is through consuming long chain PUFA from dietary sources.

The intake of animal, saturated and *trans*-unsaturated fats is associated with all-cause mortality [27] and death due to prostate cancer [18,27]. The consumption of MUFA, PUFA, and vegetable fats on the other hand are associated with a decreased risk of developing prostate cancer or death from prostate cancer [18,27].

Unrepaired DNA damage can result in mutations and some mutations can lead to the development of cancerous tumours. Polymorphisms, such as the single nucleotide polymorphism rs2853826 found in the mitochondrial gene *NADH dehydrogenase subunit*, can influence oxidative stress in women carrying the G allele (G10398), and who also consume alcohol [28]. In such an instance, genotype and alcohol consumption may therefore have an impact on the risk of breast cancer development. Unsurprisingly, cancers such as prostate cancer have been found to be associated with raised levels of DNA damage [29], and raised antioxidant levels can help activate the expression of the *glutathione S-transferase* gene and thereby help protect against this damage [30]. The measurement of DNA damage is regarded as an ideal biomarker for the assessment of the influence of foods or food components on cancer, and the alkaline comet assay (single cell gel electrophoresis) is regarded as a suitable technique for such an assessment [31,32].

The aim of this study was to determine the association between fat and oil intake, as part of a modified Mediterranean style dietary intervention study, and whole blood fatty acid profiles and their association with markers of inflammation and DNA damage in men with prostate cancer. It was hypothesised that the proposed diet would be associated with improvements in PSA, CRP, DNA damage and whole blood fatty acid levels. Evidence obtained could be used to support the prescribed diet as the basis for dietary guidelines that may benefit men with prostate cancer in the future.

2. Experimental Section

Ethical approval was obtained from the Northern B Health and Disability Ethics Committee, Auckland, New Zealand (Ethics number NTY/11/11/109) to perform this study. Study volunteers were selected from an existing cohort of men with prostate cancer based on their Gleason scores, such that those with a Gleason score of 6 (3 + 3) and 7 (3 + 4) were invited to participate in this dietary intervention. Neither a control group free from prostate cancer, nor a control group with prostate cancer and following a standard diet were included. The dietary intervention was explained in detail and a hardcopy of the guidelines and a lengthy compilation of recipes were provided [33]. From the point of view of fat intake, volunteers were asked to adhere to the following guidelines: to include 30–50 g of mixed, unsalted seeds and nuts daily; to include 15 mL or more of extra virgin olive oil and to avoid exposure of the oil to medium and high heat; to reduce dairy intake to one portion daily (information on alternative sources of dietary calcium was provided); to substitute butter and/or margarine with an olive oil based spread; to limit intake of red meat to less than 400 g a week and

to substitute with oily fish and white meat; to avoid high temperature cooking of protein; to avoid processed meats; and to include oily fish in the diet at least once a week. The intention was not to change calorie intake, although there was a concern that this may increase due to nut and olive oil intake. Exercise was monitored at baseline and study end through the use of activity diaries. Light to moderate exercise was encouraged during the enrollment interview to encourage general well-being, but no support or resources were provided in this regard. Volunteers were provided with food samples due to the expense and novelty of some of the items, and blood samples were collected at baseline and at three months from volunteers in a non-fasting state. The blood samples were collected into vaccutainers and either kept on ice or at room temperature (plain, EDTA, Heparin and SST II Advance tubes were used). All blood tubes were processed within two hours of blood draw. The food samples supplied included 200 g of frozen vacuum packed salmon per week (Aoraki Smokehouse Salmon, Twizel, New Zealand) and 1 L of extra virgin olive oil (oleic acid content of 78.3%) per month (Seed Oil Extraction Ltd., Ashburton, New Zealand). Adherence to various aspects of the dietary intervention was assessed using a modified, validated questionnaire [34].

The fatty acid profiles were determined using the Holman Bloodspot fatty acid profile test (Lipid Technologies LLC (Austin, MN, USA) via Functional and Integrative Medicine Ltd. (Napier, New Zealand)). Frozen whole blood was thawed and approximately 75 μL was spotted onto the supplied filter cards. The composition of the fatty acids in the samples was derivatised to form fatty acid methyl esters and thereafter assessed using gas chromatography (Lipid Technologies LLC).

The comet assay can be used to detect lesions in DNA strands [35], and was used herein to assess change in DNA damage over time. Results were also obtained by additionally challenging DNA with hydrogen peroxide (H_2O_2) as described by Olive & Banáth [36]. This involved treating 20 μL of whole blood with 1 mL of a 200 μM solution of H_2O_2 in phosphate buffered saline solution, placing on ice for 30 min and discarding the supernatant after centrifugation. Thereafter the comet assay was performed on heparinised blood as outlined in Karunasinghe *et al.* [37,38]. DNA damage was quantitated using the Komet® version 6.0 digital imaging system (Andor Technology, Belfast, UK). The first 50 leucocytes suitable for capturing were scored. Leucocytes were visualised using an Axioskop 2 fluorescent microscope (Zeiss, Goettingen, Germany) and a CCD camera (Evolution VF, QI Imaging, Media Cybernetics, Warrendale, PA, USA). In this way DNA damage was induced wherever significant weakness was present in the DNA strands and hence H_2O_2-induced DNA damage was considered as an indicator of "DNA fragility". Data for percentage tail DNA were log-transformed as they were not normally distributed. The back-transformed mean of the log-transformed values was used for the statistical analysis.

Statistical analysis was carried out using SAS (V9.2 SAS Institute, Cary, NC, USA) as follows: the Students paired *t*-test was used for the comparison of variables at the baseline and three month time points and Spearman bivariate correlations were used to measure relationships between variables.

3. Results

The characteristics of the study participants are presented in Table 1 and summarised as follows: participants were aged between 52 and 74 years; 80% had a body mass index (BMI) of $\geq$25 kg/m^2 and over the course of the study mean body weight reduced by 2.3 kg ($p = 0.0007$); 60% had undergone prostatectomy, whilst 30% of participants were on watchful wait or active surveillance. All participants had a Gleason score of 6 (3 + 3) or 7 (3 + 4) at the time of prostatectomy or most recent biopsy.

Table 1. Baseline characteristics of the study participants.

Baseline Characteristics		*n*
Age (years) (range 52–74 years)	50–59	3
	60–69	12
	≥70	5
BMI (kg/m^2) (range 23–33 kg/m^2)	≤19.9	0
	20–24.9	4
	25–29.9	12
	≥30	4
Gleason score *	3 + 3	14
	3 + 4	6
Smoking status	Never	7
	Past	13
	Present	0
Supplements	Omega 3 (from fish oil)	3
	Vitamins	4
Treatment type	None	6
	Prostatectomy	10
	Prostatectomy + ADT + DxR	1
	Prostatectomy + DxR	1
	ADT + DxR	1
	Brachytherapy	1

BMI: Body mass index; ADT: Androgen deprivation therapy; DxR: Radiotherapy (other than Brachytherapy); * The Gleason score is based on tissue obtained from the prostatectomy. Where a prostatectomy was not performed, the Gleason score was based on a biopsy sample.

A modified Mediterranean adherence score was used to assess adherence to the study diet at baseline and at three months. The intake of olive oil, nuts, dairy, fish and red meat changed significantly over the course of the study (Table 2). Saturated fat intake, as a percentage of total fat intake at baseline and three months, decreased significantly ($p < 0.0001$). As expected, the source of dietary fat changed in response to the recommended dietary intervention. Figure 2 shows intake of MUFA increased and SFA and total fatty acid decreased significantly over the study period. However, the intake of total fat and PUFA, when measured in grams per day, did not change (Figure 2).

The source, type and amount of fatty acid intake influenced various physiological characteristics, as well as blood levels and ratios. At study end BMI was inversely and significantly correlated to blood *n*3PUFA ($r = -0.451$; $p = 0.046$). Decreases in BMI were associated with increased measurements of PUFA ($r = -0.484$; $p = 0.031$) and LA ($r = -0.463$; $p = 0.040$). In addition, increased whole blood arachidonic acid (AA) ($r = -0.455$; $p = 0.044$) levels were associated with weight loss but not a significant decrease in BMI.

Table 2. Changes in the sources of dietary fat from baseline to three months.

Dietary Component (Unit of Measure)	Mean (SE)		Mean Difference (95% CI)	*p*
	Baseline	Three Months		
Olive oil (mL/day)	14.5 (3.8)	28.8 (4.7)	14.2 (6.8–16.0)	**0.0008**
Nuts (Servings/week)	2.3 (0.5)	5.1 (0.6)	2.9 (1.5–4.2)	**0.0003**
Butter/cream/margarine (Servings/day)	2.1 (0.3)	1.0 (0.3)	−1.1 (−0.6–−1.6)	**0.0002**
Dairy products (Servings/week)	7.4 (0.9)	4.4 (0.7)	−2.9 (−1.2–−4.7)	**0.0025**
Fish (Servings/week)	1.7 (0.2)	3.5 (0.5)	1.8 (0.9–2.7)	**0.0005**
Red and processed meat (Servings/week)	3.9 (0.5)	1.9 (0.4)	−2.0 (−2.6–−1.3)	**0.0005**

SE: standard error; CI: confidence interval.

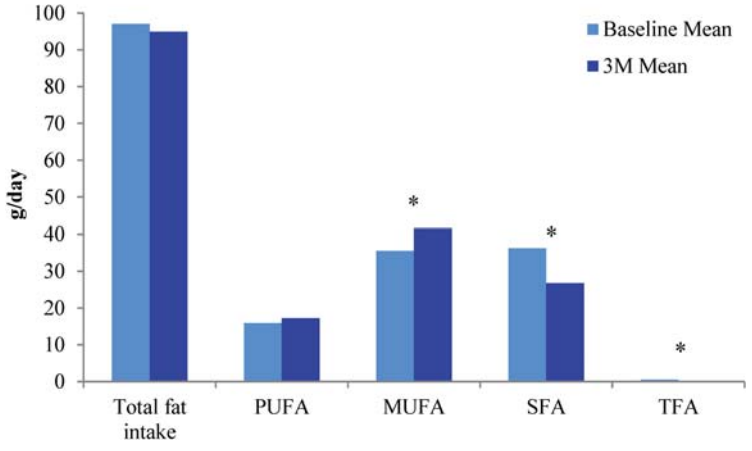

Figure 2. Changes in types of dietary fat intake from baseline to three months.* Statistically significant *p* values; PUFA: polyunsaturated fatty acids; MUFA: monounsaturated fatty acids; SFA: saturated fatty acids; TFA: trans fatty acids.

Total SFA significantly decreased at study end, partly due to a significant decrease in stearic acid intake (Table 3). Total MUFA, PUFA or any individual fatty acid within those synthesis pathways, showed no change, with the exception of DHA and DHA + EPA which showed a statistically significant increase in blood levels (Table 3). In addition, the ratios of *n*6PUFA:*n*3PUFA and AA:EPA had both decreased by study end (Table 3).

No significant correlations were noted between fatty acid measurements obtained from the blood fatty acid profile and food intake assessed via FoodWorks®7 (Xyris software Pty Ltd. 2012, Kenmore Hills, Australia). However, some statistically significant correlations were evident between various fatty acids reported from the blood fatty acid profile and food items as assessed in an adherence questionnaire (Table 4). Dairy intake in particular was inversely correlated with total *n*3PUFA, EPA and EPA + DHA, and positively correlated with the ratio of AA to EPA (Table 4).

C-reactive protein, PSA and DNA damage were measured at baseline and at three months. Neither C-reactive protein nor PSA changed significantly over the course of the study period. However, a significant, inverse relationship between adherence to the modified Mediterranean diet and basal DNA damage emerged. Spearman correlation was used to identify relationships between intake of individual food items that were recommended as part of the dietary intervention and DNA damage at three months. Foods high in animal fat were significantly positively associated with basal DNA damage (Table 5). In addition, association of DNA fragility with various fat related dietary components

was assessed and the DNA fragility was inversely correlated with fish intake ($r = -0.452$; $p = 0.045$) whilst dairy intake was found to be positively associated with DNA fragility ($r = 0.571$; $p = 0.008$).

Significant correlations were observed between basal DNA damage and dietary fat sources, as measured by an adherence questionnaire, as well as various fatty acids reported from the blood fatty acids profile at three months (Table 5). No associations were evident when analysing fatty acid intake, as measured by the diet diaries and analysed via FoodWorks®7 (Xyris software Pty Ltd. 2012), and basal DNA damage. A representative example of various levels of DNA damage is evident in Figure 3.

Results show that total MUFA and *n*9MUFA (particularly oleic acid), were inversely associated with DNA damage while total *n*6PUFA, and a higher ratio of *n*6PUFA to *n*3PUFA, were associated with increased DNA damage.

Table 3. Whole blood fatty acid profile expressed as mean percent, at baseline and three months.

Blood Fatty Acids	Mean (SE)		Mean Difference (95% CI)	*p*
	Baseline	Three Months		
Total SFA	34.7 (0.3)	33.7 (0.4)	−1.0 (0.4–1.5)	**0.002**
16:0 Palmitic acid	22.6 (0.3)	22.3 (0.4)	−0.3 (−0.1–0.7)	0.161
18:0 Stearic acid	10.5 (0.2)	10.0 (0.2)	−0.5 (0.2–0.9)	**0.002**
Total MUFA	23.4 (0.4)	23.7 (0.4)	0.3 (0.4–1.0)	0.366
Total *n*9MUFA	23.1 (0.4)	23.4 (0.4)	0.3 (−0.4–1.0)	0.380
18:1ω9 Oleic acid	22.7 (0.4)	23.2 (0.4)	0.5 (−0.2–1.1)	0.162
Total PUFA	39.5 (0.5)	40.3 (0.5)	0.9 (−0.1–1.8)	0.079
Total *n*6PUFA	32.8 (0.4)	33.0 (0.5)	0.2 (−0.7–1.2)	0.636
18:2ω6 LA	19.6 (0.7)	19.4 (0.9)	−0.2 (−1.7–1.4)	0.832
20:4ω6 AA	9.1 (0.3)	8.9 (0.3)	−0.2 (−0.7–0.3)	0.379
Total *n*3PUFA	6.6 (0.4)	7.3 (0.3)	0.6 (−0.0–1.3)	0.057
18:3ω3 LNA	0.5 (0.0)	0.6 (0.1)	0.0 (−0.1–0.2)	0.689
20:5ω3 EPA	1.4 (0.9)	1.5 (0.7)	0.1 (−0.2–0.5)	0.463
22:6ω3 DHA	3.0 (0.9)	3.5 (0.1)	0.5 (0.2–0.8)	**0.001**
EPA + DHA	4.4 (0.4)	5.0 (0.2)	0.6 (0.3–1.2)	**0.042**
Modified WBS *n*3 Index	6.1 (0.5)	7.0 (0.3)	0.9 (0.0–1.7)	**0.043**
*n*6PUFA:*n*3PUFA	5.2 (0.3)	4.7 (0.2)	−0.6 (−1.0–−0.1)	**0.019**
AA:EPA	8.58 (0.9)	6.9 (0.6)	−1.6 (−3.1–−0.2)	**0.030**

Abbreviations: AA: Arachidonic acid; CI: confidence interval; DHA: docosahexaneoic acid; DPA: docosapentaenoic acid; EPA: eicosapentaenoic acid; LA: linoleic acid; LNA: linolenic acid; MUFA: monounsaturated fatty acids; *n*9MUFA: omega 9 monounsaturated fatty acids; *n*3PUFA: omega 3 polyunsaturated fatty acids; *n*6PUFA: omega 6 polyunsaturated fatty acids; *p*: probability value; PUFA: polyunsaturated fatty acids; SE: standard error; SFA: saturated fatty acids; WBS: whole blood spot.

Table 4. Correlation between various whole blood fatty acid levels and intake of selected food items at three months.

Blood Fatty Acids	Dietary Fat Source	Correlation	*p*
Total *n*3PUFA	Fish intake	0.210	0.374
	Nut intake	0.341	0.141
	Dairy intake	−0.433	0.057
	Red meat intake	0.082	0.732
EPA	Fish intake	0.172	0.468
	Nut intake	0.147	0.535
	Dairy intake	−0.580	**0.007**
	Red meat intake	−0.475	**0.034**
EPA + DHA	Fish intake	0.123	0.605
	Nut intake	0.222	0.347
	Dairy intake	−0.609	**0.004**
	Red meat intake	0.055	0.817
*n*6PUFA:*n*3PUFA	Fish intake	0.192	0.418
	Nut intake	−0.349	0.132
	Dairy intake	−0.147	0.537
	Red meat intake	0.486	**0.029**
AA:EPA	Fish intake	0.233	0.323
	Nut intake	0.084	0.725
	Dairy intake	0.409	0.073
	Red meat intake	−0.029	0.904

Abbreviations: EPA: eicosapentanoiec acid; DHA: docosahexaneoic; *n*3PUFA: omega 3 polyunsaturated fatty acid, *n*6PUFA: omega 6 polyunsaturated fatty acid; AA: arachidonic acid.

Table 5. Correlation between DNA damage and dietary fatty acid intake and blood fatty acids.

Outcome of Interest	Dietary Fat Sources	Baseline		Three Months	
		Correlation	*p*	Correlation	*p*
DNA damage	Olive oil	0.002	0.995	−0.370	0.109
	Servings of butter, cream, margarine	0.278	0.235	0.456	**0.043**
	Servings of fish	0.202	0.393	0.510	0.829
	Servings of red meat	0.066	0.783	0.576	**0.007**
Blood Fatty Acid	Total MUFA	0.200	0.3988	−0.565	**0.009**
	Total *n*9MUFA	0.211	0.371	−0.561	**0.010**
	Oleic acid	0.220	0.352	−0.514	**0.020**
	Total *n*6PUFA	−0.116	0.627	0.536	**0.015**
	Total *n*3PUFA	−0.314	0.178	−0.224	0.342
	*n*6PUFA:*n*3PUFA ratio	0.330	0.155	0.507	**0.023**

Abbreviations: MUFA: mono-unsaturated fatty acids; *n*9MUFA: omega 9 polyunsaturated fatty acids; *n*6PUFA: omega 6 polyunsaturated fatty acids; *n*3PUFA: omega 3 polyunsaturated fatty acids.

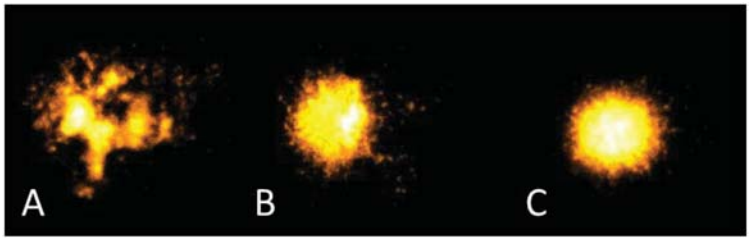

Figure 3. Representative images of different levels of DNA damage as measured by the Comet assay. **A**: extensive damage; **B**: moderate damage; **C**: minor damage.

4. Discussion

Dietary fat intake was measured by assessing whole blood fatty acid levels, as well as by using four-day food diaries and assessing intake via FoodWorks®7 software (Xyris software Pty Ltd. 2012). A modified Mediterranean diet adherence questionnaire was used to evaluate conformity to a Mediterranean style dietary pattern, which is generally high in both *n*3 and *n*6PUFA, and to measure intake and change in intake of specific high fat foods in response to the dietary intervention.

The Holman Bloodspot fatty acid profile test (Lipid Technologies LLC), requiring whole blood samples, was used to assess fatty acid profiles at baseline and study end. Although other fatty acid profile tests can be used to measure fatty acid levels from other components of blood samples, whole blood was regarded as preferable as it can be used to assess fatty acid intake over the previous two months. Both Rise *et al.* [39] and Sun *et al.* [40] state that erythrocyte fatty acid profiles provide a better reflection of long term PUFA intake than plasma fatty acid profiles, and this view is supported by work carried out by Katan *et al.* [41]. In their study, Katan *et al.* concluded that erythrocyte fatty acid profiles reflected intake over the past one to two months [41]. It is clear that plasma and serum fatty acid profiles reflect more recent fatty acid intake than erythrocyte, whole blood or adipose tissue fatty acid profiles [39,40,42]. Based on this evidence it is likely that the Holman Bloodspot test captures fatty acid intake over the two months prior to blood collection.

A number of statistically significant changes in fatty acid profiles (Table 3) were noted such as the increase in DHA whole blood levels from 3.0% to 3.5% ($p = 0.001$). However, although the changes in EPA levels were not statistically significant, they did increase from 1.36% to 1.5% over the three month study period. Together, these changes contributed to a statistically significant increase in the modified WBS *n*3 index ($p = 0.043$) (Table 3).

Fatty acid profiles were measured from whole blood spots (WBS) and this presents challenges with regards to calculating a red blood cell (RBC) *n*3 index. A RBC *n*3 index is typically calculated from the sum of EPA and DHA (from RBC membranes) as a percentage of total RBC fatty acids [43]. Bailey-Hall *et al.* compared DHA and EPA levels from whole blood obtained from a finger prick with values obtained from RBCs (venipuncture) [44]. Although the mean percentage for DHA was approximately 150% lower from capillary whole blood than in RBCs, the DHA and EPA values from the two sample types were highly correlated [44]. As mentioned in the "Experimental" section, the fatty acid profiles were determined by Lipid Technologies LLC (Austin Minnesota) using Holman Bloodspot fatty acid profile tests. Results were presented as a percentage of total lipid content from WBS. An RBC *n*3 index was reported and as this percentage was calculated from WBS, it was therefore necessary to apply a conversion factor wherein the relationship between DHA from whole blood *versus* DHA from red blood cells, was taken into account [44]. The actual algorithm used is proprietary information [45]. Rather than refer to this value as the RBC *n*3 index, which could be viewed as misleading, the authors have used the term "modified WBS *n*3 index" (Table 3). Due to the relationship between blood fatty acids in whole blood *vs.* RBC from venipuncture established by Bailey-Hall [44] upon which the algorithm developed by Lipid Technologies is based [45], the authors have considered the modified WBS *n*3

index as equivalent to the widely used RBC *n*3 index. The RBC *n*3 index is negatively associated with death, particularly sudden death, from coronary heart disease [43]. Although the most desirable levels might be influenced by cultural background, maximal cardioprotection and slowest rate of telomere loss takes place at an RBC *n*3 index $\geq$8% and 8.7% respectively [43]. The increase in the modified WBS *n*3 index found in this study (from 6.10% to 6.98%) was significant, yet it remained below the target value for the reduction of coronary heart disease risk. However, it is believed that any increase in the modified WBS *n*3 index would be beneficial as *n*3 fatty acids can alter membrane biophysical properties and in addition to lipid metabolism, this may impact on inflammatory responses [46]. In addition, the dietary intervention continued for only three months and it is possible that the modified WBS *n*3 index may have continued to increase until target levels were reached.

Fatty acid profiles have predominantly been analysed from either plasma [47–49] or serum [50] and therefore results from these studies are not comparable with our own due to the different substrates used. However, in a recent Australian study data were collected on fasting whole blood fatty acids, but only intake in grams of SFA, MUFA and PUFA were shown, as well as a limited number of blood fatty acid ratios [51]. Total fat, as well as SFA, MUFA and PUFA intake (all measured in grams per day) were all comparatively higher in our study, relative to the study carried out by Alhazmi *et al.* [51].

An association between a change in dietary pattern over a three month time period, and whole blood fatty acids was investigated. Importantly, overall fat intake did not change despite a Mediterranean dietary pattern being traditionally high in fat. This lack of change is due to a substitution in source of fats, such that meats high in saturated fat were replaced by oily fish, and although dairy intake decreased significantly, olive oil and nut intake also increased significantly (Table 2). These changes are consistent with the adoption of a Mediterranean style dietary pattern. The change in fat source is supported by the statistically significant decrease in total SFA (Figure 2), particularly stearic acid (Table 3). Changes in blood fatty acid profiles, although physiologically small, were statistically significant (Table 3). This is largely due to the fact that the percentage values are small and therefore a large physiological change is unlikely.

In this dietary intervention study the intake of olive oil, oily fish, seeds and nuts was promoted, and therefore the dietary intake of MUFA and PUFA increased (Table 2). This increase is partly reflected in the change in blood fatty acid levels (Table 3). The intake of dietary sources of MUFA increased significantly ($p = 0.0243$) (Figure 2), as did the whole blood levels of *n*3PUFAs DHA ($p = 0.001$) and EPA + DHA ($p = 0.042$) (Table 3).

The increase in the modified WBS *n*3 index was consistent with the reported intake of dietary items containing *n*3PUFAs. Increased intake of *n*3PUFA is often associated with a reduction in *n*6PUFA blood levels partially due to competitive inhibition of rate limiting desaturase enzymes [23] (Figure 1), although there is some debate regarding this perhaps overly simplistic view [24,26]. However, there were no correlating significant changes in percentage *n*6PUFA in our study. While we expected that intake of some sources of *n*6PUFA, such as the cheaper vegetable oils that are often found in processed foods (e.g., soybean, sunflower, rice-bran, cottonseed and corn oils) would decrease due to substitution with olive oil, which is much lower in *n*6PUFA, there is no evidence that this occurred. Although we assessed for olive oil intake, we did not question the intake of other oils. An alternative explanation could lie in the fatty acid composition of nuts. We recommended and observed an increased consumption of nuts. Nut consumption increased from a mean of 2.2 to 5.2 servings per week. Many nuts are high in *n*6PUFA, thus off-setting the decrease of *n*6PUFA from other sources. In spite of this, the increase in *n*3PUFA contributed to a statistically significant decrease in the *n*6:*n*3 ratio, indicating a shift towards a less inflammatory profile. Correlations between intake of dietary fatty acids and blood fatty acids were not evident (values not reported). The levels of blood fatty acids are not only affected by intake [48,49], but also by the rate at which fatty acids are transformed (Figure 1). This transformation is often inefficient and influenced by rate limiting enzymes such as the delta-6-desaturase enzymes [52].

It is also important to consider whether *n3*PUFAs from plant sources decreased as this might counter-balance the increase in fish intake. However, one would expect this to be evident from the blood fatty acid profiles, as sources of EPA and DHA would largely be from oily fish and the limited conversion of alpha linolenic acid via elongation and desaturation reactions to stearidonic acid, EPA and finally DHA [23]. EPA and DHA can also be obtained from certain algal species [23], but only one of the study participants took algae-based supplements. The output from FoodWorks®7 software (Xyris software Pty Ltd. 2012) is in the form of food components and therefore we are comparing measurements of whole foods, such as fish, from the adherence tool, with measurement of food components such as *n*3PUFA, which can be sourced from a number of different foods including fish, refined vegetable oils and nuts for example.

What is of particular interest and relevance is that EPA intake was inversely associated with intake of dairy products ($p = 0.007$) and red meat ($p = 0.034$); and blood percentage EPA + DHA was significantly inversely associated with dairy intake. Due to the study design, the effect of dietary intake on prostate cancer risk could not be assessed. However, it is interesting to note that the above mentioned association between increased EPA and DHA intake with decreased dairy was also reported in a study where the influence of various dietary components on prostate cancer risk was assessed [53]. These results support evidence obtained from the adherence questionnaires that fish, as the primary dietary source of EPA, partially replaced the intake of meat, and some dairy products.

The Comet assay is a standard method for measuring DNA damage in eukaryotic cells, regardless of how that damage has been caused [54]. Leucocytes, as in this study, are usually used for the analysis of comets, but one of the drawbacks is that these cells are not usually a target tissue for cancer [54]. However, DNA damage in leucocytes, as measured in a Comet assay, may still present as a reliable marker for increased cancer risk as genomic instability is a common and widely accepted characteristic amongst cancers. A number of studies have been reported wherein DNA damage has been used to assess response to genotoxic stress in terms of cancer risk or effect on cancer related pathways [55–57]. Machowetz *et al.* and Colomer *et al.* both reported a reduction in DNA damage in response to olive oil consumption [58,59]. For this reason it was anticipated that a similar reduction in DNA damage would be observed in our own study participants as their consumption of extra virgin olive oil had increased significantly from 14.83 mL/day to 28.75 mL/day (Table 2). While an inverse association was seen between olive oil consumption and DNA damage, this was not significant ($p = 0.109$) (Table 5). However, the percentage of oleic acid in the blood (along with total MUFA and total omega 9), was inversely associated with basal DNA damage at the end of the study, which is consistent with published results [60]. As this association occurred in spite of only a minor increase in the blood oleic acid ratio, the relationship may serve as a marker for an unmeasured, associated factor, such as olive oil polyphenols.

When investigating food sources of fatty acids, it was clear that DNA damage was associated with a higher intake of dairy products and red meat (Table 5). Increased MUFA intake (Figure 2), supported by statistically significant MUFA blood levels (Table 3) were inversely correlated with basal DNA damage at three months (Table 5). Total *n*6PUFA and *n*6PUFA:*n*3PUFA on the other hand were positively correlated with DNA damage (Table 5) and this was not unexpected as *n*6PUFA is believed to be pro-inflammatory and low *n*6PUFA:*n*3PUFA ratios are believed to be anti-inflammatory.

While we did not question participants to obtain detailed information about culinary fats at baseline, we predicted that most participants would have been consuming olive oil with a lower level of polyphenols than that provided by the extra virgin olive oil supplied for this study (Oil Seed Extractions Ltd., Ashburton, New Zealand). Furthermore, our requirement of just one or more tablespoons of olive oil daily was perhaps too low to boost oleic acid levels sufficiently. In a study by Mitjavila *et al.* [61] olive oil was supplemented at a rate of a litre per week (equivalent to just over 140 mL/day), the usual Mediterranean diet includes 60 mL/day of extra virgin olive oil [34]. However, this was thought to be too high an expectation for a New Zealand population that does not have a tradition of olive oil consumption.

The inclusion of oily fish was an important component of the modified Mediterranean diet. The diet was modified to promote the inclusion of oily fish due to the *n*3PUFA content in fish being a good source of the anti-inflammatory fatty acids, EPA and DHA. From the adherence questionnaire the reported intake of fish doubled (Table 2), and this increase was statistically significant ($p = 0.0005$). No significant correlation was seen between any of the blood fatty acids and fish intake (correlations ranged from $r = 0.017$ to 0.21 (Table 4)). These results are not entirely inconsistent with those reported by Norrish *et al.*, in which fish intake was "moderately correlated" to EPA and DHA when measured from red blood cells obtained from New Zealand men ($r = 0.26$ and 0.32 respectively, the p values were not reported) [62]. The whole blood fatty acid profile is a reflection of oily fish intake over the preceding two months, whilst the diet diaries are a measure of intake the week prior to the blood draw. Some of the volunteers indicated that they had consumed all their salmon donations by this stage and may have been unwilling to purchase additional oily fish. This highlights the advantage of blood biomarkers that reflect both short and longer-term intake over diet diaries or food frequency questionnaires to assess dietary intake.

In addition to changes in the consumption of fish, it can be seen that intake from other sources of dietary fat also changed. Statistically significant changes were seen in the consumption of olive oil and nuts, where consumption increased, and dairy products, where consumption decreased (Table 2). This could result in the increased intake of *n*3PUFA and the decreased intake of *n*6PUFA, depending on the type and quantity of nuts consumed. The type of nuts consumed was not recorded.

Although the authors cannot speculate as to whether the modified Mediterranean diet detailed herein would increase longevity, it is clear that indicators of general health were enhanced. This view is supported by the fact that many of the men who were carrying excess weight, decreased their body weight during the study period; that whole blood fatty acid profiles improved, specifically DHA levels and the modified WBS *n*3 index (a marker of heart health); and that DNA damage levels decreased. In addition, anecdotal reports show that one of the study volunteers reported improved sleep patterns, thought to be due to decreased nocturia (nocturia being a common side-effect of prostate cancer treatment and prostatic disease); one volunteer experienced reduced arthritic pain; another experienced a reduced need for anti-inflammatory medication, whilst a number of volunteers commented on an improved feeling of well-being.

5. Conclusions

Dietary change to promote the intake of oily fish and olive oil as part of a Mediterranean style diet can be achieved in men with prostate cancer. Both the source and type of dietary fat intake changed significantly over the course of the dietary intervention. The intake of olive oil, nuts and fish increased significantly, whilst the intake of dairy and red meat decreased significantly from baseline to three months. The whole blood levels of the SFA, stearic acid decreased significantly, whilst the levels of DHA increased significantly. Although the whole blood levels of total *n*6PUFAs did not change significantly over the course of the intervention, care should be taken to provide advice regarding the increased intake of nuts to ensure that the type and quantity of nuts consumed maintains *n*6PUFA within levels associated with reduced health risks. Whilst dietary fat intake significantly changed over the course of the study, this change was not statistically associated with the significant changes in blood fatty acid profiles. However, total MUFA and oleic acid levels in the volunteers adhering to this dietary intervention were associated with a significant reduction in DNA damage. DNA damage was positively correlated with the ratio of *n*6PUFA to *n*3PUFA, as well as to the intake of red and processed meats, and dairy products.

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Author Contributions: K.S.B. planned and initiated the study; planned and wrote the manuscript; S.E. and K.S.B. carried out the study; N.K. and S.E. carried out the Comet assays and interpreted the results; planned and edited drafts of the manuscript; D.Y.H. carried out the statistical analysis and edited drafts of the manuscript; A.J. processed and stored the samples; S.Z. performed the phlebotomy; L.R.F. helped plan the study and edited drafts of the manuscript.

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References

1. Cancer: New Registrations and Deaths 2010. Available online: http://www.health.govt.nz/publication/cancer-new-registrations-and-deaths-2010 (accessed on 22 August 2014).
2. Bishop, K.S.; Chi, H.-J.K.; Han, D.Y.; Ferguson, L.R. Prostate cancer prevention in the developing world—What are we waiting for? *Curr. Pharmacogenomics Pers. Med.* **2012**, *10*, 70–86. [CrossRef]
3. Itsiopoulos, C.; Hodge, A.; Kaimakamis, M. Can the Mediterranean diet prevent prostate cancer? *Mol. Nutr. Food Res.* **2009**, *53*, 227–239. [CrossRef] [PubMed]
4. Melnik, B.C.; John, S.M.; Schmitz, G. Over-stimulation of insulin/IGF-1 signaling by western diet may promote diseases of civilization: Lessons learnt from Laron syndrome. *Nutr. Metab.* **2011**, *8*, 41. [CrossRef]
5. Serra-Majem, L.; de la Cruz, J.N.; Ribas, L.; Salleras, L. Mediterranean diet and health: Is all the secret in olive oil? *Pathophysiol. Haemost. Thromb.* **2003**, *33*, 461–465. [CrossRef] [PubMed]
6. Breen, A.P.; Murphy, J.A. Reactions of oxyl radicals with DNA. *Free Radic. Biol. Med.* **1995**, *18*, 1033–1077. [CrossRef] [PubMed]
7. Sugimura, T.; Wakabayashi, K.; Nakagama, H.; Nagao, M. Heterocyclic amines: Mutagens/carcinogens produced during cooking of meat and fish. *Cancer Sci.* **2004**, *95*, 290–299. [CrossRef] [PubMed]
8. Norrish, A.E.; Ferguson, L.R.; Knize, M.G.; Felton, J.S.; Sharpe, S.J.; Jackson, R.T. Heterocyclic amine content of cooked meat and risk of prostate cancer. *J. Natl. Cancer Inst.* **1999**, *91*, 2038–2044. [CrossRef] [PubMed]
9. John, E.M.; Stern, M.C.; Sinha, R.; Koo, J. Meat consumption, cooking practices, meat mutagens, and risk of prostate cancer. *Nutr. Cancer* **2011**, *63*, 525–537. [CrossRef] [PubMed]
10. Joshi, A.D.; Corral, R.; Catsburg, C.; Lewinger, J.-P.; Koo, J.; John, E.M.; Ingles, S.; Stern, M.C. Red meat and poultry, cooking practices, genetic susceptibility and risk of prostate cancer: Results from the California Collaborative Prostate Cancer Study. *Carcinogenesis* **2012**, *33*, 2108–2118. [CrossRef]
11. Van Hemelrijck, M.; Rohrmann, S.; Steinbrecher, A.; Kaaks, R.; Teucher, B.; Linseisen, J. Heterocyclic aromatic amine (HCA) intake and prostate cancer risk: Effect modification by genetic variants. *Nutr. Cancer* **2012**, *64*, 704–713.
12. Zemel, M.B.; Sun, X. Dietary calcium and dairy products modulate oxidative and inflammatory stress in mice and humans. *J. Nutr.* **2008**, *138*, 1047–1052. [PubMed]
13. Ganmaa, D.; Li, X.M.; Wang, J.; Qin, L.Q.; Wang, P.Y.; Sato, A. Incidence and mortality of testicular and prostatic cancers in relation to world dietary practices. *Int. J. Cancer* **2002**, *98*, 262–267. [CrossRef] [PubMed]
14. Torfadottir, J.E.; Steingrimsdottir, L.; Mucci, L.; Aspelund, T.; Kasperzyk, J.L.; Olafsson, O.; Fall, K.; Tryggvadottir, L.; Harris, T.B.; Launer, L.; *et al.* Milk intake in early life and risk of advanced prostate cancer. *Am. J. Epidemiol.* **2012**, *175*, 144–153. [CrossRef] [PubMed]
15. Pettersson, A.; Kasperzyk, J.L.; Kenfield, S.A.; Richman, E.L.; Chan, J.M.; Willett, W.C.; Stampfer, M.J.; Mucci, L.A.; Giovannucci, E.L. Milk and dairy consumption among men with prostate cancer and risk of metastases and prostate cancer death. *Cancer Epidemiol. Biomark. Prev.* **2012**, *21*, 428–436. [CrossRef]
16. Giovannucci, E.; Rimm, E.B.; Colditz, G.A.; Stampfer, M.J.; Ascherio, A.; Chute, C.G.; Willett, W.C. A prospective study of dietary fat and risk of prostate cancer. *J. Natl. Cancer Inst.* **1993**, *85*, 1571–1579. [CrossRef] [PubMed]
17. Salem, S.; Salahi, M.; Mohseni, M.; Ahmadi, H.; Mehrsai, A.; Jahani, Y.; Pourmand, G. Major dietary factors and prostate cancer risk: A prospective multicenter case-control study. *Nutr. Cancer* **2011**, *63*, 21–27. [PubMed]
18. Pelser, C.; Mondul, A.M.; Hollenbeck, A.R.; Park, Y. Dietary fat, fatty acids, and risk of prostate cancer in the NIH-AARP diet and health study. *Cancer Epidemiol. Biomark. Prev.* **2013**, *22*, 697–707. [CrossRef]

19. Ohwaki, K.; Endo, F.; Kachi, Y.; Hattori, K.; Muraishi, O.; Nishikitani, M.; Yano, E. Relationship between dietary factors and prostate-specific antigen in healthy men. *Urol. Int.* **2012**, *89*, 270–274. [CrossRef] [PubMed]
20. Erkkilä, A.; de Mello, V.D.F.; Risérus, U.; Laaksonen, D.E. Dietary fatty acids and cardiovascular disease: An epidemiological approach. *Prog. Lipid Res.* **2008**, *47*, 172–187. [CrossRef] [PubMed]
21. White, H.M.; Richert, B.T.; Latour, M.A. Impacts of Nutrition and Environmental Stressors on Lipid Metabolism. In *Lipid Metabolism*; Baeze, R.V., Ed.; InTech: Rijeka, Croatia, 2013; Chapter 10. [CrossRef]
22. Fatty Acids: Methylene-Interrupted Double Bonds. Available online: http://lipidlibrary.aocs.org/Lipids/fa_poly/index.htm (accessed on 22 August 2014).
23. Lenihan-Geels, G.; Bishop, K.S.; Ferguson, L.R. Alternative sources of omega-3 fats: Can we find a sustainable substitute for fish? *Nutrients* **2013**, *5*, 1301–1315. [CrossRef] [PubMed]
24. Plourde, M.; Cunnane, S.C. Extremely limited synthesis of long chain polyunsaturates in adults: Implications for their dietary essentiality and use as supplements. *Appl. Physiol. Nutr. Metab.* **2007**, *32*, 619–634. [CrossRef] [PubMed]
25. Plourde, M.; Fortier, M.; Vandal, M.; Tremblay-Mercier, J.; Freemantle, E.; Begin, M.; Pifferi, F.; Cunnane, S.C. Unresolved issues in the link between docosahexaenoic acid and Alzheimer's disease. *Prostaglandins Leukot. Essent. Fat. Acids* **2007**, *77*, 301–308. [CrossRef]
26. Barcelo-Coblijn, G.; Murphy, E.J. Alpha-linolenic acid and its conversion to longer chain *n*-3 fatty acids: Benefits for human health and a role in maintaining tissue *n*-3 fatty acid levels. *Prog. Lipid Res.* **2009**, *48*, 355–374. [CrossRef] [PubMed]
27. Richman, E.; Kenfield, S.A.; Chavarro, J.E.; Stampfer, M.J.; Giovannucci, E.L.; Willett, W.C.; Chan, J.M. Fat intake after diagnosis and risk of lethal prostate cancer and all-cause mortality. *JAMA* **2013**, *173*, 1–8.
28. Blein, S.; Berndt, S.; Joshi, A.D.; Campa, D.; Ziegler, R.G.; Riboli, E.; Cox, D.G.; Gaudet, M.M.; Stevens, V.L.; Diver, W.R.; *et al.* Factors associated with oxidative stress and cancer risk in the breast and prostate cancer cohort consortium. *Free Radic. Res.* **2014**, *48*, 380–386. [CrossRef]
29. Lockett, K.L.; Hall, M.C.; Clark, P.E.; Chuang, S.C.; Robinson, B.; Lin, H.Y.; Su, L.J.; Hu, J.J. DNA damage levels in prostate cancer cases and controls. *Carcinogenesis* **2006**, *27*, 1187–1193. [CrossRef] [PubMed]
30. Kanwal, R.; Pandey, M.; Bhaskaran, N.; Maclennan, G.T.; Fu, P.; Ponsky, L.E.; Gupta, S. Protection against oxidative DNA damage and stress in human prostate by glutathione *S*-transferase P1. *Mol. Carcinog.* **2014**, *53*, 8–18. [CrossRef] [PubMed]
31. Wasson, G.R.; McKelvey-Martin, V.J.; Downes, C.S. The use of the comet assay in the study of human nutrition and cancer. *Mutagenesis* **2008**, *23*, 153–162. [CrossRef] [PubMed]
32. Liao, W.; McNutt, M.A.; Zhu, W.G. The comet assay: A sensitive method for detecting DNA damage in individual cells. *Methods* **2009**, *48*, 46–53. [CrossRef] [PubMed]
33. Erdrich, S.; Bishop, K. The Modified Mediterranean Diet for Men: Recipe Collection. Available online: https://cdn.auckland.ac.nz/assets/fmhs/sms/nutrition/pcd/docs/recipes.png (accessed on 16 June 2014).
34. Martinez-Gonzalez, M.A.; Garcia-Arellano, A.; Toledo, E.; Salas-Salvado, J.; Buil-Cosiales, P.; Corella, D.; Covas, M.I.; Schroder, H.; Aros, F.; Gomez-Gracia, E.; *et al.* A 14-item Mediterranean diet assessment tool and obesity indexes among high-risk subjects: The PREDIMED trial. *PLoS One* **2012**. [CrossRef]
35. Dhillon, V.S.; Thomas, P.; Fenech, M. Comparison of DNA damage and repair following radiation challenge in buccal cells and lymphocytes using single-cell gel electrophoresis. *Int. J. Radiat. Biol.* **2004**, *80*, 517–528. [CrossRef] [PubMed]
36. Olive, P.L.; Banath, J.P. The comet assay: A method to measure DNA damage in individual cells. *Nat. Protoc.* **2006**, *1*, 23–29. [CrossRef] [PubMed]
37. Karunasinghe, N.; Ryan, J.; Tuckey, J.; Masters, J.; Jamieson, M.; Clarke, L.C.; Marshall, J.R.; Ferguson, L.R. DNA stability and serum selenium levels in a high-risk group for prostate cancer. *Cancer Epidemiol. Biomark. Prev.* **2004**, *13*, 391–397.
38. Ferguson, L.R.; Han, D.Y.; Fraser, A.G.; Huebner, C.; Lam, W.J.; Morgan, A.R.; Duan, H.; Karunasinghe, N. Genetic factors in chronic inflammation: Single nucleotide polymorphisms in the STAT-JAK pathway, susceptibility to DNA damage and Crohn's disease in a New Zealand population. *Mutat. Res.* **2010**, *690*, 108–115. [CrossRef]

39. Rise, P.; Eligini, S.; Ghezzi, S.; Colli, S.; Galli, C. Fatty acid composition of plasma, blood cells and whole blood: Relevance for the assessment of the fatty acid status in humans. *Prostaglandins Leukot. Essent. Fat. Acids* **2007**, *76*, 363–369. [CrossRef]
40. Sun, Q.; Ma, J.; Campos, H.; Hankinson, S.E.; Hu, F.B. Comparison between plasma and erythrocyte fatty acid content as biomarkers of fatty acid intake in US women. *Am. J. Clin. Nutr.* **2007**, *86*, 74–81. [PubMed]
41. Katan, M.B.; Deslypere, J.P.; van Birgelen, A.P.; Penders, M.; Zegwaard, M. Kinetics of the incorporation of dietary fatty acids into serum cholesteryl esters, erythrocyte membranes, and adipose tissue: An 18-month controlled study. *J. Lipid Res.* **1997**, *38*, 2012–2022. [PubMed]
42. Katan, M.B.; Grundy, S.M.; Willett, W.C. Should a low-fat, high-carbohydrate diet be recommended for everyone? Beyond low-fat diets. *N. Engl. J. Med.* **1997**, *337*, 563–566; discussion 566–567. [PubMed]
43. Harris, W.S. The omega-3 index: Clinical utility for therapeutic intervention. *Curr. Cardiol. Rep.* **2010**, *12*, 503–508. [CrossRef] [PubMed]
44. Bailey-Hall, E.; Nelson, E.B.; Ryan, A.S. Validation of a rapid measure of blood PUFA levels in humans. *Lipids* **2008**, *43*, 181–186. [CrossRef] [PubMed]
45. Bibus, D.M.; Lipid Technologies, LLC, Austin, MN, USA. Personal communication, 2014.
46. Harris, W.S. The omega-3 index as a risk factor for coronary heart disease. *Am. J. Clin. Nutr.* **2008**, *87*, 1997S–2002S. [PubMed]
47. Gann, P.H.; Hennekens, C.H.; Sacks, F.M.; Grodstein, F.; Giovannucci, E.L.; Stampfer, M.J. Prospective study of plasma fatty acids and risk of prostate cancer. *J. Natl. Cancer Inst.* **1994**, *86*, 281–286. [CrossRef] [PubMed]
48. Ma, J.; Folsom, A.R.; Shahar, E.; Eckfeldt, J.H. Plasma fatty acid composition as an indicator of habitual dietary fat intake in middle-aged adults. The Atherosclerosis Risk in Communities (ARIC) Study Investigators. *Am. J. Clin. Nutr.* **1995**, *62*, 564–571. [PubMed]
49. Saadatian-Elahi, M.; Slimani, N.; Chajes, V.; Jenab, M.; Goudable, J.; Biessy, C.; Ferrari, P.; Byrnes, G.; Autier, P.; Peeters, P.H.; *et al.* Plasma phospholipid fatty acid profiles and their association with food intakes: Results from a cross-sectional study within the European Prospective Investigation into Cancer and Nutrition. *Am. J. Clin. Nutr.* **2009**, *89*, 331–346. [CrossRef] [PubMed]
50. Holub, B.J.; Wlodek, M.; Rowe, W.; Piekarski, J. Correlation of omega-3 levels in serum phospholipid from 2053 human blood samples with key fatty acid ratios. *Nutr. J.* **2009**, *8*, 58. [CrossRef] [PubMed]
51. Alhazmi, A.; Stojanovski, E.; Garg, M.L.; McEvoy, M. Fasting whole blood fatty acid profile and risk of type 2 diabetes in adults: A nested case control study. *PLoS One* **2014**. [CrossRef]
52. Surette, M.E. Dietary omega-3 PUFA and health: Stearidonic acid-containing seed oils as effective and sustainable alternatives to traditional marine oils. *Mol. Nutr. Food Res.* **2013**, *57*, 748–759. [CrossRef] [PubMed]
53. Leitzmann, M.F.; Stampfer, M.J.; Michaud, D.S.; Augustsson, K.; Colditz, G.C.; Willett, W.C.; Giovannucci, E.L. Dietary intake of *n*-3 and *n*-6 fatty acids and the risk of prostate cancer. *Am. J. Clin. Nutr.* **2004**, *80*, 204–216. [PubMed]
54. Collins, A. The comet assay for DNA damage and repair. *Mol. Biotechnol.* **2004**, *26*, 249–261. [CrossRef] [PubMed]
55. Waters, D.J.; Shen, S.; Glickman, L.T.; Cooley, D.M.; Bostwick, D.G.; Qian, J.; Combs, G.F.; Morris, J.S. Prostate cancer risk and DNA damage: Translational significance of selenium supplementation in a canine model. *Carcinogenesis* **2005**, *26*, 1256–1262. [CrossRef] [PubMed]
56. Mroz, R.M.; Schins, R.P.F.; Li, H.; Jimenez, L.A.; Drost, E.M.; Holownia, A.; MacNee, W.; Donaldson, K. Nanoparticle-driven DNA damage mimics irradiation-related carcinogenesis pathways. *Eur. Respir. J.* **2008**, *31*, 241–251. [CrossRef] [PubMed]
57. Karanika, S.; Karantanos, T.; Li, L.; Corn, P.G.; Thompson, T.C. DNA damage response and prostate cancer: Defects, regulation and therapeutic implications. *Oncogene* **2014**. [CrossRef]
58. Machowetz, A.; Poulsen, H.E.; Gruendel, S.; Weimann, A.; Fito, M.; Marrugat, J.; de la Torre, R.; Salonen, J.T.; Nyyssonen, K.; Mursu, J.; *et al.* Effect of olive oils on biomarkers of oxidative DNA stress in Northern and Southern Europeans. *FASEB J.* **2007**, *21*, 45–52. [CrossRef] [PubMed]
59. Colomer, R.; Menendez, J.A. Mediterranean diet, olive oil and cancer. *Clin. Transl. Oncol.* **2006**, *8*, 15–21. [CrossRef] [PubMed]

60. Weinbrenner, T.; Fito, M.; de la Torre, R.; Saez, G.T.; Rijken, P.; Tormos, C.; Coolen, S.; Albaladejo, M.F.; Abanades, S.; Schroder, H.; *et al.* Olive oils high in phenolic compounds modulate oxidative/antioxidative status in men. *J. Nutr.* **2004**, *134*, 2314–2321. [PubMed]
61. Mitjavila, M.T.; Fandos, M.; Salas-Salvado, J.; Covas, M.I.; Borrego, S.; Estruch, R.; Lamuela-Raventos, R.; Corella, D.; Martinez-Gonzalez, M.A.; Sanchez, J.M.; *et al.* The Mediterranean diet improves the systemic lipid and DNA oxidative damage in metabolic syndrome individuals. A randomized, controlled, trial. *Clin. Nutr.* **2013**, *32*, 172–178. [CrossRef] [PubMed]
62. Norrish, A.E.; Skeaff, C.M.; Arribas, G.L.; Sharpe, S.J.; Jackson, R.T. Prostate cancer risk and consumption of fish oils: A dietary biomarker-based case-control study. *Br. J. Cancer* **1999**, *81*, 1238–1242. [CrossRef] [PubMed]

nutrients

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Macro- and Micronutrients Consumption and the Risk for Colorectal Cancer among Jordanians

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Abstract: Objective: Diet and lifestyle have been reported to be important risk factors for the development of colorectal cancer (CRC). However, the association between total energy and nutrient intake and the risk of developing CRC has not been clearly explained. The aim of our study is to examine the relationship between total energy intake and other nutrients and the development of CRC in the Jordanian population. Research Methods and Procedures: Dietary data was collected from 169 subjects who were previously diagnosed with CRC, and 248 control subjects (matched by age, gender, occupation and marital status). These control subjects were healthy and disease free. Data was collected between January 2010 and December 2012, using interview-based questionnaires. Logistic regression was used to evaluate the association between quartiles of total energy, macro- and micronutrient intakes with the risk of developing CRC in our study population. Results: Total energy intake was associated with a higher risk of developing CRC (OR = 2.60 for the highest *versus* lowest quartile of intake; 95% CI: 1.21–5.56, *p*-trend = 0.03). Intakes of protein (OR = 3.62, 95% CI: 1.63–8.05, *p*-trend = 0.002), carbohydrates (OR = 1.41, 95% CI: 0.67–2.99, *p*-trend = 0.043), and percentage of energy from fat (OR = 2.10, 95% CI: 0.38–11.70, *p*-trend = 0.009) significantly increased the risk for the development of CRC. Saturated fat, dietary cholesterol and sodium intake showed a significant association with the risk of developing CRC (OR = 5.23, 95% CI: 2.33–11.76; OR = 2.48, 95% CI: 1.18–5.21; and OR = 3.42, 95% CI: 1.59–7.38, respectively), while vitamin E and caffeine intake were indicative of a protective effect against the development of CRC, OR = 0.002 (95% CI: 0.0003–0.011) and 0.023 (95%CI: 0.008–0.067), respectively. Conclusion: Our results suggest an increased risk for the development of CRC in subjects with high dietary intake of energy, protein, saturated fat, cholesterol, and sodium, and diets high in vitamin E and caffeine were suggestive of a protective effect against the risk of developing CRC. Impact: This is the first study in Jordan to suggest that it may be possible to reduce CRC risk by adjusting the intake of some macro-and micronutrients.

Keywords: colorectal cancer; total energy; macronutrient; micronutrients

1. Introduction

Published report suggests that colorectal cancer (CRC) is one of the three most common forms of cancer with nearly 1.4 million new cases diagnosed in the year 2012 [1]. The report noted that the Republic of Korea, followed by Slovakia and Hungary had the highest incidence of diagnosed CRC, while the lowest incidence of diagnosed CRC was in Africa and Asia [1]. The National Cancer Registry of Jordan reported 554 diagnosed cases of CRC in the year 2009. This total number of cases was calculated to be 11.9% of all newly diagnosed cancer cases in the kingdom of Jordan [2]. Cancer is described in part by an abnormal cell growth that is believed to be initiated either by internal or external (environmental) factors [3]. Diet and physical activity are external factors that may play a role in the development of CRC disease [3].

Dietary intakes of energy, macro- and micro-nutrients have been implicated in the etiology of CRC [4]. Several studies have shown that high dietary intakes of energy and energy-supplying macronutrients (fat, protein and carbohydrate) may have a positive association with the risk of developing CRC [4,5]. Additionally, fruits and vegetables as sources of dietary fiber, folate, phytoestrogens, vitamin C, selenium, carotenoids, phenols, and flavonoids could protect against the development of CRC [6,7]. Antioxidants are reported to function by trapping free radicals and reactive oxygen molecules at the cellular level, thus acting as a protective mechanism against oxidative damage [6,7]. Free radicals in the body are generally produced during metabolic processes, such as those involving digestion. For example, iron found in red meat is reported to be a source of free radicals present in the body [8]. However, evidence suggesting red meat as a possible cause of colon cancer has been questioned by Santorelli *et al.* [9] in the CRC debate. Generally, in many households, meals high in fat are usually low in fruits, vegetables and fiber. Therefore, it is unclear if this increase risk in developing CRC is attributable to the high fat intake or the low fruit, vegetable and fiber intake. Free radical-induced lipid peroxidation has been implicated in malignant transformation [10]. The formation of lipid peroxidation products is normally prevented or scavenged by host antioxidants. Low levels of antioxidant nutrients in circulation have been associated with an increased risk of cancer [10].

One local Jordanian study published by Arafa *et al.* [11], showed in descriptive terms that traditional Jordanian foods are cooked with a high quantity of saturated fats and oils, and more importantly, anecdotal evidence exists that the fruits and vegetables component of the local diet is very low, while red meat and saturated fat components are quite high. More so, in the study by Arafa *et al.* [11], difference in the dietary macro- and micronutrient intake in the traditional diets were reported. The study results were descriptive in nature and did not investigate any association between diet and the risk of developing CRC. In addition, it is important to identify risk factors that could be modified to decrease CRC incidence among Jordanians. Based on current knowledge, the risk of developing CRC in the Jordanian population should be investigated in more detail. Accordingly, the aim of the present study is to investigate the association between macro-and micronutrient intake and colorectal cancer risk using data from a case-control study conducted in Jordan.

2. Materials and Methods

2.1. Study Population and Methods

The study sample consisted of 503 participants; with 232 diagnosed CRC cases and 271 controls (262 males and 241 females). Participants were enrolled in the study from January 2010 to December 2012. Participating subjects were patients diagnosed with CRC (cases) who were recruited from five Jordanian hospitals specializing in oncology diagnosis and treatment. The hospitals included King Hussein Cancer Center (KHCC), King Abdullah University Hospital, Prince Hamzeh Hospital, Jordan University Hospital, and Al-Basheer Hospital. The control group was recruited from hospital personnel, outpatients, visitors and was matched as closely as possible for age, gender, occupation and marital status. Control subjects were excluded if any first- or second degree relatives were diagnosed

with CRC. The study protocol was approved by KHCC Institutional Review Board (IRB) Committee (09 KHCC 10; May 2009) and other hospitals gave their approval accordingly. Written informed consent was obtained from all subjects before their interview. The following inclusion criteria for controls were used: Jordanian nationals aged 18 years or older, ability to communicate clearly and verbally, free of any type of diagnosed cancer, diabetes mellitus, liver disease and rheumatoid arthritis. For inclusion in the diagnosed CRC cancer group, subjects must have received their diagnosis less than 1 year prior to the time of the first interview. The exclusion criteria for this group included those who were considered "critically ill", such as an in-patient at any facility and those who were unable to communicate verbally and clearly.

2.2. Data Collection

Socio-demographic, health and dietary data were collected by trained research assistants using interview-based questionnaires. The socio-demographic data included age, marital status, household income, education (illiterate, primary and secondary, diploma and B.Sc., and postgraduate degrees), occupation and tobacco usage (current and previous smokers were categorized as smokers and those who never smoked were set as non-smokers). The comprehensive health data included the participant's full medical history to confirm that only CRC diagnosed subjects and healthy disease free subjects were included. A validated Arabic quantitative Food Frequency Questionnaire (FFQ), adapted from the Diet History Questionnaire (DHQ I) of the National Cancer Institute of the United States of America [12], was used for dietary assessment. The FFQ questions sought to obtain information on the dietary history of study participants prior to CRC diagnosis, and to confirm the dietary habits of control participants. We selected a period of one year prior to the study inception date, to account for seasonal variation in food types. We noted a fixed dietary pattern for the period, with some participants suggesting this pattern existed for at least five years. A qualified dietitian asked participants, during face-to-face interviews, how frequently, on average, during the past year they had consumed one standard serving of specific food items in nine categories (<1/month, 2–3/month, 1–2/week, 3–4/week, 5–6/week, 1/day, 2–3/day, 4–5/day, or 6/day). Food lists in the modified FFQ questions were classified based on types of foods: 21 items of vegetables; 16 items of meat such as red meat (lamb and beef), chicken, fish, cold meat, and others; 21 items of fruits and juices; nine items of milk and dairy products; eight items of cereals; four items of beans; four items of soups and sauces; five items of drinks; nine items of snacks and sweets; and 14 items of herbs and spices [12]. Food models and standard measuring tools were used to help participants estimate portion size. Responses on frequency of consumption and serving size for each food item were converted into average daily intake. Data was collected from a total of 503 participants. However, the data from 86 participants was excluded due to incomplete response to required questions (n = 58); over-estimation of calorie intake (>5000 kcal for male and >4000 kcal for female) (n = 12); and under-estimation of calorie intake (<500 kcal for females and <800 kcal for males) (n = 16) [13]. Dietary intakes were analyzed using dietary analysis software (ESHA Food Processor SQL version 10.1.1; ESHA, Salem, OR, USA) with additional data on foods consumed in Jordan [14].

The 7-day Physical Activity Recall (PAR), developed by Sallis *et al.* [15] was used to measure physical activity level. 7-Day PAR is a structured interview that depends on participant's recall of time spent engaging in physical activity over a seven day period. Our participants were asked specific and probing questions in order to obtain a complete history of their physical activities. They were asked to recall their physical activities for the previous year before their enrollment into the study. PAR covers different levels of physical activity and intensity such as aerobic exercise, work-related activities, gardening, walking, recreation, and leisure-time activities. The PAR interview focuses on collecting data on intensity, time or duration, and type of activity. The number of hours spent in different activity levels were obtained and converted into Metabolic Equivalents (METs). Average METs for walking = 3.3 METs, for moderate activity = 4.0 METs, for vigorous activity = 8.0 METs. The score expressed as MET-min/week was calculated as: (MET level × minutes of activity/day × days per week). Total Physical Activity MET-min/week is obtained by METs summation and categorized

as inactive (below 600 MET-min/week), minimally active and Health Enhancing Physical Activity (HEPA) active. Minimally active category included subjects who reported a minimum of at least 600 MET-min/week. The category HEPA active included any subject who performed vigorous-intensity activity on at least 3 days a week and accumulated at least 1500 MET-min/week or who performed any combination of walking, moderate-intensity or vigorous intensity activities on 5 or more days achieving a minimum of at least 3000 MET-min/week [15].

Body weight was measured to the nearest 0.1 kg, with minimal clothing and without shoes, using a calibrated portable scale. Height was measured to the nearest 0.5 cm with participants in the full standing position without shoes using a calibrated portable measuring rod. Body mass index (BMI) was calculated as the ratio of weight in kilograms to the square of height in meters [16].

2.3. Statistical Analyses

Statistical analysis was performed with SPSS IBM-20 software. The significance level was set at $p = 0.05$. For descriptive statistics, mean $\pm$ standard deviation (SD) and percentages were used. T-tests evaluated the differences between cases and controls in continuous variables, and Chi-square was used to detect differences among categorical variables.

Because all nutrients were correlated with energy intake, variation due to energy intake and its associated measurement error was minimized by energy adjustment of the nutrients using the regression method [17]. This method of energy adjustment is computed from the residuals of the regression model with total energy intake as the independent variable and the nutrient as the dependent variable. Regression equation was used to calculate the expected mean of nutrient intake of the study population. Next, for each participant, the energy-adjusted intake was calculated by adding the expected mean nutrient intake of the study population to the residual derived from the regression analysis. Shapiro-Wilk test was used to assess the normality of the distributions of dietary intake variables. Non-normally distributed variables were log transformed [17].

Nutrient intakes were modeled using quartiles of distribution in the study population with quartile 1 being the lowest intake and quartile 4 the highest. Odds ratios (ORs) and 95% CIs (95% CIs) for CRC were calculated by using logistic regression models for quartiles of nutrient intakes, with the lowest quartile as the reference category. Confounders were selected based on known risk factors for CRC reported in the literature. Potential confounders were chosen based on previous studies [4,18] including the Cancer Prevention Study II [18]. Confounders included in data analysis included age, gender, BMI, physical activity (MET-min/week), family history (beyond the second degree) of CRC, household income, educational level, marital status and smoking. Trend tests were calculated using linear regression with nutrient intakes as continuous data.

3. Results

Table 1 shows participants' age, anthropometric measurements, socio-demographic and health characteristics, stratified by gender. Average age for controls was 51.4 $\pm$ 10.9 years and 53.8 $\pm$ 12.2 years for cases. Significant differences were found between cases and controls in male height, and in female BMI. No significant differences were detected in employment, marital status, monthly income, smoking and physical activity levels between the CRC diagnosed and control participants. However, family history (beyond the second degree) of CRC and having other health problems in female participants was significantly higher in the CRC group compared to the controls.

The mean daily intakes of total energy, macronutrients, and micronutrients appear in Table 2. The CRC group reported significantly higher intakes of total energy, protein, fat, saturated fat and cholesterol ($p < 0.05$) compared to the control group. In addition, the CRC group had significantly higher intakes of folate, Iron, selenium as well as omega-3 ($p < 0.05$) when compared to the control group, and the control group had a higher percentage of calories from carbohydrate when compared to the CRC group ($p < 0.05$).

Table 1. Age, anthropometrics measurements and selected characteristics of the study participants.

Characteristics	Males (n = 193)			Females (n = 224)		
	Control (n = 113)	Case (n = 80)	p-value	Control (n = 135)	Case (n = 89)	p-value
Age years (mean ± SD)	55.2 ± 11.6	57.9 ± 12.1	0.110	48.1 ± 8.9	50.0 ± 11.0	0.161
Height cm (mean ± SD)	164.3 ± 9.6	169.5 ± 9.2	0.001	170.1 ± 40.5	166.8 ± 10.6	0.458
Weight kg (mean ± SD)	81.3 ± 16	81.1 ± 14.6	0.092	79.9 ± 14.9	77.5 ± 16.5	0.271
BMI kg/m^2 (mean ± SD)	27.3 ± 4.8	27.8 ± 5.4	0.558	30.2 ± 6.0	27.6 ± 7.4	0.004
Age Category n (%)						
<40 years	9 (8)	10 (12.7)		22 (16.9)	14 (15.9)	
40–49 years	25 (22.1)	12 (15.2)	0.397	57 (43.8)	32 (36.4)	0.095
50–59 years	36 (31.9)	15 (19.0)		35 (26.9)	20 (22.7)	
≥60 years	43 (38.1)	42 (53.2)		16 (12.3)	22 (25.0)	
Total	113 (100.0)	79 (100.0)		130 (100.0)	88 (100.0)	
Employed (%)						
Yes	63 (55.8)	30 (37.5)		26 (19.3)	21 (23.6)	
No	50 (44.2)	50 (62.5)	0.081	109 (80.7)	68 (76.4)	0.510
Total	113 (100.0)	80 (100.0)		133 (100.0)	89 (100.0)	
Marital status n (%)						
Married	106 (93.8)	76 (95)		113 (83.7)	74 (83.2)	
Single	4 (3.5)	2 (2.6)		10 (7.4)	2 (2.2)	
Divorced	-	1 (1.3)	0.695	1 (0.7)	1 (1.1)	0.364
Widowed	3 (2.7)	1 (1.3)		11 (8.1)	12 (13.5)	
Total	113 (100.0)	80 (100.0)		135 (100.0)	89 (100.0)	
Family history (beyond the second degree) of CRC n (%)						
Yes	45 (40.2)	27 (33.8)		45 (34.1)	42 (47.7)	
No	67 (59.8)	53 (66.3)	0.475	87 (65.9)	46 (52.3)	0.016
Total	112 (100.0)	80 (100.0)		132 (100.0)	88 (100.0)	
Other health problem n (%) (excluding diabetes, liver disease, rheumatoid arthritis)						
Yes	45 (40.2)	45 (57.0)		47 (34.8)	40 (44.9)	
No	67 (59.8)	34 (43.0)	0.367	88 (65.2)	48 (53.9)	0.043
Total	112 (100.0)	79 (100.0)		135 (100.0)	88 (100.0)	
Tobacco use n (%)						
Yes	36 (34.0)	17 (23.0)		4 (3.0)	7 (8.0)	
No	70 (66.0)	57 (77.0)	0.113	131 (97.0)	81 (92.0)	0.093
Total	106 (100.0)	74 (100.0)		135 (100.0)	88 (100.0)	
Household income n (%)						
<300 USA $	10 (8.8)	6 (7.4)		15 (11.0)	5 (5.6)	
300–750 USA $	27 (23.9)	19 (23.8)	0.663	41 (30.4)	24 (27.0)	0.562
>750 USA $	65 (57.6)	36 (45.0)		44 (32.6)	24 (27.0)	
Unknown	11 (9.7)	19 (23.8)		35 (26.0)	36 (40.4)	
Total	113 (100.0)	80 (100.0)		135 (100.0)	89 (100.0)	
Physical activity levels n (%)						
Inactive *	30 (27.0)	27 (33.8)		10 (9.1)	16 (18.0)	
Minimally Active †	40 (36.0)	25 (31.3)	0.469	33 (30.0)	23 (25.8)	0.183
HEPA active ‡‡	41 (36.9)	28 (35.0)		67 (60.9)	50 (56.2)	
Total	111 (100.0)	80 (100.0)		110 (100.0)	89 (100.0)	

BMI: Body Mass Index; * Inactive: not fitting in "Minimally Active" or "HEPA active"; Significance is at $p \leq 0.05$. † Minimally Active: at least 600 MET per week; ‡‡ Health Enhancing Physical Activity: HEPA active: more than 3000 MET per week.

Table 2. Mean intake per day ± SD of nutrients for the study participants.

Nutrient	Control	Case	Difference (Case − Control)	*p*-value *
Energy (kcal)	3476.0 ± 1172.9	3719.4 ± 1018.1	243.4	0.029
Protein (g)	109.6 ± 42.5	120.9 ± 52.4	11.3	0.016
Protein %	12.5 ± 2.3	13 ± 3.7	0.4	NS
Carbohydrate (g)	593.9 ± 203.1	608.1 ± 164.6	14.2	NS
Carbohydrate %	68.9 ± 8.6	66.1 ± 8.4	−2.8	0.001
Fiber (g)	48.4 ± 23.3	48.1 ± 21.3	−0.3	NS
Soluble Fiber (g)	6.0 ± 4.5	5.4 ± 3.5	−0.7	NS
Insoluble Fiber (g)	14.7 ± 10.8	13.1 ± 9.1	−1.5	NS
Fat (g)	80.2 ± 39.1	93.8 ± 41.3	13.6	0.001
Fat %	20.4 ± 6.6	22.1 ± 6.5	1.7	0.009
Saturated Fat (g)	29.8 ±16.3	35.4 ± 17.1	5.6	0.001
Saturated Fat %	7.6 ± 3.2	8.4 ± 3.2	0.8	0.009
Cholesterol (mg)	340.2 ± 229.0	409.3 ± 273.7	69.1	0.005
Vitamin A (RE)	1116.5 ± 850.5	1199.8 ± 871.6	83.3	NS
Beta-carotene (µg)	6478.7 ± 5259.1	6822.6 ± 556.3	343.9	NS
Vitamin B_{12} (µg)	3.9 ± 3.5	4.5 ± 4.3	0.6	NS
Vitamin C (mg)	239.2 ± 173.1	259.7 ± 207.4	20.5	NS
Vitamin D (mg)	0.8 ± 0.7	0.9 ± 0.7	0.1	NS
Vitamin E (α-Tocopherol) (mg)	6.4 ± 3.9	6.7 ± 4.1	0.2	NS
Folate (mcg)	461.3 ± 217.2	506.3 ± 186.6	44.9	0.029
Vitamin K (µg)	193.3 ± 203.8	197.5 ± 174.3	4.1	NS
Calcium (mg)	1171.1 ± 526.9	1230.5 ± 459.2	59.4	NS
Iron (mg)	25.2 ± 9.9	27.4 ± 10.1	2.3	0.022
Sodium (mg)	4796.2 ± 2837.5	5112.0 ± 2218.6	315.8	NS
Selenium (µg)	109.3 ± 53.3	120.1 ± 48.9	10.9	0.033
Phosphate (mg)	1334.4 ± 561.7	1416.6 ± 619.5	82.2	NS
Omega-3 (mg)	0.6 ± 0.4	0.7 ± 0.5	0.1	0.014
Caffeine (mg)	3036.6 ± 2829.8	2980.7 ± 3041.8	−55.9	NS

* Significance is at $p < 0.05$.

Table 3 shows the ORs and corresponding 95% CI of the CRC group by intake quartile of associated macronutrients. After adjusting for potential confounders, increasing intakes (in the highest *versus* the lowest quartile of intake) of total energy (OR = 2.60, 95% CI: 1.22–5.56, *p*-trend = 0.030), and protein (OR = 3.62, 95% CI: 1.63–8.04, *p*-trend = 0.002) were significantly associated with CRC. A significant positive trend in risk was found for carbohydrate ($p = 0.043$), but none of the quartiles are different from the reference category. The odds ratios for quartiles of fat intake as g/day were not calculated due to distribution issues (the bottom quartile had only 1 participant and the top quartile only 2 control participants). As noted in Table 3, saturated fat and cholesterol intakes show significant direct associations with CRC risk (OR = 5.23, 95% CI: 2.33–11.76 and OR = 2.48, 95% CI: 1.18–5.21, respectively) in the highest *versus* the lowest quartile of intake, and the trend tests were also significant. No association for intake of total fiber with CRC was detected, (*p*-trend = 0.979, Table 3). However, the upper quartile of insoluble fiber was found to be protective against CRC (OR = 0.42, 95% CI: 0.19–0.91) but the trend test was not significant (*p*-trend = 0.162).

Table 3. Adjusted ORs [a] and CIs of CRC risk by macronutrient intake quartiles.

Nutrient	Adjusted				
	Q1	**Q2**	**Q3**	**Q4**	***p*-Trend**
Energy (Kcal)					
No. of Cases (169)/Controls (248)	32/72	45/60	44/60	48/56	0.030
OR	1	1.51	1.83	2.60 *	
95% CI	-	0.70–3.27	0.85–3.93	1.22–5.56	
Protein (g)					
No. of Cases (169)/Controls (248)	30/74	37/67	46/58	56/48	0.002
OR	1	1.66	1.74	3.62 *	
95% CI	-	0.74–3.69	0.78–3.90	1.63–8.04	
Carbohydrate (g)					
No. of Cases (169)/Controls (248)	36/68	37/67	43/62	53/51	0.043
OR	1	0.77	1.24	1.41 *	
95% CI	-	0.36–1.64	0.58–2.64	0.68–2.99	
Fiber (g)					
No. of Cases (169)/Controls (248)	46/58	49/56	33/71	41/63	0.979
OR	1	1.29	0.48	0.57	
95% CI	-	0.62–2.69	0.22–1.04	0.27–1.21	
Soluble Fiber (g)					
No. of Cases (169)/Controls (248)	43/61	53/52	39/65	34/70	0.551
OR	1	1.86	0.85	0.58	
95% CI	-	0.84–4.15	0.39–1.84	0.26–1.27	
Insoluble Fiber (g)					
No. of Cases (169)/Controls (248)	46/58	51/54	40/64	32/72	0.162
OR	1	1.04	0.66	0.42	
95% CI		0.49–2.22	0.30–1.41	0.19–0.91	
Fat [b] *(g)*					
No. of Cases (169)/Controls (248)	0/104	4/100	63/42	102/2	
OR	-	-	-	-	
95% CI	-	-	-	-	
Saturated Fat (g)					
No. of Cases (169)/Controls (248)	28/76	36/69	52/52	53/51	0.009
OR	1	2.23	3.61	5.23 *	
95% CI	-	1.00–4.98	1.68–7.77	2.33–11.76	
Cholesterol (mg)					
No. of Cases (169)/Controls (248)	33/71	32/72	49/56	55/49	0.027
OR	1	0.94	1.84	2.48 *	
95% CI	-	0.43–2.05	0.87–3.91	1.18–5.21	

[a] Adjusted for total energy intake normality of the distributions of dietary intake variables was assessed by the Shapiro-Wilk test. Non-normally distributed variables were log transformed. Other potential confounders included age, gender, BMI, physical activity (METs/week), family history (beyond the second degree) of CRC, education attainment, household income, marital status and tobacco use; [b] Odds ratios were also calculated for percentage of energy from fat using the following categories: 1 ($\leq$20% of energy), 2 (20%–35% of energy), 3 ($\geq$35% of energy). The ORs for category 2 and category 3 relative to category 1 were 1.80 (95% CI: 1.07–3.04), and 2.10 (95% CI: 0.38–11.70), respectively, with *p*-trend = 0.009. * Significant different from reference category, $p \leq 0.05$.

Vitamin E intake showed significant protective effect against CRC with OR = 0.02 and 95% CI: 0.0003–0.011, (Table 4). Neither quartile analysis nor the trend test was significant for vitamins A, C, B_{12}, D, K, and folate, beta-carotene, phosphate, and omega-3. Calcium showed a significant risk in the top two quartiles.

Table 4. Adjusted ORs [a] and CIs of CRC risk by micronutrient intake quartiles.

Nutrient	Adjusted				
	Q1	**Q2**	**Q3**	**Q4**	***p*-Trend**
Vitamin A (RAE)					
No. of Cases (169)/Controls (248)	42/62	38/67	43/61	46/58	0.769
OR	1	0.90	0.87	0.77	
95% CI	-	0.43–1.89	0.43–1.77	0.37–1.58	
Beta-carotene (µg)					
No. of Cases (169)/Controls (248)	42/62	39/66	63/41	47/57	0.575
OR	1	0.78	0.78	0.71	
95% CI	-	0.38–1.59	0.38–1.63	0.33–1.55	
Vitamin B_{12} (µg)					
No. of Cases (169)/Controls (248)	45/59	31/73	40/65	53/51	0.493
OR	1	0.38	0.66	1.07	
95% CI	-	0.17–0.83	0.32–1.35	0.52–2.222	
Vitamin C (mg)					
No. of Cases (169)/Controls (248)	41/63	40/65	40/64	48/56	0.359
OR	1	0.81	0.63	0.89	
95% CI	-	0.40–1.62	0.29–1.34	0.42–1.89	
Vitamin D (mg)					
No. of Cases (169)/Controls (248)	39/68	43/59	40/64	47/57	0.163
OR	1	1.07	1.33	1.47	
95% CI	-	0.50–2.31	0.64–2.76	0.70–3.08	
Vitamin E (α-Tocopherol) (mg)					
No. of Cases (169)/Controls (248)	95/9	62/43	12/92	-/104	0.001
OR	1	0.05	0.02	-	
95% CI	-	0.01–0.23	0.0003–0.011	-	
Folate (µg)					
No. of Cases (169)/Controls (248)	33/71	41/64	46/58	49/55	0.057
OR [a]	1	1.32	1.24	1.14	
95% CI	-	0.62–2.84	0.58–2.66	0.54–2.42	
Vitamin K (µg)					
No. of Cases (169)/Controls (248)	37/67	41/63	48/57	43/61	0.612
OR	1	0.95	1.12	0.95	
95% CI	-	0.68–2.13	0.88–2.70	0.74–2.29	
Calcium (mg)					
No. of Cases (169)/Controls (248)	29/75	40/65	55/49	45/59	0.146
OR	1	1.80	3.92	2.39	
95% CI	-	0.82–3.96	1.81–8.50	1.04–5.52	

[a] Adjusted for total energy intake normality of the distributions of dietary intake variables was assessed by the Shapiro-Wilk test. Non-normally distributed variables were log transformed. Other potential confounders included age, gender, BMI, physical activity (METs/week), family history (beyond the second degree) of CRC, education attainment, household income, marital status and tobacco use.

4. Discussion

The results from of the present study provide further evidence for an association between CRC risk and diet. Generally, the results of this case-control study on CRC risk illustrate a relationship between macro- and micronutrients intake and this type of cancer among Jordanians.

As BMI was obtained at the time of interview for both patients and controls, the association between obesity and CRC in this study could not be evaluated. The lower BMI in cases may reflect the effect of chemotherapy and other therapies which cancer patients were exposed to before the interview time.

Our study revealed a direct association between total energy intake and the risk of developing CRC, as supported by several other studies [4,19]. Caloric restriction was found to reduce cancer

incidence in rodents and colorectal cell proliferation in humans [20]. The potential mechanism could be through insulin growth factor-1 (IGF-1), where increasing energy could be responsible for glycemic overload and a compensatory increase of serum insulin and related IGF-1, a promoter of tumor cell growth *in vitro* [21,22]. Elevated circulating insulin and IGF level may increase CRC risk, possibly by decreasing IGF-binding proteins (IGFBP-1) and increasing the bioactivity of IGF-I [23,24]. Insulin may increase the circulating IGF-1/IGFBP-3 ratio by increasing hepatic growth hormone sensitivity which could be implicated in increasing the risk for CRC [23,24].

High carbohydrate intake may increase glycemic load, insulin levels, and IGF-1 [20,21]. A significant trend for higher intake of carbohydrate was detected among cases compared to controls. This observation is consistent with some studies [25–27] but not all [4,28]. Borugian *et al.* [26] reported a significant positive association between carbohydrate intake and risk of CRC in both men (OR = 1.7; 95% CI: 1.1–2.7) and women (OR = 2.7; 95% CI: 1.5–4.8) among Chinese in North America. While, Franceschi *et al.* [27] found a direct association between dietary glycemic load and CRC risk, with OR of 1.7 (95% CI: 1.5–2.2).

The effect of fiber on CRC incidence is inconsistent; some studies report a significant inverse association between total fiber intake and CRC risk [4,29–31], whereas other studies found no association between fiber intake and CRC incidence [27,32–34]. Although our results showed no association for the intake of total fiber with CRC, a significant protective effect of insoluble fiber on the risk of CRC development at the highest quartile has been detected [32]. A prospective cohort study of women in the United States, found that total fiber was not associated with CRC risk, with relative risk (RR) for the highest relative to lowest quintile of 0.75 (95% CI: 0.48–1.17, *p*-trend = 0.12). In the other two mentioned studies, significant associations in age-adjusted models disappeared after adjustment for other risk factors. In the Pooling Project analysis including data from 13 cohort studies the report showed statistically significant inverse associations for colorectal cancer in the age adjusted models (Quintile 5 *vs.* Quintile 1, RR 0.84, 95% CI: 0.77–0.92), but not after multivariable adjustment (Quintile 5 *vs.* Quintile 1, RR 0.94, 95% CI: 0.86–1.03) [33]. Similarly, in an NIH-AARP analysis the statistically significant inverse association in the age adjusted model (Quintile 5 *vs.* Quintile 1, HR 0.73, 95% CI: 0.65–0.82) disappeared after multivariable adjustment (Quintile 5 *vs.* Quintile 1, RR 0.99, 95% CI: 0.85–1.15) [34].

Similar to other studies [4,12,26,35], our study results show that total fat, saturated fat and cholesterol have a significant direct effect on CRC risk. The total consumption of fat was much higher in our CRC participants than controls, with only one control in the first quartile. Our observation on fat, saturated fat and cholesterol is in agreement with the report of Arafa *et al.* [11] who reported the daily intake from saturated, mono and polyunsaturated fats and cholesterol is significantly higher among CRC diagnosed subjects as compared to controls, ($p < 0.05$). The proposed mechanism for fat involvement in colorectal carcinogenesis appears to be complex. However, Endo *et al.* [35], showed that the molecular mechanisms underlying the promotion of colorectal carcinogenesis by a high-fat diet (HFD) is through its effect on the role of the insulin-signal pathway and the c-Jun *N*-terminal kinase (JNK) pathway, which was reported to play a crucial role in insulin resistance during colorectal carcinogenesis in the presence of hyperinsulinaemia induced by a HFD. They found that colonic cell proliferation was promoted via the JNK pathway in the presence of a HFD providing an explanation of the effect of dietary fat intake on colon carcinogenesis through the JNK pathway [35].

In our study, protein intake was found to have a significant direct association with CRC. Arafa *et al.* [11] indicated that the consumption of protein among CRC diagnosed patients was higher than intake in a control group, and they speculated that this may be associated with a higher risk for development of CRC. In contrast, one other study by Sun *et al.* [5], reported an inverse association for intake of protein (OR: 0. 85, 95% CI: 0.69–1.00, *p*-trend = 0.002, 4th *versus* 1st quartile). However, Egeberg *et al.* [36] reported a significant association between specific red meat subtypes intake and the risk of developing colon and rectal cancers. They found that consuming lamb meat was significantly related to risk of developing colon cancer, while consuming pork meat was significantly related to

the risk of developing rectal cancer [36]. No associations were found between intake of red meat, processed meat, fish, or poultry and risk for colon cancer or rectal cancer [36]. Fifty percent of participants consumed red meat more than 1–2 times per week (results not shown) in serving size ranges from 90–120 gm. However, the majority (80%) consumed poultry more than 3–4 times weekly. This may partially explain why protein intake in this study was a CRC risk factor rather than protective. In a previous report, Tayyem *et al* [37], we reported that the Jordanian population consumes more animal proteins than plant proteins [37]. In fact, meat intake increased from 7.68 kg/year per-capita in 1961 to 35.85 kg/year per-capita in 2005 [37].

In our study, vitamin E was found to have a significant inverse association with the risk of CRC development. The remarkable effect of vitamin E consumption on protecting against CRC could be attributed to the comparative ratio of 95 case/9 control participants at the lowest quartile compared to 12 case/92 control participants at the 3rd quartile of vitamin E consumption. These results are in agreement with other studies [31,38–41]. A study of Satia-Abouta *et al.* [29], that had been conducted on African Americans, revealed that vitamin E intake was strongly and inversely associated with a 70% reduced risk for colon cancer (OR 0.3; 95% CI (0.1–0.6)). This trend of association was not demonstrated in the same study in Whites (OR 1.0; 95% CI (0.6–1.6)). This could be attributed to ethnic differences [29]. Perhaps, this may be due to genetic makeup or the influence of genes in the metabolic processes. This effect may arise from vitamin E activity as an antioxidant against free radicals and reactive oxygen molecules [39].

The association between folate intake and CRC disease state is being debated and no significant association between folate and CRC risk has been reported [26,42–45], similar to results obtained in our study. However, recent research indicates that folate may have a role in the metabolism of colon carcinogenesis, perhaps by increasing 5, 10-methylenetetrahydrofolate levels for DNA synthesis [44,45].

Iron (Fe) intake was found to have a positive weak association with CRC risk [24]. Our results are consistent with those from other studies [46–48]. In Larsson *et al.* [46] study, the RR of colon cancer, comparing extreme categories of heme iron intake, was 2.29 (95% CI: 1.25–4.21) among women who consumed at least 20 g/week of alcohol, and 1.05 (95% CI: 0.74–1.48) among women who consumed less than 20 g/week of alcohol. The molecular mechanisms of iron carcinogenesis may be explained by the actions of auto-oxidation of iron involving only $Fe^{2+} + O_2$ in oxidant formation in biological systems and its pH dependency, activation of oxidative responsive transcription factors and pro-inflammatory cytokines, and iron-induced hypoxia signaling [47,48].

In our study, the intake of selenium was found to have a significant trend for direct association with CRC risk. Other studies reported the presence of an inverse association between selenium intake and plasma levels with CRC risk [49–51]. Our results may be explained by the finding of Whanger [51] who conducted a study on the form of selenium compounds with CRC risk. His results suggested that Selenomethionine (Semet), the major seleno-compound in cereal grains and enriched yeast, may be the effective form of selenium against CRC. However, Whanger [51] found that Se-methylselenocysteine (SeMCYS), the major seleno-compound in Se-accumulator plants and some plants of economic importance such as garlic and broccoli, may be ineffective against CRC and only protect against mammary tumors. Our results suggest that the effective form of selenium that could protect against CRC disease may have been limited in our participants' diets.

Some studies have shown an association between high sodium intake and CRC development [52–54], and Zhivotovskiy *et al.* [54] have reported that the risk of CRC increased almost 3.5-fold as the dietary intake of salt increased with *p*-trend of 0.008.The results of our study is in agreement with these reports. One potential explanation for this is the presence of chemical carcinogens such as *N*-nitroso compounds in salted foods such as processed meats, dairy products and canned foods, which can be formed by the reaction of sodium nitrate or sodium nitrite in the curing process or in the body, or heterocyclic amines, which have been detected in fish or meat cooked in high temperatures, such as grilling, which is commonly used for grilled meat [54].

Regarding caffeine intake, our results are in agreement with other studies. Caffeine has been shown to have a negative association with CRC risk [55,56]. It inhibits colon cancer cell growth, by acting as antioxidant and effectively scavenging hydroxyl radicals (·OH) [55]. Additionally, caffeine can decrease insulin sensitivity, possibly as a result of elevated plasma epinephrine levels [56].

5. Study Limitation

In a study of this type we rely greatly on the ability and memory recall of participants to accurately and carefully provide information from a period when it was not necessarily important to remember the details of long digested meals, or physical activities that were undertaken. It is understandable that some individuals may have a greater recall than others, and that biases may exist in the minds of those being interviewed, and indeed, by the interviewer. Traumatic events such as the diagnosis of a life threatening disease condition may ultimately have a very significant role in the memory recall of some participants. Because of the obvious limitations placed on the recall of memory, we are using the only means currently available to us, the FFQ, which although prone to errors, is nevertheless an accepted and validated form used in many research studies.

We did not take into account the possible effects of cooking on the bioavailability of the various nutrients, and although we attempted to control for a range of potential confounders, we did not measure alcohol use (culturally discouraged). Nor did we consider the use of food dietary supplements; however, we are aware that the use of dietary supplements is not common or widespread. Finally, the one year dietary recall time frame may not be sufficient to determine an association with a disease state that may take years to develop; nevertheless, we see this study as a pointer to the need for further long term studies involving journal and diary entries of nutritional intakes along with physical activities for designated period of time, whether it be five to fifteen years.

A major strength of our study is the validated and detailed FFQ used to collect dietary data from our study population. Even though dietary data were collected at only one time, the FFQ has been reported to be an adequate instrument for measuring macro- and micronutrient intake [12]. Confirmative studies should verify and extend the presented data on Jordanian dietary habits in order to establish recommendations for people in Jordan to decrease colorectal cancer incidence.

6. Conclusions

This study, conducted in a Jordanian population group provides additional evidence that diets containing high energy, protein, total fat, saturated fat, cholesterol, and sodium intakes may increase the risk of CRC development, whereas high intakes of insoluble fiber, vitamin E, and caffeine may decrease the risk of these diseases. These results suggest that dietary changes could help to reduce the incidences of CRC in the Jordanian population.

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Abbreviation

CRC	Colorectal cancer
FFQ	Food Frequency Questionnaire
DHQ	Diet History Questionnaire I
PAR	7-day Physical Activity Recall
HEPA	Health Enhancing Physical Activity

METs	Metabolic Equivalents
BMI	Body mass index

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References

1. World Cancer Research Fund and American Institute for Cancer Research. *Food, Nutrition, Physical Activity, and the Prevention of Cancer: A Global Perspective*; American Institute for Cancer Research: Washington, DC, USA, 2007.
2. Cancer Prevention Directorate: National Cancer Registry. *Incidence of Cancer in Jordan*; Cancer Prevention Directorate: National Cancer Registry: Amman, Jordan, 2009.
3. Georgakilas, A. Cancer Prevention—From Mechanisms to Translational Benefits. Available online: http://www.intechopen.com/books/cancer-prevention-from-mechanisms-to-translational-benefits (Accessed on 22 December 2014).
4. Harshman, M.R.; Aldoori, W. Diet and colorectal cancer: Review of the evidence. *Can. Fam. Physician* **2007**, *53*, 1913–1920. [PubMed]
5. Sun, Z.; Liu, L.; Wang, P.P.; Roebothan, B.; Zhao, J.; Dicks, E.; Cotterchio, M.; Buehler, S.; Campbell, P.T.; McLaughlin, J.R.; *et al.* Association of total energy intake and macronutrient consumption with colorectal cancer risk: Results from a large population-based case-control study in Newfoundland and Labrador and Ontario, Canada. *Nutr. J.* **2012**, *11*, 18. [CrossRef] [PubMed]
6. Millen, A.E.; Subar, A.F.; Graubard, B.I.; Peters, U.; Hayes, R.B.; Weissfeld, J.L.; Yokochi, L.A.; Ziegler, R.G.; PLCO Cancer Screening Trial Project Team. Fruit and vegetable intake and prevalence of colorectal adenoma in a cancer screening trial. *Am. J. Clin. Nutr.* **2007**, *86*, 1754–1764.
7. Sato, Y.; Tsubono, Y.; Nakaya, N.; Ogawa, K.; Kurashima, K.; Kuriyama, S.; Hozawa, A.; Nishino, Y.; Shibuya, D.; Tsuji, I. Fruit and vegetable consumption and risk of colorectal cancer in Japan: The Miyagi Cohort Study. *Public Health Nutr.* **2005**, *8*, 309–314. [CrossRef] [PubMed]
8. Kabat, G.C.; Miller, A.B.; Jain, M.; Rohan, T.E. A cohort study of dietary iron and heme iron intake and risk of colorectal cancer in women. *Br. J. Cancer* **2007**, *97*, 118–122. [CrossRef] [PubMed]
9. Santarelli, R.L.; Vendeuvre, J.L.; Naud, N.; Taché, S.; Guéraud, F.; Viau, M.; Genot, C.; Corpet, D.E.; Pierre, F.H. Meat processing and colon carcinogenesis: Cooked, nitrite-treated, and oxidized high-heme cured meat promotes mucin-depleted foci in rats. *Cancer Prev. Res. (Phila)* **2010**, *3*, 852–864. [CrossRef]
10. Gonzalez, R.A. Free radicals, oxidative stress and DNA metabolism in human cancer. *Cancer Investig.* **1999**, *17*, 376–377. [CrossRef]
11. Arafa, M.A.; Waly, M.I.; Jriesat, S.; Al Khafajei, A.; Sallam, S. Dietary and lifestyle characteristics of colorectal cancer in Jordan: A case-control study. *Asian Pac. J. Cancer Prev.* **2011**, *12*, 1931–1936. [PubMed]
12. Tayyem, R.F.; Abu-Mweis, S.S.; Bawadi, H.A.; Agraib, L.; Bani-Hani, K. Validation of a Food Frequency Questionnaire to assess macronutrient and micronutrient intake among Jordanians. *J. Acad. Nutr. Diet.* **2014**, *114*, 1046–1052. [CrossRef] [PubMed]
13. Willett, W. *Nutritional Epidemiology*, 2nd ed.; Oxford University Press: New York, NY, USA, 1998.
14. Pellet, P.; Shadarevian, S. *The Food Composition Tables for Use in the Middle East*, 2nd ed.; American University of Beirut: Beirut, Lebanon, 1970.
15. Sallis, J.F.; Haskell, W.L.; Wood, P.D. Physical activity assessment methodology in the Five-City Project. *Am. J. Epidemiol.* **1985**, *121*, 91–106. [PubMed]
16. Lee, R.D.; Nieman, D.C. *Nutritional Assessment*, 6th ed.; McGraw Hill: New York, NY, USA, 2012.
17. Willett, W.; Stampfer, M.J. Total energy intake: Implications for epidemiologic analyses. *Am. J. Epidemiol.* **1986**, *124*, 17–27. [PubMed]
18. Chao, A.; Thun, M.J.; Jacobs, E.J.; Henley, S.J.; Rodriguez, C.; Calle, E.E. Cigarette smoking and colorectal cancer mortality in the cancer prevention study II. *J. Natl. Cancer Inst.* **2000**, *92*, 1888–1896. [CrossRef] [PubMed]
19. Jarvandi, S.; Davidson, N.O.; Schootman, M. Increased risk of colorectal cancer in type 2 diabetes is independent of diet quality. *PLoS One* **2013**, *8*, e74616. [CrossRef] [PubMed]
20. Longo, V.D.; Fontana, L. Calorie restriction and cancer prevention: Metabolic and molecular mechanisms. *Trends Pharmacol. Sci.* **2010**, *31*, 89–98. [CrossRef] [PubMed]

21. Meinhold, C.L.; Dodd, K.W.; Jiao, L.; Flood, A.; Shikany, J.M.; Genkinger, J.M.; Hayes, R.B.; Stolzenberg-Solomon, R.Z. Available carbohydrates, glycemic load, and pancreatic cancer: Is there a link? *Am. J. Epidemiol.* **2010**, *171*, 1174–1182. [CrossRef] [PubMed]
22. Lin, J.K.; Shen, M.Y.; Lin, T.C.; Lan, Y.T.; Wang, H.S.; Yang, S.H.; Li, A.F.; Chang, S.C. Distribution of a single nucleotide polymorphism of insulin-like growth factor-1 in colorectal cancer patients and its association with mucinous adenocarcinoma. *Int. J. Biol. Markers* **2010**, *25*, 195–199. [PubMed]
23. Peters, G.; Gongoll, S.; Langner, C.; Mengel, M.; Piso, P.; Klempnauer, J.; Rüschoff, J.; Kreipe, H.; von Wasielewski, R. IGF-1R, IGF-1 and IGF-2 expression as potential prognostic and predictive markers in colorectal-cancer. *Virchows Arch.* **2003**, *443*, 139–145. [CrossRef]
24. Kaaks, R.; Toniolo, P.; Akhmedkhanov, A.; Lukanova, A.; Biessy, C.; Dechaud, H.; Rinaldi, S.; Zeleniuch-Jacquotte, A.; Shore, R.E.; Riboli, E. Serum C-peptide, insulin-like growth factor (IGF)-I, IGF-binding proteins, and colorectal cancer risk in women. *J. Natl. Cancer Inst.* **2000**, *92*, 1592–1600. [CrossRef] [PubMed]
25. McCarl, M.; Harnack, L.; Limburg, P.J.; Anderson, K.E.; Folsom, A.R. Incidence of colorectal cancer in relation to glycemic index and load in a cohort of women. *Cancer Epidemiol. Biomark. Prev.* **2006**, *15*, 892–896. [CrossRef]
26. Borugian, M.J.; Sheps, S.B.; Whittemore, A.S.; Wu, A.H.; Potter, J.D.; Gallagher, R.P. Carbohydrates and colorectal cancer risk among Chinese in North America. *Cancer Epidemiol. Biomark.* **2002**, *11*, 187–193.
27. Franceschi, S.; Dal Maso, L.; Augustin, L.; Negri, E.; Parpinel, M.; Boyle, P.; Jenkins, D.J.; la Vecchia, C. Dietary glycemic load and colorectal cancer risk. *Ann. Oncol.* **2001**, *12*, 173–178. [CrossRef] [PubMed]
28. Dray, X.; Boutron-Ruault, M.C.; Bertrais, S.; Sapinho, D.; Benhamiche-Bouvier, A.M.; Faivre, J. Influence of dietary factors on colorectal cancer survival. *Gut* **2003**, *52*, 868–873. [CrossRef] [PubMed]
29. Satia-Abouta, J.; Galanko, J.A.; Potter, J.D.; Ammerman, A.; Martin, C.F.; Sandler, R.S. Associations of total energy and macronutrients with colon cancer risk in African Americans and Whites: Results from the North Carolina colon cancer study. *Am. J. Epidemiol.* **2003**, *158*, 951–962. [CrossRef] [PubMed]
30. Nomura, A.M.Y.; Hankin, J.H.; Henderson, B.E.; Wilkens, L.R.; Murphy, S.P.; Pike, M.C.; le Marchand, L.; Stram, D.O.; Monroe, K.R.; Kolonel, L.N. Dietary fiber and colorectal cancer risk: The multiethnic cohort study. *Cancer Causes Control* **2007**, *18*, 753–764. [CrossRef] [PubMed]
31. Tantamango, Y.M.; Knutsen, S.F.; Beeson, L.; Fraser, G.; Sabate, J. Association between dietary fiber and incident cases of colon polyps: The adventist health study. *Gastrointest. Cancer Res.* **2011**, *4*, 161–167. [PubMed]
32. Lin, J.; Zhang, S.M.; Cook, N.R.; Rexrode, K.M.; Liu, S.; Manson, J.E.; Lee, I.M.; Buring, J.E. Dietary intakes of fruit, vegetables, and fiber, and risk of colorectal cancer in a prospective cohort of women (United States). *Cancer Causes Control* **2005**, *16*, 225–233. [CrossRef] [PubMed]
33. Park, Y.; Hunter, D.J.; Spiegelman, D.; Bergkvist, L.; Berrino, F.; van den Brandt, P.A.; Buring, J.E.; Colditz, G.A.; Freudenheim, J.L.; Fuchs, C.S.; *et al.* Dietary fiber intake and risk of colorectal cancer: A pooled analysis of prospective cohort studies. *JAMA* **2005**, *294*, 2849–2857. [CrossRef] [PubMed]
34. Schatzkin, A.; Mouw, T.; Park, Y.; Subar, A.F.; Kipnis, V.; Hollenbeck, A.; Leitzmann, M.F.; Thompson, F.E. Dietary fiber and whole-grain consumption in relation to colorectal cancer in the NIH-AARP Diet and Health Study. *Am. J. Clin. Nutr.* **2007**, *85*, 1353–1360. [PubMed]
35. Endo, H.; Hosono, K.; Fujisawa, T.; Takahashi, H.; Sugiyama, M.; Yoneda, K.; Nozaki, Y.; Fujita, K.; Yoneda, M.; Inamori, M.; *et al.* Involvement of JNK pathway in the promotion of the early stage of colorectal carcinogenesis under high-fat dietary conditions. *Gut* **2009**, *58*, 1637–1643. [CrossRef] [PubMed]
36. Egeberg, R.; Olsen, A.; Christensen, J.; Halkjær, J.; Jakobsen, M.U.; Overvad, K.; Tjønneland, A. Associations between red meat and risks for colon and rectal cancer depend on the type of red meat consumed. *J. Nutr.* **2013**, *143*, 464–472. [CrossRef] [PubMed]
37. Tayyem, R.F.; Bawadi, H.; Shawawreh, A.; Jad-Allah, H.; Abu-Oleim, S.; Khader, Y. Changes in eating pattern among Jordanians. *Dirasat Agric. Sci.* **2010**, *37*, 46–55.
38. Bostick, R.M.; Potter, J.D.; McKenzie, D.R.; Sellers, T.A.; Kushi, L.H.; Steinmetz, K.A.; Folsom, A.R. Reduced risk of colon cancer with high intake of vitamin E: The Iowa Women's Health Study. *Cancer Res.* **1993**, *53*, 4230–4237. [PubMed]
39. Ferraroni', M.; la Vecchia, C.; D'Avanzo, B.; Negri, E.; Francesc, S.; Decarli, A. Selected micronutrient intake and the risk of colorectal cancer. *Br. J. Cancer* **1994**, *70*, 1150–1155.

40. Tseng, M.; Murray, S.C.; Kupper, L.L.; Sandier, R.S. Micronutrients and the risk of colorectal adenomas. *Am. J. Epidemiol.* **1996**, *144*, 1005–1014. [CrossRef] [PubMed]
41. Williams, C.D.; Satia, J.A.; Adair, L.S.; Stevens, J.; Galanko, J.; Keku, T.O.; Sandler, R.S. Antioxidant and DNA methylation-related nutrients and risk of distal colorectal cancer. *Cancer Causes Control* **2010**, *21*, 1171–1181. [CrossRef] [PubMed]
42. De Vogel, S.; Dindore, V.; van Engeland, M.; Goldbohm, R.A.; van den Brandt, P.A.; Weijenberg, M.P. Dietary folate, methionine, riboflavin, and vitamin B-6 and risk of sporadic colorectal cancer. *J. Nutr.* **2008**, *138*, 2372–2378. [CrossRef] [PubMed]
43. Harnack, L.; Jacobs, D.R., Jr.; Nicodemus, K.; Lazovich, D.; Anderson, K.; Folsom, A.R. Relationship of folate, vitamin B-6, vitamin B-12, and methionine intake to incidence of colorectal cancers. *Nutr. Cancer* **2002**, *43*, 152–158. [CrossRef] [PubMed]
44. Ma, J.; Stampfer, M.J.; Giovannucci, E.; Artigas, C.; Hunter, D.J.; Fuchs, C.; Willett, W.C.; Selhub, J.; Hennekens, C.H.; Rozen, R. Methylenetetrahydrofolate reductase polymorphism, dietary interactions, and risk of colorectal cancer. *Cancer Res.* **1997**, *57*, 1098–1102. [PubMed]
45. Kennedy, D.A.; Stern, S.J.; Moretti, M.; Matok, I.; Sarkar, M.; Nickel, C.; Koren, G. Folate intake and the risk of colorectal cancer: A systematic review and meta-analysis. *Cancer Epidemiol.* **2011**, *35*, 2–10. [CrossRef] [PubMed]
46. Larsson, S.C.; Adami, H.O.; Giovannucci, E.; Wolk, A. Correspondence. Re: Heme iron, zinc, alcohol consumption, and risk of colon cancer. *J. Natl. Cancer Inst.* **2005**, *97*, 232–234. [CrossRef] [PubMed]
47. Sesink, A.; Termont, D.; Kleibeuker, J.H.; Meer, R.V. Red meat and colon cancer: Dietary haem, but not fat, has cytotoxic and hyperproliferative effects on rat colonic epithelium. *Carcinogenesis* **2000**, *21*, 1909–1915. [CrossRef] [PubMed]
48. Huang, X. Iron overload and its association with cancer risk in humans: Evidence for iron as a carcinogenic metal. *Mutat. Res.* **2003**, *533*, 153–171. [CrossRef] [PubMed]
49. Jacobs, E.T.; Jiang, R.; Alberts, D.S.; Greenberg, E.R.; Gunter, E.W.; Karagas, M.R.; Lanza, E.; Ratnasinghe, L.; Reid, M.E.; Schatzkin, A.; *et al.* Selenium and colorectal adenoma: Results of a pooled analysis. *J. Natl. Cancer Inst.* **2004**, *96*, 1669–1675. [CrossRef] [PubMed]
50. Me´plan, C.; Hesketh, J. The influence of selenium and selenoprotein gene variants on colorectal cancer risk. *Mutagenesis* **2012**, *27*, 177–186. [CrossRef] [PubMed]
51. Whanger, P.D. Selenium and its relationship to cancer: An update. *Br. J. Nutr.* **2004**, *91*, 11–28. [CrossRef] [PubMed]
52. Murata, A.; Fujino, Y.; Pham, T.M.; Kubo, T.; Mizoue, T.; Tokui, N.; Matsuda, S.; Yoshimura, T. Prospective cohort study evaluating the relationship between salted food intake and gastrointestinal tract cancer mortality in Japan. *Asia Pac. J. Clin. Nutr.* **2010**, *19*, 564–571. [PubMed]
53. Tuyns, A.J. Salt and gastrointestinal cancer. *Nutr. Cancer* **1988**, *11*, 229–232. [CrossRef] [PubMed]
54. Zhivotovskiy, A.S.; Kutikhin, A.G.; Azanov, A.Z.; Yuzhalin, A.E.; Magarill, Y.A.; Brusina, E.B. Colorectal cancer risk factors among the population of South-East Siberia: A case-control study. *Asian Pac. J. Cancer Prev.* **2012**, *13*, 5183–5188. [CrossRef] [PubMed]
55. Sinha, R.; Cross, A.J.; Daniel, C.R.; Graubard, B.I.; Wu, J.W.; Hollenbeck, A.R.; Gunter, M.J.; Park, Y.; Freedman, N.D. Caffeinated and decaffeinated coffee and tea intakes and risk of colorectal cancer in a large prospective study. *Am J Clin Nutr.* **2012**, *96*, 374–381. [CrossRef] [PubMed]
56. Shi, X.; Dalal, N.S.; Jain, A.C. Antioxidant behaviour of caffeine: Efficient scavenging of hydroxyl radicals. *Food Chem. Toxicol.* **1991**, *29*, 1–6. [CrossRef] [PubMed]

nutrients

MDPI

Review

Inflammaging and Cancer: A Challenge for the Mediterranean Diet

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Abstract: Aging is considered the major risk factor for cancer, one of the most important mortality causes in the western world. Inflammaging, a state of chronic, low-level systemic inflammation, is a pervasive feature of human aging. Chronic inflammation increases cancer risk and affects all cancer stages, triggering the initial genetic mutation or epigenetic mechanism, promoting cancer initiation, progression and metastatic diffusion. Thus, inflammaging is a strong candidate to connect age and cancer. A corollary of this hypothesis is that interventions aiming to decrease inflammaging should protect against cancer, as well as most/all age-related diseases. Epidemiological data are concordant in suggesting that the Mediterranean Diet (MD) decreases the risk of a variety of cancers but the underpinning mechanism(s) is (are) still unclear. Here we review data indicating that the MD (as a whole diet or single bioactive nutrients typical of the MD) modulates multiple interconnected processes involved in carcinogenesis and inflammatory response such as free radical production, NF-κB activation and expression of inflammatory mediators, and the eicosanoids pathway. Particular attention is devoted to the capability of MD to affect the balance between pro- and anti-inflammaging as well as to emerging topics such as maintenance of gut microbiota (GM) homeostasis and epigenetic modulation of oncogenesis through specific microRNAs.

Keywords: aging; inflammation; inflammaging; cancer; mediterranean diet; nutrients; microRNAs; NU-AGE project

1. Inflammaging as a Major Component of Aging, Age-Related Diseases and Cancer

Human aging is a complex, extremely heterogeneous and dynamic trait determined by a number of environmental, genetic, epigenetic, and stochastic factors [1]. A pervasive feature of human aging and probably one of its major causes, is represented by the chronic, low-level state of systemic and sterile (in the absence of overt infection) inflammation called "inflammaging" [2,3]. Indeed,

inflammation has been recently included among the seven pillars of aging [4]. It can be beneficial as an acute, transient immune response to harmful conditions, facilitating the repair, turnover and adaptation of many tissues. However, during aging, inflammatory response tends to become chronic and of low grade, leading to tissue degeneration.

Indeed, inflammaging is characterized by a general increase in plasma levels and cell capability to produce pro-inflammatory cytokines such as Interleukin-6 (IL-6), Interleukin-1 (IL-1) and Tumour Necrosis Factor-α (TNF-α) and by a subsequent increase of the main inflammatory markers, such as C-reactive protein (CRP) and serum amyloid A (A-SAA) [2,5]. This generalized pro-inflammatory status, interacting with the genetic background and environmental factors, potentially triggers the onset of the most important age-related diseases, such as cardiovascular diseases, atherosclerosis, metabolic syndrome, type 2 diabetes, obesity, neurodegeneration, arthrosis and arthritis, osteoporosis and osteoarthritis, sarcopenia, major depression, frailty and cancer [6,7].

The hypothesis of a possible correlation between cancer and inflammation was firstly formulated by the Greek physician Galenus [8,9]. In 1863 Rudolph Virchow, a pioneer of cellular pathology, noted inflammatory cells within tumor mass and that tumors arise at sites of chronic inflammation [10,11]. A functional framework developed by Hanahan and Weinberg (2000) characterizes cancer by six biological hallmarks, able to regulate the conversion of normal cells in cancer cells: self-sufficiency in growth signals, insensitivity to growth inhibitory signals, limitless replicative potential, the ability to evade programmed cell-death (apoptosis), the ability to sustain angiogenesis, the ability to invade tissues and metastasize [12]. Studies have also supported the important role of inflammatory cells and cytokines in the tumor microenvironment [13–15]. In 2011, Weinberg and Hanahan proposed four additional new cancer hallmarks: ability to evade the immune system, presence of inflammation, tendency towards genomic instability and dysregulated metabolism [16]. The correlation between chronic inflammation and cancer has been supported by epidemiological and experimental studies on humans and animal models [13,15,17] along with the observation that preventive treatments with anti-inflammatory drugs such as aspirin or cyclooxygenase-2 (COX-2) inhibitors reduce the risk of developing colorectal and breast cancer and even mortality [15,18,19]. Chronic inflammation affects all cancer stages, increasing the onset risk, supporting the initial genetic mutation or epigenetic mechanism leading to cancer initiation [20–22], promoting tumor progression, and supporting metastatic diffusion [9,22–25].

We recently hypothesised that it is important to distinguish between systemic inflammaging and local inflammaging. While the pathological and pathogenetic role of circulating pro-inflammatory compounds and cytokines is unclear and possibly negligible (a marker of inflammation rather than an active player), there are a number of observations and papers suggesting that the local production of inflammatory cytokines can have strong deleterious effects, as we recently suggested in the case of breast cancer niche as a paradigmatic example [26,27]. Therefore, it is tempting to speculate that the important aspect to be considered in inflammaging and cancer is not the mere increase in inflammatory mediators but rather the source, and therefore the local targets, of these mediators. The tangled interplay among local immune responses and systemic inflammation and their influence on clinical outcomes in cancer has been recently reviewed [28].

Different types of tissues (muscle, adipose tissue), organs (brain and liver), systems (immune system) and ecosystems (gut microbiota, GM) may contribute to the systemic inflammatory state, through altered production of pro-inflammatory and/or anti-inflammatory mediators [5,7,26,29,30].

Inflammaging can be influenced by many other factors, such as microRNAs (miRs) and agalactosylated *N*-glycans, together with the products and metabolites of the intestinal microbiota.

Additionally, some mitochondrial components, including mtDNA and other "cellular debris" released outside of the cells, as a consequence of natural cell turnover/damage, could trigger and sustain a sort of "physiological inflammatory tone" that increases with age [31]. This conceptualization can be extended to nutrients, whereby an excess of nutrients could be therefore capable of triggering

an inflammatory response [32–34] that has been dubbed "metaflammation" [35], contributing to the above-mentioned physiological inflammatory tone.

Inflammatory Sources for Cancer Development

Apart from inflammaging, viruses, bacteria and parasite infections as well as the exposure to chemical or physical agents can support chronic inflammation and have been linked to several cancer types [36–38]. Similarly, unresolved inflammation unrelated to infections can also contribute to carcinogenesis as observed in Barrett's metaplasia, chronic pancreatitis or esophagitis [21,38–43] or in autoimmune diseases [21].

Obesity plays a central role in carcinogenesis since adipose tissue has been recognized as an endocrine source of mediators (hormones, acute-phase proteins, cytokines, adipokines and growth factors, [44]) able to sustain a chronic low-grade inflammation. During the last fifteen years, obesity has been associated with several types of tumors such as breast, endometrium, prostate, kidney, esophagus, stomach, colon, pancreas, gallbladder, and liver [45–49] and also with an increased cancer aggressiveness, risk of relapse and mortality [49].

A large amount of data indicates that inflammation is closely connected to oxidative stress. Reactive oxygen species (ROS) are continuously produced by our cells as a by-product of oxidative metabolism and are essential for several physiological functions and signalling pathways. However, an excessive accumulation of ROS may cause cellular oxidative damage to nucleic acids and proteins in cells of several systems including the endocrine and the immune systems [26,50]. We have recently hypothesised that most of the deleterious effects of excessive oxidative stress in tissues and organs can be mediated by the induction of unwanted inflammatory reactions [50].

Indeed, an important characteristic of tumor promoters is their ability to recruit inflammatory cells and to stimulate them to generate ROS [51]. Mast cells and leukocytes recruited to the site of damage lead to a "respiratory burst" due to an increased uptake of oxygen and, thus, an increased release and accumulation of ROS at the site of damage [20]. On the other hand, inflammatory cells also produce soluble mediators, such as metabolites of arachidonic acid, CRP, cytokines (IL-1, IL-6), and chemokines, which act by further recruiting inflammatory cells to the site of damage and producing more reactive species. This sustained inflammatory/oxidative environment leads to a vicious cycle, which can affect healthy neighboring epithelial and stromal cells, by inducing DNA damage and activating epigenetic mechanisms, and over a long period of time may lead to carcinogenesis.

During tumor progression, immune and inflammatory cells produce cytokines and chemokines, which facilitate cancer cell survival and proliferation, and promote the angiogenic switch enhancing tumor growth [52]. Cytokines and chemokines also induce further recruitment and differentiation of immune cells in the tumor microenvironment [53]. The key mediators can activate signal transduction cascades and induce changes in transcription factors, such as nuclear factor-κB (NF-κB), signal transducer and activator of transcription 3 (STAT3), PPAR-γ, β-catenin, p53, hypoxia-inducible factor-1α (HIF-1α), activator protein-1 (AP-1), nuclear factor of activated T cells (NFAT), and NF-E2 related factor-2 (Nrf2), which mediates immediate cellular stress responses [54]. All these molecules are regulated by the transcription factor NF-κB [55], which could be considered as a "hub" in tumorigenesis linking cellular senescence, inflammaging and cancer [23]. As summarized in Figure 1, almost all gene products involved in inflammation are indeed regulated by the activation of NF-κB (e.g., TNF-α, IL-1, IL-6, chemokines, COX-2, 5LOX, CRP) [56] and NF-κB is activated in response to several well known cancer risk factors such as smoke, stress, dietary agents, obesity, infectious agents and irradiation. Moreover, NF-κB has been associated with transformation of cells [57] and is constitutively active in most tumor cells. Cellular senescence, a tumour suppressive stress response, is associated with a secretory phenotype that might be an important additional contributor to chronic inflammation [58]. Senescent cells are in fact characterized by the capability to produce high amounts of pro-inflammatory proteins [59,60]. The senescent phenotype is also accompanied by an upregulation of the DNA damage-response system and recently it has been proposed that the accrual of DNA damage with age

can contribute significantly to inflammaging via the production of IL-6 [27]. In the cancer field this phenomenon, related to the propagation to bystander cells of DNA damage, DNA damage response and inflammation, has been conceptualized as "para-flammation" [61].

All these exogenous and endogenous danger signals (viruses, bacteria, including the GM and its products, damaged and senescent cells, cell debris, altered/modified proteins, *N*-glycans, mtDNA, ROS, *etc.*) are overall conceptualized by our group as "garbage", able to trigger inflammaging and inflammation. Indeed, all these "dysfunctional" molecules can be sensed by receptors of the innate immune response and thus are potential stimulants of pro-inflammatory responses. This "garbage" is an inevitable byproduct of the normal metabolism, but its accumulation becomes evident with advancing age and/or in pathological conditions [3], due to the lifelong exposure to exogenous/endogenous insults on one side, and to the decreased capacity of the ubiquitin-proteasome system [62,63] and autophagy [64] to cope with these products on the other side.

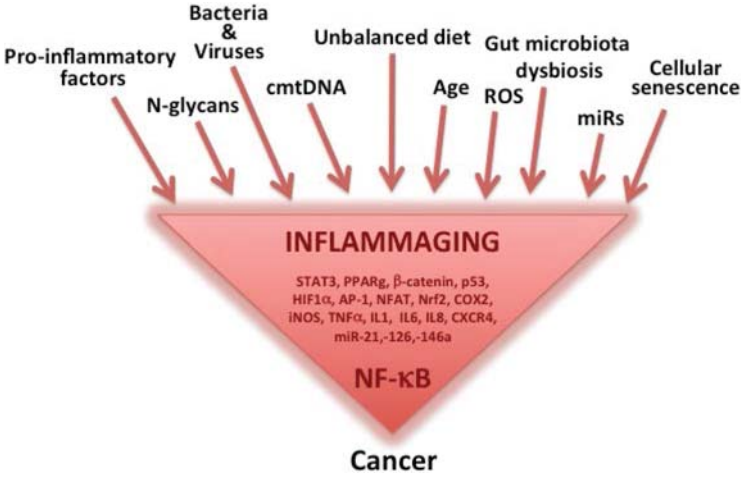

Figure 1. Among the main causes of inflammaging, we found the accumulation of pro-inflammatory factors, viruses and bacteria, age, reactive oxygen species (ROS) and cellular senescence. Inflammaging can also be influenced by many other factors, including non-immunological ones, and those not directly related to inflammation, such as microRNAs (miRs), circulating mitochondrial DNA (cmtDNA) and agalactosylated *N*-glycans, together with the products and related metabolites of the intestinal microbiota. Several pathways and molecules are triggered by these factors, which then are able to activate the nuclear transcription factor NF-κB which could be considered as a hub in carcinogenesis, linking inflammaging, cellular senescence and cancer.

2. The Mediterranean Diet

2.1. The Mediterranean Diet: Definitions and Characteristics

In literature, there are various definitions of the Mediterranean Diet (MD) but they generally share the main components: a high consumption of vegetables, fruits, whole grains, legumes, olive oil and fish (especially marine species), a low intake of saturated fats such as butter and other animal fats, red meat, poultry, dairy products and a regular but moderate consume of ethanol mainly consisting of red wine during meals. Some of these features overlap with other healthy dietary patterns, whereas other aspects are unique to the MD.

The MD is the typical dietary pattern of the populations bordering the Mediterranean area. The traditional MD has been accepted and acknowledged by the scientific community following the publication by Ancel Keys and colleagues showing results from the Seven Countries Study. The

purpose of this longitudinal epidemiological study, started in the late 1950s, was to examine the relationships between lifestyle and dietary factors and cardiovascular diseases in populations from different regions of the world (the USA, Northern Europe, Southern Europe and Japan). Resulting data indicated that the mortality rate for coronary heart disease was higher in the USA and Northern Europe in comparison to Southern Europe. In particular, subjects from Greece and Italy showed the lowest mortality for cardiovascular diseases [65]. In 2003, the PREDIMED study found that the MD supplemented with extra virgin olive oil or tree nuts was able to prevent cardiovascular diseases in comparison to a low-fat diet [66].

Between 2005 and 2010, the Moli-sani study showed that higher adherence to the MD was associated with a reduction of leukocytes and platelets suggesting that the set of foods composing the MD could have an anti-inflammatory action and a protective effect on many diseases (primarily atherosclerosis) with an inflammatory pathogenesis [67]. Overall, these and other studies indicated the existence of inverse associations between MD and total mortality [68], the incidence of coronary heart disease [69,70], thrombotic stroke [71], and with the development of various forms of cancer [72,73].

The international scientific community has accepted the role of the MD in increasing life expectancy and improving general health contributing to the spread of the MD pattern as a central pillar of programs and public health policy in many countries, from the USA to Europe. The MD is not only a diet but represents a lifestyle. The "Mediterranean Diet Foundation" developed a chart of the food pyramid, which includes information closely related to the Mediterranean lifestyle, cultural and social order as well as the importance of exercise and conviviality. Figure 2 highlights the importance of the Mediterranean lifestyle, including factors not associated with the use of particular foods.

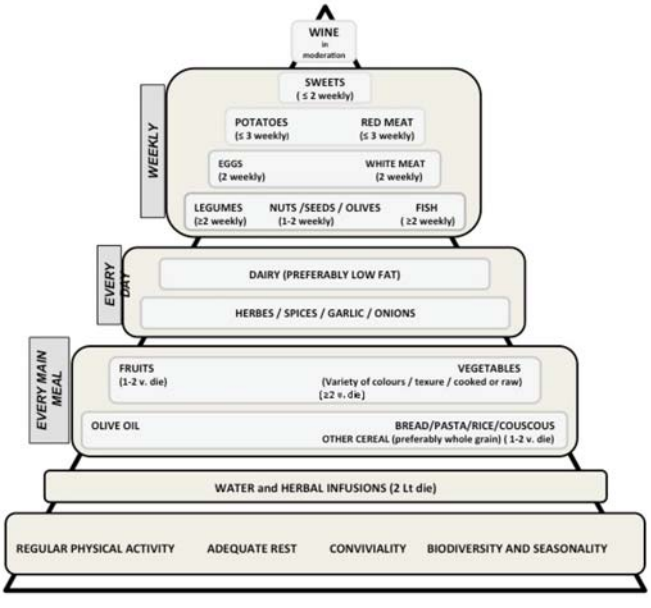

Figure 2. Pyramid of Mediterranean lifestyle (inspired by the "Mediterranean Diet Foundation" Barcelona Spain). The size of different sectors of the pyramid is directly proportional to the frequency of use of that particular food or food group. At the base of the pyramid there are healthy habits and groups of foods to be eaten daily and in large quantities (*i.e.*, fruit, vegetables, *etc.*). The upper levels show the foods to be eaten moderately (*i.e.*, sweets, red meat, *etc.*).

The carbohydrate composition of the MD deserves special attention. Consumption of unrefined whole grain carbohydrates as a preferred choice has a double action: it limits the elevation of postprandial blood glucose and ensures a good supply of fiber. In fact, whole grain cereals have a lower glycemic index (GI) than refined products made with white flour, white rice and sugar. The consumption of low-GI foods avoids sudden increases in blood glucose, limits the secretion of insulin, and, therefore, inflammation [74]. Moreover, low-GI products have an anti-atherogenic action, decreasing the production of atherogenic lipoproteins, oxidized LDL and inflammatory markers [75]. The consumption of whole grains, legumes and other plant foods recommended by the MD brings a high amount of fiber (β-glucans, arabinoxylans, galactomannans, pectins) that increases satiety and helps to control weight. Numerous scientific results showed that dietary fiber promotes gut health and prevents cardiovascular disease, cancer, obesity and diabetes [76]. In the gut, prebiotic fiber (inulin, lactulose and galactooligosaccharides) can be selectively fermented by Bifidobacteria and/or Lactobacilli. The growth of these microorganisms maintains homeostasis and functionality of the intestinal microbiota and reduces the risk of dysbiosis [77]. Moreover, fiber is an effective "carrier" of bioactive antioxidants (vitamins C and E, carotenoids, and polyphenols).

The MD is characterized by a high content of "good fats", monounsaturated (MUFA) and polyunsaturated (PUFA) fatty acids, present in marine fish, vegetable oils (especially olive oil), in nuts and seeds, and by a low intake of saturated fatty acids and hydrogenated oils (trans fats). In particular, the MD provides an optimal dietary fat profile characterized by a low intake of saturated and ω-6 fatty acids and a moderate intake of ω-3 fatty acids [78]. The ratio between ω-6 and ω-3 PUFAs plays an important role in the modulation of inflammation and blood coagulation [79] and is one of the most powerful anti-inflammatory features of this diet.

In terms of micronutrients, the MD is rich in B vitamins (B1, B2, niacin, B6, folate or B12), antioxidant vitamins (vitamins E and C) and minerals, especially iron, selenium, phosphorus and potassium.

Plant foods constitute the core of MD and are characterized by a high content of "non-nutritive" components (phytochemicals), including polyphenols, phytosterols and carotenoids. Phytochemicals are bioactive substances known to combat cellular inflammation due to their powerful antioxidant action. Data from PREDIMED and other studies suggested that the efficiency of the dietary antioxidants in modulating the plasma antioxidant capacity depends on the health status of individuals. In fact, the best results are obtained in people with some risk factors (for example, smokers) or cardiovascular disease or in subjects with a low initial plasma antioxidant capacity. The healthy subjects with low levels of oxidative stress showed a reduced responsiveness to antioxidants. In addition, some studies indicate a negative effect of antioxidant supplements in overall mortality and mortality caused by cardiovascular disease, diabetes and some types of cancer. This is certainly due to the complexity of the interactions between endogenous and exogenous antioxidants and to the existence of homestatic mechanisms of control intended to prevent an overload of reducing agents maintaining the physiological state of homeostasis [80,81]. Therefore, although an adequate intake of antioxidants is needed to counteract oxidative stress, these compounds should be introduced through plant foods such as fruits, vegetables, whole grains, nuts and seeds naturally present in a healthy and complete nutritional model such as the MD.

Diet scores are increasingly being employed to define MD adherence in epidemiological studies [82]. Trichopoulou and colleagues proposed in 1995, and subsequently updated, the Mediterranean Diet Score (MDS) [83]. This simple score was constructed by assigning a value of 0 or 1 for each of the nine components. Therefore, the total MDS ranges from 0 (minimal adherence to the traditional Mediterranean diet) to 9 (maximum adherence) [70]. The NIH-AARP Diet, the Health Study, the European Prospective Investigation into Cancer and Nutrition (EPIC) and other studies used the MDS and other scores later derived from it to confirm the correlation between adherence to MD and mortality reduction, demonstrating the MD protective role in regard to the prevention of cancer, cardiovascular and other chronic diseases [72,73,84,85].

2.2. The Preventive Role of the Mediterranean Diet on Cancer

2.2.1. Epidemiological Studies

Nutrition represents an easily modifiable factor able to contrast inflammation and oxidative stress. Growing evidence indicates the beneficial and preventive role of the Mediterranean Diet (MD) in the onset of cancer and other diseases associated with increased level of inflammation, oxidative damage and angiogenesis. A recent metanalysis of all the observational studies regarding the adherence to MD in relation to cancer risk [86] showed that MD is associated with a significant reduction of overall risk of cancer incidence and mortality by 10%. In particular, increased adherence to the MD reduces the likelihood of having colorectal cancer CRC, even among obese and diabetic patients suggesting potential benefits of this dietary model on CRC risk factors [87–89]. Contrasting data are reported on other forms of neoplasms. While the metanalysis by Schwingshackl and Hoffmann indicates a reduction of the risk of prostate cancer by 4%, a recent paper reported that a higher Mediterranean Diet Score (MDS, see Section 2.1 for details) was not associated with risk of advanced prostate cancer or disease progression in a cohort of 47867 men prospectively followed for 24 years. However, in the same subjects, the adherence to the MD was associated with lower overall mortality after diagnosis of nonmetastatic prostate cancer [90]. Some studies investigating the role of the MD on oral and pharyngeal cancer reported an inverse association between the risk of this neoplasm and adherence to the MD, as measured by various indexes, and indicated a stronger effect in younger subjects [91–93]. A meta-analysis by Schwingshackl and Hoffmann did not seem to confirm an effect of the MD on the risk of breast cancer, even if a subgroup of case-control study showed that the risk of this cancer could be reduced by 18% in women adhering to the MD. In particular, a recent study on 500 Greek middle-aged women showed that one unit increase in MDS was associated with 9% lower risk of breast cancer. It is worth noting that the protective effect of the MD against breast cancer seemed to depend on individual's characteristics and potential risk factors, *i.e.* obesity, physical activity, smoking, age at the menarche, menopausal status. In the above described cohort, the beneficial effect of MD on breast cancer risk is observed only in normal weight, non smoking women and in women who did not present an early menarche (<12 year old) [94]. The studies describing the MD preventive action against various types of cancer suggested that this healthful dietary pattern acts through several mechanisms, decreasing the dysregulated free radical production and inflammation [80,95,96].

2.2.2. Chemoprotective Effects of Polyphenols on Inflammation and Cancer

The abundant consumption of fruit, vegetables, grains, legumes, olive oil and the moderate intake of red wine introduces, in the organism, high levels of different polyphenols and plant bioactive compounds that initially were known as antioxidants but later were studied for their anti-inflammatory, anti-tumor, anti-atherogenic abilities that could not be explained solely on the basis of their antioxidant properties. In fact, a series of investigations into the mechanism of action of these molecules have shed light on the fact that polyphenols do not merely exert their effects only as free radical scavengers, but may also modulate cellular signaling processes involved in inflammatory response or may themselves serve as signaling agents [97]. In particular, dietary polyphenols from olive oil (oleuropein, hydroxyltyrosol) and from red wine (resveratrol) were shown to modulate the eicosanoids pathway through the inhibition of cellular enzymes such as phospholipase A2 (PLA2), cyclooxygenase (COX-1 and COX-2) and lipoxigenase (LOX). This action reduces the cellular production of arachidonic acid and inflammatory prostaglandins and leukotrienes [98]. Other studies showed that also quercitin, the most abundant and widespread natural flavonoid present in a variety of fruit and vegetables, inhibited COX and LOX in different cellular animal models exerting an anti-inflammatory action [99–101]; quercitin is also able to rejuvenate senescent fibroblasts by activating proteasome function [102]. Olive oil and red wine polyphenols reduce inflammatory angiogenesis, a key pathogenic process in cancer and atherosclerosis, in human cultured endothelial cell through the inibithion of COX-2 protein expression, prostaglandin production and MMP-9 release. This effect is accompanied by a

substantial reduction of ROS levels and NF-κB activation [103]. A variety of polyphenols (quercitin, apigenin, luteolin, kaempferol, myricetin) are able to modulate the inflammatory process through the inhibition of nitric oxide (NO) production by supressing nitric oxide synthase (NOS) enzyme expression and/or activity [104–106]. A plethora of studies on human and animal cellular models have shown that different flavonoids such as quercitin and phenolic compounds from extra virgin olive oil interfere with the expression, production and/or function of cytokines/chemokines such as TNF-α, IL-1β, IL-6, IL-8, MCP-1, IFN-γ and IL-10, contributing to the control of the balance between pro- and anti-inflammatory mediators and exerting a potent anti-inflammatory activity. These compounds, as natural antioxidants are able to efficiently modulate the redox status of cells and strictly regulate the inducible gene expression of inflammatory mediators [107–112]. Moreover, polyphenols are involved in multiple steps of the NF-κB activation process, which represent an important and very promising pathway for the treatment and prevention of inflammatory diseases and cancer [113]. A recent review described the action played by dietary polyphenols in the inhibition of cancer cell growth due to their ability to modulate the activity of multiple targets involved in carcinogenesis through simultaneous direct interaction or modulation of gene expression. In particular, polyphenols are able to reduce and prevent the cross-talk between ErbB receptors, NF-κB and the Hedgehog (HH)/glioma-associated oncogene (GLI) pathways representing three of the main signal transduction pathways for neoplastic transformation [114].

Phenolic compounds are able to modulate the pathways of mitogen-activated protein kinases (MAPKs). These specific transcription factors play a central role in cell growth, proliferation, death and differentiation by modulating gene transcription in response to changes in the cellular environment. MAPKs regulate the transcription and traslocation of inflammatory mediators and represent potential targets for new anti-inflammatory molecules. Kaempferol, chrysin, apigenin and luteolin inhibit the activity of the three mitogen activated protein kinases, ERK, JNK and p38, blocking TNF-α stimulated ICAM-1 expression in respiratory epithelial cells [115]. Even quercitin inhibits a wide range of pro-inflammatory genes through the regulation of the MAPK pathway. In particular, this compound inhibits ERK, JNK and their phosphorylated forms, suppressing the transcription and the production of TNF-α in human monocytes [116].

In this context, quercitin and other dietary polyphenols typical in the MD, are able to reduce inflammation through a series of different but interconnected mechanisms, and may represent very attractive anti-inflammatory agents and safe non-pharmacological tools for the prevention and treatment of cancer (Figure 3). Moreover, polyphenols exerted their anti-cancer and chemopreventive action through the regulation of mTOR (mammalian target of rapamycin) and the sirtuins pathways by mechanisms that mimic caloric restriction [117]. In particular, quercitin is able to inhibit mTOR activity by multiple pathways. The signalling pathway of mTOR stimulates cell growth and proliferation inducing protein synthesis and inhibiting autophagy in case of food wealth. When essential cellular functions are endangered by insufficient nutrient supply, as in the case of caloric restriction, mTOR activity is blocked and cytosolic compounds are recruited for degradation and recycled by autophagy. On the contrary, the mTOR complex is often hyperactivated in cancer [118] and therefore is considered to be an interesting and attractive therapeutic target for anti-cancer therapy. Sirtuins role in cancer development is very complex and contradictory since different members of the sirtuin family are implicated in various cancer types. Several studies corroborate the possibility of the inhibitory effect of sirtuins on inflammation [119,120] by influencing mainly the NF-κB pathway [121,122] or TNF-α and IL-6 expression (SIRT6) [123,124]. A series of polyphenols have been shown to induce SIRTt1, defined as a guardian against oxidative stress and DNA damage [117], acting as tumor suppressor and attenuating cellular proliferation, but also speeding up tumorigenesis activating oncoproteins. Such dual functions of SIRT1 may be determined, at least in part, by its subcellular localization [125,126]. Data from mice indicated that a diet rich in olive oil polyphenols reduced oxidative stress, inducing NRF2 and the expression of its target genes coding for antioxidant enzymes, and increasing SIRT1 gene expression [127]. Systematic molecular analysis of olive oil phenolic extracts identified secoiridoids as

a family of compounds with a strong anti-cancer activity related to the activation of anti-aging/cellular stress-like gene signatures, including endoplasmic reticulum (ER) stress and the unfolded protein response as well as SIRT1 and NRF2 signaling [128]. Several studies demonstrated that resveratrol is able to induce Sirt1 and clinical investigations indicated a role of this compound in the modulation of enzyme systems involved in carcinogen activation and detoxification, suggesting a possible mechanism by which resveratrol inhibits carcinogenesis. Unfortunately, phase II studies have failed to confirm the safety and efficacy of resveratrol in patients with relapsed/refractory multiple myeloma [129].

While many epidemiological studies have associated the consumption of polyphenols with a decreased risk of developing several diseases such as cancer, intervention studies have not always confirmed these effects. This discrepancy may in part depend on potential differences in doses, interactions with the food matrix, and differences in polyphenol bioavailability that limit their overall biological effectiveness. In addition to endogenous factors such as microbiota and digestive enzymes, the food matrix considerably affects bioaccessibility, uptake, and further metabolism of polyphenols. In particular, dietary fiber (such as hemicellulose), divalent minerals, and viscous and protein-rich meals are likely to cause detrimental effects on polyphenol bioaccessibility. In addition, certain food preparation techniques may alter nutrient composition and structure reducing polyphenol bioavailability [130].

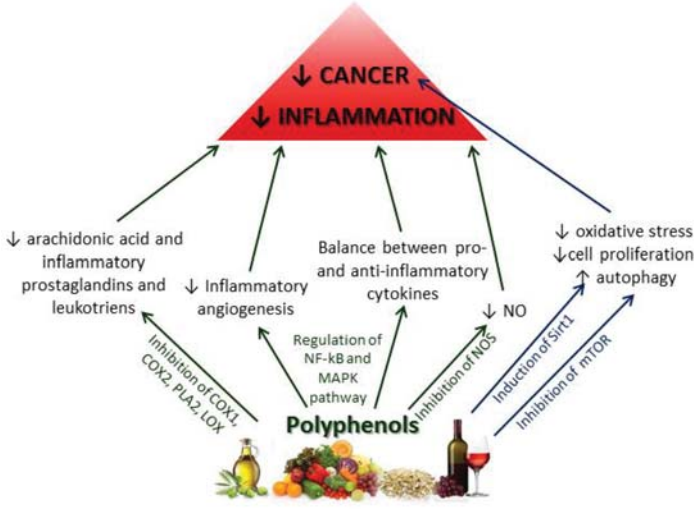

Figure 3. Polyphenols control and reduce inflammation through a series of pathways preventing cancer and other age-related diseases with an inflammatory pathogenesis. Moreover, resveratrol, quercitin and other polyphenols exerted their anti-cancer and chemopreventive action through mechanism that mimic caloric restriction (sirtuin and mTOR pathways).

2.2.3. Other Mediterranean Diet Components and Their Effects on Inflammation and Cancer

Several epidemiological studies have linked the increased consumption of lycopene, the red and lipophilic carotenoid representing the most relevant functional component of tomatoes, with decreased prostate cancer risk. *In vitro*, several experiments showed that lycopene enhances the antioxidant response of prostate cells, inhibits proliferation, induces apoptosis and decreases the metastatic capacity of prostate cancer cells. However, the *in vivo* effectiveness of lycopene as a chemoprotective has still to be proven [131]. Several clinical trials have shown that carotenoids, vitamin A, and vitamin E based antioxidant supplements do not possess preventive effects on cancer and may be harmful and increase

mortality especially in well-nourished populations. Therefore, the optimal source of antioxidants seems to come from diet, not from antioxidant supplements in pills or tablets [132].

The high presence of fiber typical of the MD is probably at the basis of the reduced incidence of colorectal cancer. Dietary fibers possess a proven anti-inflammatory action decreasing systemic inflammation-associated biomarkers such as CRP, IL-6, and TNF-α [133–135], as well as inhibiting COX-2 and iNOS activities and gene expression [136].

Fermentable dietary fibers shift the gut microbial populations by providing substrates for bacterial fermentation. In particular, fructooligosaccharides and galactooligosaccharides increase the fecal populations of *Bifidobacteria* and *Lactobacillus* [137] and these changes in gut flora population can result in a modulation of inflammatory processes [138]. Moreover, the consumption of fiber-rich foods avoids glycemic "spikes" and rapid insulin release that may adversely regulate multiple mechanisms, including (acutely and/or chronically) oxidative stress, inflammation, low-density lipoprotein oxidation, protein glycation, and blood coagulation [74]. Finally, dietary fibers favour an enlargement of the bulk of stool fasting intestinal transit and reducing the contact of potentially carcinogenic and toxic coumpounds with gastrointestinal epithelium [139].

Data from PREDIMED and other studies indicated an association between walnut consumption and reduced risk of cancer and mortality particularly in the context of the MD. Numerous components of walnuts, including α-linolenic acid (ALA), ellagitannins, γ-tocopherol, melatonin, β-sisterol and fiber may counter inflammation-related cancer mechanisms [140]. In particular, one-year of the MD supplemented with either extra virgin olive oil or mixed nuts (walnuts, almonds, and hazelnuts) *versus* a low fat diet decreased intercellular adhesion molecule-1 (ICAM-1), IL-6, TNFR60, and TNFR80 levels in adults [141].

The frequent consumption of marine fish typical in the MD provides a high quantity of ω-3 polyunsatured fatty acids. A high ω-3 to ω-6 fatty acids ratio has been associated with a reduced risk of cancer, especially breast cancer, and with improved prognosis [78,142]. ω-3 Fatty acids exert anti-angiogenic effects and have anti-inflammatory and immunosuppressive properties reducing inflammation through different mechanisms. In particular, EPA (eicosapentaenoic) and DHA (docosahexanoic) ω-3 fatty acids partially replace arachidonic acid as eicosanoid substrate in all cell membranes but especially in erythrocytes, neutrophils, monocytes and liver cells, thus suppressing the production of ω-6 pro-inflammatory eicosanoids. In addition, EPA and DHA suppress the NF-κB pathway and modulate plasma membrane micro-organization (lipid rafts), in particular relatively to the function of Toll-like receptors (TLRs), and T-lymphocyte signaling molecule recruitment to the immunological synapse [143].

The traditional MD is characterized by a low consumption of red and processed meat, which is often associated with an increased risk of colorectal cancer. Processed meat intake is linked to cancer risk through different mechanisms including the production of carcinogenic heterocyclic amines, polycyclic aromatic hydrocarbons and N-nitroso compounds as well as the high content of saturated fatty acids that enhances the prostaglandin system feeding the arachidonic acid and PGE_2 pro-inflammatory pathways [144].

In contrast to the MD, the Western diet, characterized by a low intake of nutrient-rich food (fruit, vegetables, whole grain cereals, legumes and fish) and by an over consumption of salt, refined sugars, saturated fatty acids and a low ω-6:ω-3 fatty acids ratio, damages the immune system leading to an increased level of inflammation and increased onset of cancer [145].

2.3. The Mediterranean Diet Epigenetic Regulation: the Role of microRNAs

MiRs are small non-coding RNAs involved in the post-transcriptional regulation of gene expression and are recently recognized as diagnostic and prognostic biomarkers for many age-related diseases and aging [59,146].

MiRs play a critical role in basic biological processes such as cellular differentiation, apoptosis, cell proliferation, metabolism, inflammation, stem cells development, immune modulation and

carcinogenesis [147,148]. It has also been shown that miR expression is tissue specific, altered with age, and can define the physiological context of the cell, including disease [149]. Alterations in the expression of specific miRs have also been reported to play a role in oxidative stress-induced inflammation [20,51–54,150]. Recently, our team identified three miRs we named "inflamma-miRs": miR-21, -126 and -146a, which target mRNAs belonging to the NF-κB pathway [151].

Several patterns of expression of miRs were exclusive of certain tumors and reflect the differentiation state of tumor development [152]. Especially in the last 10 years, many studies have shown that dysregulation of miR expression underlies many human cancers, both as oncogenes (oncomirs) or tumor suppressors [153–155]. Therefore, the possibility of using miRs to block accumulation of senescent cells to inhibit the establishment of a microenvironment favoring cancer development and progression could be a potential new approach to cancer prevention.

In this regard, it is extremely important to know which type of miRs can be modulated by nutrients typical of MD, and to evaluate the possible role of nutrient-induced miRs as chemoprevention therapy.

MiRs are also present in all the biological fluids of our body. The possibility to monitor the changes of metabolic miR profiling in the blood stream after prolonged diet intervention in humans could be a relevant achievement. Currently, this topic is in its infancy; in cancer, the study of the association between miRs expression and diet has been carried out mainly using tumor cell lines or animal models. The absorption and metabolism of nutrients at the molecular level have been studied with high-throughput "omics" technologies. The results obtained led to the recognition of certain nutrients able to regulate gene expression, at the base of nutrigenomics [156]. It is expected that miRs expression may also change in response to certain dietary bioactive agents, such as PUFAs, vitamins and phytochemicals and some important data are reviewed as follows:

2.3.1. Fatty Acids

The development of tumors such as colon cancer [157–160], breast cancer [161], and glioblastoma [162] is inversely related to the intake of ω-3 PUFA. In contrast, diets rich in ω-6 PUFA (linoleic acid, arachidonic acid and LA, AA) favor both the initiation and promotion of colon cancer [163,164]. In mice, Davidson and colleagues studied the effect of a diet based on corn oil-cellulose compared with a diet based on fish oil (EPA and DHA) and pectin in the presence of carcinogens: their results demonstrated an increased expression of miR-16, miR-19b, miR-21, miR-26b, miR-27b, miR -93, 200c, and miR-203 and the decreased expression of some of their direct targets, such as, PTK2B, TCF4, PDE4B, HDAC4, and IGF1 [158], thus suggesting some different molecular mechanisms involving the fish oil diet. Vinciguerra *et al.* observed that unsaturated fatty acids inhibit PTEN expression in human hepatocytes by up-regulating miR-21 synthesis via mTOR/NF-κB-dependent signaling, exemplifying a regulatory mechanism by which fatty acids affect PTEN expression and trigger liver disorders [165].

Butyrate has chemoprotective properties acting as an inhibitor of histone deacetylase, decreasing proliferation and increasing apoptosis in tumor cells of the colon-rectum [166–169]. Human colon cancer cells (HCT116) treated with butyrate reduce the expression of different miRs belonging to the miR-17-92-18b, miR-106a and miR-106b-25 clusters [170] mediated by p21, which is a direct target of miR-106b. Experiments in rats showed that butyrate, generated by fermentable fiber and fish oil (EPA and DHA) have a synergistic protective action towards colon tumorigenesis. The increased expression of miR-19b, -26b, -27b, -200c, -203 and the concomitant decrease of their targets expression mediate the tumor suppression mechanisms [171].

2.3.2. Vitamins

All-trans-retinoic acid, the most biologically active metabolite of vitamin A, acts as a tumor suppressor factor in lung, liver, bladder, prostate, breast, and pancreatic cancer models [172]. In breast cancer cells (MCF-7), exposure to retinoic acid inhibited cell proliferation by inducing miR-21 [173].

Recent studies show that vitamin D may exert its protective effects by modulating the expression of miRs and their targets. Vitamin D3 down-regulated miR-181a and miR-181b in human myeloid leukemia cells, resulting in an up-regulation of p27Kip1 and p21Cip1 and cell cycle arrest [174].

2.3.3. Phytochemicals

As described in previous paragraphs, many studies showed that the consumption of foods rich in polyphenols was associated with the prevention of chronic diseases [175–178]. In particular, quercetin, hesperidin, narangin, anthocyanins, catechins, proanthocyanin, caffeic acid, ferulic acid, and curcumin, act through a common mechanism envisaging the modulation of five miRs, *i.e.*, miR-30c, miR-291b-5p, miR-296-5p, miR -373, and miR-467b [179].

The treatment of human pancreatic cancer cells with curcumin led to a significant up-regulation of eleven miRs and down-regulation of eighteen miRs [180]. Among all, miR-22 was the most significantly up-regulated and was associated with the suppression of Sp1 and estrogen receptor 1, while miR-199a* was the most significantly down-regulated miR. Curcumin and its synthetic analogue, curcumin diflourinated (CDF), alone or in combination, down-regulate miR-200 and miR-21 expression, inducing the up-regulation of its target PTEN in pancreatic cancer cells [181].

Hereafter, changes in blood levels of miRs after the consumption of specific nutrients could be used as biomarkers to monitor the metabolic effects of dietary intervention over time and thus identify dietary interventions that may protect our body from the development of cancer.

3. Gut Microbiota, Inflammation and Cancer

Inflammaging and immunosenescence are the main culprits of the changes in microbiota composition in older people [182,183]. It is well known that the human digestive tract is colonized by over 100 trillions of bacteria, which constitute the so-called "gut microbiota" (GM) [184]. These microorganisms, responsible for the degradation of certain complex substances ingested by diet, allowing their digestion and the absorption of certain micronutrients, contribute substantially to our metabolism, and are also essential for the normal development of the immune system. Longitudinal studies have shown that the intestinal microbiota is extremely malleable and could be altered in response to changes in the environment, geography, genetics, metabolism, age, antibiotic treatments, stress, and diet. This plasticity allows the human body to optimize performance while preserving metabolic and immune homestasis as well as health [185].

The impact of the habitual diet on the GM of the elderly [186,187] has been recently highlighted in a study demonstrating the correlation of diet with the inflammatory status, residence and a different rate of health decline upon aging [188]. The balance of carbohydrates, proteins and fats has a profound influence on the maintenance of GM homeostasis [189–191]. The adoption of a specific diet, of animal origin, rather than a predominantly vegetable one, determines a different composition of the GM at the expense of interindividual differences in microbial gene expression [192]. Moreover, a diet rich in animal fats induces the development of inflammation and intestinal diseases by modification of the GM.

Our group has shown that centenarians have a different composition of the GM in respect to young subjects and that this is associated with an increase of the "inflammatory state" represented by high levels of pro-inflammatory cytokines (IL-6 and IL-8) [193].

The dysbiosis condition of GM in the elderly can trigger the development of carcinogenic processes in the intestinal mucosa. The number of cases of colorectal cancer (CRC), in fact, increases in the elderly population. The greatest number of CRC occurs in the elderly, with nearly 70% of cases diagnosed in those older than age 65 and 40% diagnosed in those over 75 years of age [194].

It has been demonstrated that mice with a compromised GM homeostasis are prone to developing an inflammatory state in the intestinal mucosa, which, in turn, predisposes them to cancer development [195].

The next generation sequencing techniques allowed, with extreme accuracy, the identification of the composition of GM associated with CRC. The feces of CRC patients compared with those of healthy subjects are particularly rich in pro-inflammatory opportunistic pathogens and microorganisms responsible for metabolic disorders. The "good" GM, with protective function on the mucous membrane, is rather very poor. It is therefore possible to assume that there are "families" of microorganisms able to perform a pro-carcinogenic function (*Fusobacterium*, *Prevotella*, *Coprobacillus*), as well as other families able to perform a protective function of the intestinal mucosa such as *Bifidobacterium* and *Faecalibacterium* [196].

The mechanisms by which microorganisms induce the development of the CRC are varied and include the development of a chronic inflammatory process, the production of toxic metabolites, the development of genotoxins that acts directly on the cell cycle, on the development of DNA and the activation of food heterocyclic amine which are pro-carcinogenic compounds [197].

Among the various metabolites produced by the intestinal microbiota, pro-carcinogenic molecules and molecules with important protective functions have been identified. Secondary bile acids, ammonia, certain amines, phenols and hydrogen sulfide are toxic metabolites, while butyrate plays an anti-proliferative action, energizes the intestinal cells and has shown an apoptotic action against CRC cells *in vitro* [198].

Thus, due to the strong influence of nutrition on GM composition and metabolism [187], the adoption of an anti-inflammatory dietary pattern such as the Mediterranean Diet will contribute to the maintenance of a "good" GM with healthy outcomes.

4. A Systems Biology Approach to Diet, Inflammation and Cancer

Systems biology aims to understand how a biological system as a whole responds to internal and external stimuli. Metabolomics [199], instead, aims to measure the global dynamic metabolic response of a living system to biological stimuli or genetic manipulation as a whole, and represents the cutting-edge methodology to fully understand the system-wide effects that diet has on any living organism. The latter approach neatly superimposes to the first: the final result is in both cases a "top-down" view of all the biochemistry processes involved in a complex organism, even if at distinct levels.

Important efforts were made to allow researchers to readily process metabolomics data. The Human Metabolome Project [200] seeks to reproduce what the Human Genome Project did in the genome field, hosting a rapidly growing database of thousands of human metabolites, along with their spectroscopic data. Similarly, the LIPID Metabolites and Pathway Strategy [201] is progressively characterizing human lipids. Metabolomics represents a first-class tool to understand diseases where other approaches are falling short [202]. Metabolome-wide association (MWA) studies, deeply intertwining genome-derived concepts to the "metabolome" world, will in the near future represent a standard approach [203–206]. A recent study by Watson *et al.* [207] successfully identified metabolites that affect *Caenorhabditis elegans* gene expression and physiology, exploiting an interspecies systems biology approach. Influence of a herring-based diet on sterol metabolism and protein turnover in mice was recently identified, with clear implications also on disease development [208].

The common thread connecting cancer, inflammation and diet is now the focus of metabolomics studies, where the goal is to identify individual metabolites representing end-points of perturbed molecular pathways. These altered molecular pathways may then be further investigated in depth, exploiting other "-omics" techniques [209–211]. In a work by Sreekumar *et al.* (2009), the authors were able to successfully identify 87 metabolites distinguishing prostate cancer from benign prostate tissue [212]. More recently, de Boer *et al.* designed a protocol to easily screen the population to detect CRC [213] (or its precursor, advanced adenoma) relying only on volatile organic compound (VOC) analysis. This finding, if confirmed, will pave the way to new, effective and cost-saving methods to perform preventative, large-scale screening, of the population.

To strengthen the proofs in favour of this inter-systems link, focused cohort-based studies are needed. The European Prospective Investigation into Cancer and Nutrition (EPIC) did exactly that: starting from a cohort of 2380 subjects they analysed a panel of 127 serum metabolites [214], looking for correlations among metabolite networks and different conditions, *i.e.*, physical activity energy expenditure, obesity, and waist circumference, shedding light on the possible adoption of some of the metabolites under analysis as markers to evaluate subjects health. Additionally, an independent cohort study investigating the effect of a diet based on healthy Nordic foods [208], found a lower incidence of colorectal cancer in women following such a diet. The same study also suggests that the Nordic population could better improve their health following diets centered on Nordic food, breaking the dogma that a "Mediterranean Diet" represents the best possible nutritional intervention in all cases, independently from anthropological considerations.

In our bird's eye voyage through the three different worlds of diet, inflammation and cancer, we showed how these worlds are indeed deeply intertwined. The modulatory effect that unsaturated fatty acids has on the immune systems, the inflammatory response of which can then fuel the micro-environment propelling tumour progression, is just one of the examples of these extensive and branched connections. Any effort to understand the functioning of one of these phenomena must take into consideration all of them, or faces a highly probable failure. In some of the just cited articles, authors fruitfully applied systems biology approaches to unravel the mechanistic reasons underlining the effects they observed. Most of these efforts led to results indicating important advances in the field. In the future, widespread adoption of these analysis techniques will permit breakthrough discoveries that may eventually have a deep impact on public health.

5. The Mediterranean Diet and Healthy Aging: the European Project NU-AGE Targeted on Inflammaging to Prevent Age-Related Diseases as a Whole

EU member states are experiencing an extraordinary increase in the life expectancy, which is predicted to reach 84.6 years for women and 82.5 years for men in 2060 [215]. As a direct consequence of the improvement of socio-economic-environmental conditions, the increasing longevity of European citizens will also imply a wide range of societal responsibilities, such as the increased incidence of age-related diseases and the resulting impact on health care cost. Consequently, there is an urgent need to provide policies for preventing aging and related disorders, such as cancer and neurodegenerative disorders among others, allowing the maintenance of reasonably good health as long as possible.

One of the most fascinating anti-aging strategies seems to be the possibility of reducing inflammaging without compromising the physiological role of inflammation, which is essential for survival [5]. At present, nutrition represents the most powerful and flexible tool that we have to reach a chronic and systemic modulation of the aging process in order to improve the health status of the elderly population.

Several studies analized the effect of an individual food or nutrient on a particular form of cancer. However, the overall dietary pattern is more than the sum of the single foods or nutrients eaten and the effect of a dietary intervention on any health outcome is strongly influenced by genetic and environmental factors. Studies regarding the role of the MD on cancer prevention are often conducted analyzing the association between MD adherence and the onset of a specific neoplasia. Studies considering in a comprehensive and integrated way the effect of a balanced whole MD, followed for a consistent period of time, on the chronic and systemic inflammation typical of aging are still scant in the scientific literature.

To rectify this gap in knowledge, the European Project NU-AGE (ClinicalTrials.gov Identifier, NCT01754012) [216,217] will study in a comprehensive and integrated way, the effect of a whole MD newly designed according to the nutritional needs of people over 65 years of age called "NU-AGE diet" [218] on the health status of elderly people in the EU.

The specific rationale of NU-AGE is to enroll in the study free-living, apparently healthy, elderly people including pre-frail subjects (a large segment of the elderly population having the

potential to benefit from diet change). All the volunteers will be characterized before and after dietary intervention by measuring a number of robust parameters capable of providing reliable data about different domains/subsystems (health and nutritional status, physical and cognitive functions, immunological, biochemical and metabolic parameters). A sub-group of subjects will be further characterized by advanced techniques (genetics, epigenetics) and highthroughput "omics" (transcriptomics, metagenomics, pyrosequencing, HITChip array) in order to identify cellular and molecular targets and mechanisms responsible for the effects of the whole diet intervention.

The massive amount of data collected from the NU-AGE nutritional intervention will be stored in an ad-hoc built database that, based on an integrated statistical analysis and a system biology approach, will allow the identification of nutritional risk factors in the elderly associated with inflammaging, and to identify the pathways and networks responsive to the NU-AGE diet [219].

This approach will allow an evaluation of the whole-organism response considering several tissues and organs/systems as a functional network instead of assessing the single tissue and organ responses separately, as in previously funded projects, which thereby missed the fundamental cross-talk between tissues and organs/systems. For a detailed description of the entire project it is suggested to read the recently published NU-AGE project dedicated special issue [220].

6. Conclusions

Chronic inflammation plays a pivotal role in each stage of carcinogenesis from the initial genetic or epigenetic changes to tumour progression and metastatic diffusion [23,25]. Thus, the chronic, low-level inflammatory state typical in the elderly, that we have named "inflammaging", likely represents one of the links between aging and cancer.

Inflammaging is both local and systemic, and a variety of organs and systems provide inflammatory stimuli that accumulate lifelong [3]. The key mediators of the inflammatory response activate, in turn, the NF-κB pathway that can be considered the link among cellular senescence, inflammaging and cancer.

A suitable intervention to combat inflammation is a modification of dietary habits. Actually, several studies reported that a healthy lifestyle and a balanced diet might provide benefits to health not only by preventing the risk of diseases but also through facilitating recovery and improving survival. The MD represents one of the best examples of a healthy diet, considered a heritage of humanity by UNESCO, and is considered pivotal in many public health programs in Europe. The acceptance of a Mediterranean lifestyle indeed has a beneficial and preventive role in the onset of cancer and other diseases associated with increased level of inflammation, oxidative damage and angiogenesis.

However, even if the outcomes of the MD on health are well known, knowledge of the reasons of these outcomes is still rather poor. While recent nutrition research focused on the effects of specific dietary constituents, it is still unclear which are the molecular and cellular pathways triggered by the MD as a whole. To this aim, the NU-AGE project is studying in depth the effects of a one-year whole MD in a representative sample of four geographic and cultural areas of Europe (Northern, Eastern, Central and Southern) by measuring an unprecedented number of parameters, including those obtained from "omic" high-throughput analyses, and considering them in a systems biology perspective in order to get a comprehensive view of this dietary intervention on inflammation in old age. This strategy could also hopefully provide insight on diet as cancer prevention; NU-AGE and its study design were noted, in a special issue of the journal *Nature*, devoted to aging studies, as "the kind of large, longitudinal study that scientists the world over are clamouring for" [221].

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wrote on systems biology; C.B., C.Fa., F.B., M.M.: reviewed the literature with particular attention to inflammation and cancer; EB: reviewed the literature on aging and inflammaging; S.S. and M.C.: critically revised the whole manuscript; C.Fr. and A.S.: designed the structure of the paper and wrote on aging and inflammaging.

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References

1. López-Otín, C.; Blasco, M.A.; Partridge, L.; Serrano, M.; Kroemer, G. The hallmarks of aging. *Cell* **2013**, *153*, 1194–1217. [CrossRef] [PubMed]
2. Franceschi, C.; Bonafè, M.; Valensin, S.; Olivieri, F.; de Luca, M.; Ottaviani, E.; de Benedictis, G. Inflamm-aging. An evolutionary perspective on immunosenescence. *Ann. N.Y. Acad. Sci.* **2000**, *908*, 244–254. [CrossRef] [PubMed]
3. Franceschi, C.; Campisi, J. Chronic inflammation (inflammaging) and its potential contribution to age-associated diseases. *J. Gerontol. A Biol. Sci. Med. Sci.* **2014**, *69*, 4–9. [CrossRef]
4. Kennedy, B.K.; Berger, S.L.; Brunet, A.; Campisi, J.; Cuervo, A.M.; Epel, E.S.; Franceschi, C.; Lithgow, G.J.; Morimoto, R.I.; Pessin, J.E.; *et al.* Geroscience: Linking aging to chronic disease. *Cell* **2014**, *159*, 709–713. [CrossRef]
5. Franceschi, C. Inflammaging as a major characteristic of old people: Can it be prevented or cured? *Nutr. Rev.* **2007**, *65*, 173–176. [CrossRef] [PubMed]
6. Franceschi, C.; Capri, M.; Monti, D.; Giunta, S.; Olivieri, F.; Sevini, F.; Panourgia, M.P.; Invidia, L.; Celani, L.; Scurti, M.; *et al.* Inflammaging and anti-inflammaging: A systemic perspective on aging and longevity emerged from studies in humans. *Mech. Ageing. Dev.* **2007**, *128*, 92–105. [CrossRef] [PubMed]
7. Cevenini, E.; Monti, D.; Franceschi, C. Inflammaging. *Curr. Opin. Clin. Nutr. Metab. Care* **2013**, *16*, 14–20. [CrossRef] [PubMed]
8. Reedy, J. Galen on cancer and related diseases. *Clio Med.* **1975**, *10*, 227–238. [PubMed]
9. Trinchieri, G. Cancer and inflammation: An old intuition with rapidly evolving new concepts. *Annu. Rev. Immunol.* **2012**, *30*, 677–706. [CrossRef] [PubMed]
10. Virchow, R. *Cellular Pathology as Based upon Physiological and Pathological Histology*; J.B. Lippincott: Philadelphia, PA, USA, 1863.
11. Balkwill, F.; Mantovani, A. Inflammation and cancer: Back to Virchow? *Lancet* **2001**, *357*, 539–545. [CrossRef] [PubMed]
12. Hanahan, D.; Weinberg, R.A. The Hallmarks of Cancer. *Cell* **2000**, *100*, 57–70. [CrossRef] [PubMed]
13. Coussens, L.M.; Werb, Z. Inflammation and cancer. *Nature* **2002**, *420*, 860–867. [CrossRef]
14. Luo, J.L.; Tan, W.; Ricono, J.M.; Korchynskyi, O.; Zhang, M.; Gonias, S.L.; Cheresh, D.A.; Karin, M. Nuclear cytokine-activated IKKalpha controls prostate cancer metastasis by repressing Maspin. *Nature* **2007**, *446*, 690–694. [CrossRef] [PubMed]
15. Mantovani, A.; Allavena, P.; Sica, A.; Balkwill, F. Cancer-related inflammation. *Nature* **2008**, *454*, 436–444. [CrossRef] [PubMed]
16. Hanahan, D.; Weinberg, R.A. Hallmarks of cancer: The next generation. *Cell* **2011**, *144*, 646–674. [CrossRef] [PubMed]
17. Karin, M. Nuclear factor-κB in cancer development and progression. *Nature* **2006**, *441*, 431–436. [CrossRef] [PubMed]
18. Chia, W.K.; Ali, R.; Toh, H.C. Aspirin as adjuvant therapy for colorectal cancer—Reinterpreting paradigms. *Nat. Rev. Clin. Oncol.* **2012**, *9*, 561–570. [CrossRef] [PubMed]
19. Giraldo, N.A.; Becht, E.; Remark, R.; Damotte, D.; Sautès-Fridman, C.; Fridman, W.H. The immune contexture of primary and metastatic human tumours. *Curr. Opin. Immunol.* **2014**, *27*, 8–15. [CrossRef]
20. Hussain, S.P.; Hofseth, L.J.; Harris, C.C. Radical causes of cancer. *Nat. Rev. Cancer* **2003**, *3*, 276–285. [CrossRef] [PubMed]
21. Colotta, F.; Allavena, P.; Sica, A.; Garlanda, C.; Mantovani, A. Cancer-related inflammation, the seventh hallmark of cancer: Links to genetic instability. *Carcinogenesis* **2009**, *30*, 1073–1081. [CrossRef] [PubMed]
22. Grivennikov, S.I.; Greten, F.R.; Karin, M. Immunity, inflammation, and cancer. *Cell* **2010**, *140*, 883–899. [CrossRef] [PubMed]

23. Aggarwal, B.B.; Gehlot, P. Inflammation and cancer: How friendly is the relationship for cancer patients? *Curr. Opin. Pharmacol.* **2009**, *9*, 351–369. [CrossRef] [PubMed]
24. Multhoff, G.; Molls, M.; Radons, J. Chronic inflammation in cancer development. *Front. Immunol.* **2012**, *2*, 98. [CrossRef] [PubMed]
25. De Lerma Barbaro, A.; Perletti, G.; Bonapace, I.M.; Monti, E. Inflammatory cues acting on the adult intestinal stem cells and the early onset of cancer (review). *Int. J. Oncol.* **2014**, *45*, 959–968. [PubMed]
26. Salvioli, S.; Monti, D.; Lanzarini, C.; Conte, M.; Pirazzini, C.; Bacalini, M.G.; Garagnani, P.; Giuliani, C.; Fontanesi, E.; Ostan, R.; *et al.* Immune system, cell senescence, aging and longevity—inflamm-aging reappraised. *Curr. Pharm. Des.* **2013**, *19*, 1675–1679. [PubMed]
27. Bonafè, M.; Storci, G.; Franceschi, C. Inflamm-aging of the stem cell niche: Breast cancer as a paradigmatic example: Breakdown of the multi-shell cytokine network fuels cancer in aged people. *Bioessays* **2012**, *34*, 40–49. [CrossRef] [PubMed]
28. Diakos, C.I.; Charles, K.A.; McMillan, D.C.; Clarke, S.J. Cancer-related inflammation and treatment effectiveness. *Lancet Oncol.* **2014**, *15*, 493–503. [CrossRef]
29. Cevenini, E.; Caruso, C.; Candore, G.; Capri, M.; Nuzzo, D.; Duro, G.; Rizzo, C.; Colonna-Romano, G.; Lio, D.; di Carlo, D.; *et al.* Age-related inflammation: The contribution of different organs, tissues and systems. How to face it for therapeutic approaches. *Curr. Pharm. Des.* **2010**, *16*, 609–618. [CrossRef] [PubMed]
30. Ostan, R.; Bucci, L.; Capri, M.; Salvioli, S.; Scurti, M.; Pini, E.; Monti, D.; Franceschi, C. Immunosenescence and immunogenetics of human longevity. *Neuroimmunomodulation* **2008**, *15*, 224–240. [CrossRef] [PubMed]
31. Grignolio, A.; Franceschi, C. *History of Research into Aging/Senescence*; John Wiley & Sons: Chichester, UK, 2012.
32. Wellen, K.E.; Fucho, R.; Gregor, M.F.; Furuhashi, M.; Morgan, C.; Lindstad, T.; Vaillancourt, E.; Gorgun, C.Z.; Saatcioglu, F.; Hotamisligil, G.S. Coordinated regulation of nutrient and inflammatory responses by STAMP2 is essential for metabolic homeostasis. *Cell* **2007**, *129*, 537–548. [CrossRef] [PubMed]
33. Erridge, C.; Attina, T.; Spickett, C.M.; Webb, D.J. A high-fat meal induces low-grade endotoxemia: Evidence of a novel mechanism of postprandial inflammation. *Am. J. Clin. Nutr.* **2007**, *86*, 1286–1292. [PubMed]
34. Cani, P.D.; Amar, J.; Iglesias, M.A.; Poggi, M.; Knauf, C.; Bastelica, D.; Neyrinck, A.M.; Fava, F.; Tuohy, K.M.; Chabo, C.; *et al.* Metabolic endotoxemia initiates obesity and insulin resistance. *Diabetes* **2007**, *56*, 1761–1772. [CrossRef] [PubMed]
35. Gregor, M.F.; Hotamisligil, G.S. Inflammatory mechanisms in obesity. *Annu. Rev. Immunol.* **2011**, *29*, 415–445. [CrossRef] [PubMed]
36. De Martel, C.; Franceschi, S. Infections and cancer: Established associations and new hypotheses. *Crit. Rev. Oncol. Hematol.* **2009**, *70*, 183–194. [CrossRef] [PubMed]
37. De Visser, K.E.; Eichten, A.; Coussens, L.M. Paradoxical roles of the immune system during cancer development. *Nat. Rev. Cancer* **2006**, *6*, 24–37. [CrossRef] [PubMed]
38. Lu, H.; Ouyang, W.; Huang, C. Inflammation, a key event in cancer development. *Mol. Cancer Res.* **2006**, *4*, 221–233. [CrossRef] [PubMed]
39. Macarthur, M.; Hold, G.L.; El-Omar, E.M. Inflammation and Cancer II. Role of chronic inflammation and cytokine polymorphisms in the pathogenesis of gastrointestinal malignancy. *Am. J. Physiol. Gastrointest. Liver Physiol.* **2004**, *286*, G515–G520. [CrossRef] [PubMed]
40. Whitcomb, D.C. Inflammation and Cancer V. chronic pancreatitis and pancreatic cancer. *Am. J. Physiol. Gastrointest. Liver Physiol.* **2004**, *287*, 315–319. [CrossRef]
41. Baan, R.; Grosse, Y.; Straif, K.; Secretan, B.; El Ghissassi, F.; Bouvard, V. A review of human carcinogens Part F. Chemical agents and related occupations. *Lancet Oncol.* **2009**, *10*, 1143–1144. [CrossRef] [PubMed]
42. Houghton, A.M. Mechanistic links between COPD and lung cancer. *Nat. Rev. Cancer* **2013**, *13*, 233–245. [CrossRef] [PubMed]
43. Askling, J.; Grunewald, J.; Eklund, A.; Hillerdal, G.; Ekbom, A. Increased risk for cancer following sarcoidosis. *Am. J. Respir. Crit. Care Med.* **1999**, *160*, 1668–1672. [CrossRef] [PubMed]
44. Calder, P.C.; Ahluwalia, N.; Brouns, F.; Buetler, T.; Clement, K.; Cunningham, K.; Esposito, K.; Jönsson, L.S.; Kolb, H.; Lansink, M.; *et al.* Dietary factors and low-grade inflammation in relation to overweight and obesity. *Br. J. Nutr.* **2011**, *106*, S5–S78. [CrossRef] [PubMed]
45. Wolk, A.; Gridley, G.; Svensson, M.; Nyrén, O.; McLaughlin, J.K.; Fraumeni, J.F.; Adam, H.O. A prospective study of obesity and cancer risk (Sweden). *Cancer Causes Control* **2001**, *12*, 13–21. [CrossRef] [PubMed]

46. Calle, E.E.; Rodriguez, C.; Walker-Thurmond, K.; Thun, M.J. Overweight, obesity, and mortality from cancer in a prospectively studied cohort of U.S. adults. *N. Engl. J. Med.* **2003**, *348*, 1625–1638. [CrossRef] [PubMed]
47. Renehan, A.G.; Tyson, M.; Egger, M.; Heller, R.F.; Zwahlen, M. Body-mass index and incidence of cancer: A systematic review and meta-analysis of prospective observational studies. *Lancet* **2008**, *371*, 569–578. [CrossRef] [PubMed]
48. Basen-Engquist, K.; Chang, M. Obesity and cancer risk: Recent review and evidence. *Curr. Oncol. Rep.* **2011**, *13*, 71–76. [CrossRef] [PubMed]
49. Karagozian, R.; Derdák, Z.; Baffy, G. Obesity-associated mechanisms of hepatocarcinogenesis. *Metabolism* **2014**, *63*, 607–617. [CrossRef] [PubMed]
50. Vitale, G.; Salvioli, S.; Franceschi, C. Oxidative stress and the ageing endocrine system. *Nat. Rev. Endocrinol.* **2013**, *9*, 228–240. [CrossRef] [PubMed]
51. Reuter, S.; Gupta, S.C.; Chaturvedi, M.M.; Aggarwal, B.B. Oxidative stress, inflammation, and cancer: How are they linked? *Free Radic. Biol. Med.* **2010**, *49*, 1603–1616. [CrossRef] [PubMed]
52. Mantovani, A. Cancer: inflammation by remote control. *Nature* **2005**, *435*, 752–753.
53. Lin, W.W.; Karin, M.A. A cytokine-mediated link between innate immunity, inflammation, and cancer. *J. Clin. Investig.* **2007**, *117*, 1175–1183. [CrossRef] [PubMed]
54. Hoesel, B.; Schmid, J.A. The complexity of NF-κB signaling in inflammation and cancer. *Mol. Cancer* **2013**, *2*, 12–86.
55. Tieri, P.; Termanini, A.; Bellavista, E.; Salvioli, S.; Capri, M.; Franceschi, C. Charting the NF-κB pathway interactome map. *PLoS ONE* **2012**, *7*, e32678. [CrossRef] [PubMed]
56. Aggarwal, B.B.; Sung, B. NF-κB in cancer: A matter of life and death. *Cancer Discov.* **2011**, *1*, 469–471. [CrossRef] [PubMed]
57. Fan, Y.; Mao, R.; Yang, J. NF-κB and STAT3 signaling pathways collaboratively link inflammation to cancer. *Protein Cell* **2013**, *4*, 176–185. [CrossRef] [PubMed]
58. Freund, A.; Orjalo, A.V.; Desprez, P.Y.; Campisi, J. Inflammatory networks during cellular senescence: causes and consequences. *Trends Mol. Med.* **2010**, *16*, 238–246. [CrossRef] [PubMed]
59. Olivieri, F.; Lazzarini, R.; Recchioni, R.; Marcheselli, F.; Rippo, M.R.; di Nuzzo, S.; Albertini, M.C.; Graciotti, L.; Babini, L.; Mariotti, S.; *et al.* MiR-146a as marker of senescence-associated pro-inflammatory status in cells involved in vascular remodelling. *Age (Dordr)* **2012**, *35*, 1157–1172. [CrossRef]
60. Rodier, F.; Campisi, J. Four faces of cellular senescence. *J. Cell Biol.* **2011**, *192*, 547–556. [CrossRef] [PubMed]
61. Martin, O.A.; Redon, C.E.; Dickey, J.S.; Nakamura, A.J.; Bonner, W.M. Para-inflammation mediates systemic DNA damage in response to tumor growth. *Commun. Integr. Biol.* **2011**, *4*, 78–81. [CrossRef] [PubMed]
62. Bellavista, E.; Andreoli, F.; Parenti, M.D.; Martucci, M.; Santoro, A.; Salvioli, S.; Capri, M.; Baruzzi, A.; Del Rio, A.; Franceschi, C.; *et al.* Immunoproteasome in cancer and neuropathologies: a new therapeutic target? *Curr. Pharm. Des.* **2013**, *19*, 702–718. [CrossRef] [PubMed]
63. Bellavista, E.; Martucci, M.; Vasuri, F.; Santoro, A.; Mishto, M.; Kloss, A.; Capizzi, E.; Degiovanni, A.; Lanzarini, C.; Remondini, D.; *et al.* Lifelong maintenance of composition, function and cellular/subcellulardistribution of proteasomes in human liver. *Mech. Ageing Dev.* **2014**, *141–142*, 26–34. [CrossRef] [PubMed]
64. Morimoto, R.I.; Cuervo, A.M. Proteostasis and the aging proteome in health and disease. *J. Gerontol. A Biol. Sci. Med. Sci.* **2014**, *69*, 33–38. [CrossRef]
65. Keys, A.; Arvanis, C.; Blackburn, H. *Seven Countries: A Multivariate Analysis of Death and Coronary Heart Disease*; Harvard University Press: Cambridge, MA, USA, 1980.
66. Estruch, R.; Ros, E.; Salas-Salvadó, J.; Covas, M.I.; Corella, D.; Arós, F.; Gómez-Gracia, E.; Ruiz-Gutiérrez, V.; Fiol, M.; Lapetra, J.; *et al.* PREDIMED Study Investigators. Primary prevention of cardiovascular disease with a Mediterranean diet. *N. Engl. J. Med.* **2013**, *368*, 1279–1290. [CrossRef] [PubMed]
67. Bonaccio, M.; di Castelnuovo, A.; de Curtis, A.; Costanzo, S.; Persichillo, M.; Donati, M.B.; Cerletti, C.; Iacoviello, L.; de Gaetano, G. Moli-sani Project Investigators. Adherence to the Mediterranean diet is associated with lower platelet and leukocyte counts: Results from the Moli-sani study. *Blood* **2014**, *123*, 3037–3044. [CrossRef] [PubMed]
68. Trichopoulou, A.; Orfanos, P.; Norat, T.; Bueno-de-Mesquita, B.; Ocké, M.C.; Peeters, P.H.; van der Schouw, Y.T.; Boeing, H.; Hoffmann, K.; Boffetta, P.; *et al.* Modified Mediterranean diet and survival: EPIC-elderly prospective cohort study. *BMJ* **2005**, *330*, 991. [CrossRef] [PubMed]

69. Mente, A.; de Koning, L.; Shannon, H.S.; Anand, S.S. A systematic review of the evidence supporting a causal link between dietary factors and coronary heart disease. *Arch. Intern. Med.* **2009**, *169*, 659–669. [CrossRef] [PubMed]
70. Trichopoulou, A.; Costacou, T.; Bamia, C.; Trichopoulos, D. Adherence to a Mediterranean diet and survival in a Greek population. *N. Engl. J. Med.* **2003**, *348*, 2599–2608. [CrossRef] [PubMed]
71. Misirli, G.; Benetou, V.; Lagiou, P.; Bamia, C.; Trichopoulos, D.; Trichopoulou, A. Relation of the traditional Mediterranean diet to cerebrovascular disease in a Mediterranean population. *Am. J. Epidemiol.* **2012**, *176*, 1185–1192. [CrossRef] [PubMed]
72. Benetou, V.; Trichopoulou, A.; Orfanos, P.; Naska, A.; Lagiou, P.; Boffetta, P.; Trichopoulos, D.; Greek, E. Conformity to traditional Mediterranean diet and cancer incidence: The Greek EPIC cohort. *Br. J. Cancer* **2008**, *99*, 191–195. [CrossRef] [PubMed]
73. Couto, E.; Boffetta, P.; Lagiou, P.; Ferrari, P.; Buckland, G.; Overvad, K.; Dahm, C.C.; Tjonneland, A.; Olsen, A.; Clavel-Chapelon, F.; *et al.* Mediterranean dietary pattern and cancer risk in the EPIC cohort. *Br. J. Cancer* **2011**, *104*, 1493–1499. [CrossRef] [PubMed]
74. Brand-Miller, J.; Dickinson, S.; Barclay, A.; Celermajer, D. The glycemic index and cardiovascular disease risk. *Curr. Atheroscler. Rep.* **2007**, *9*, 479–485. [CrossRef] [PubMed]
75. Mirrahimi, A.; Chiavaroli, L.; Srichaikul, K.; Augustin, L.S.; Sievenpiper, J.L.; Kendall, C.W.; Jenkins, D.J. The role of glycemic index and glycemic load in cardiovascular disease and its risk factors: A review of the recent literature. *Curr. Atheroscler. Rep.* **2014**, *16*, 381. [CrossRef] [PubMed]
76. Sofi, F.; Macchi, C.; Abbate, R.; Gensini, G.F.; Casini, A. Mediterranean diet and health. *Biofactors* **2013**, *39*, 335–342. [CrossRef] [PubMed]
77. De Vrese, M.; Schrezenmeir, J. Probiotics, prebiotics, and synbiotics. *Adv. Biochem. Eng. Biotechnol.* **2008**, *111*, 1–66.
78. De Lorgeril, M.; Salen, P. New insights into the health effects of dietary saturated and omega-6 and omega-3 polyunsaturated fatty acids. *BMC Med.* **2012**, *10*, 50. [CrossRef] [PubMed]
79. Kalogeropoulos, N.; Chiou, A.; Ioannou, M.S.; Karathanos, V.T. Nutritional evaluation and health promoting activities of nuts and seeds cultivated in Greece. *Int. J. Food Sci. Nutr.* **2013**, *64*, 757–767. [CrossRef] [PubMed]
80. Zamora-Ros, R.; Serafini, M.; Estruch, R.; Lamuela-Raventós, R.M.; Martínez-González, M.A.; Salas-Salvadó, J.; Fiol, M.; Lapetra, J.; Arós, F.; Covas, M.I.; *et al.* PREDIMED Study Investigators. Mediterranean diet and non enzymatic antioxidant capacity in the PREDIMED study: Evidence for a mechanism of antioxidant tuning. *Nutr. Metab. Cardiovasc. Dis.* **2013**, *23*, 1167–1174. [CrossRef] [PubMed]
81. Kolomvotsou, A.I.; Rallidis, L.S.; Mountzouris, K.C.; Lekakis, J.; Koutelidakis, A.; Efstathiou, S.; Nana-Anastasiou, M.; Zampelas, A. Adherence to Mediterranean diet and close dietetic supervision increase total dietary antioxidant intake and plasma antioxidant capacity in subjects with abdominal obesity. *Eur. J. Nutr.* **2013**, *52*, 37–48. [CrossRef] [PubMed]
82. Trichopoulou, A.; Martínez-González, M.A.; Tong, T.Y.; Forouhi, N.G.; Khandelwal, S.; Prabhakaran, D.; Mozaffarian, D.; de Lorgeril, M. Definitions and potential health benefits of the Mediterranean diet: views from experts around the world. *BMC Med.* **2014**, *12*, 112. [CrossRef] [PubMed]
83. Trichopoulou, A.; Kouris-Blazos, A.; Wahlqvist, M.L.; Gnardellis, C.; Lagiou, P.; Polychronopoulos, E.; Vassilakou, T.; Lipworth, L.; Trichopoulos, D. Diet and overall survival in elderly people. *BMJ* **1995**, *311*, 1457–1460. [CrossRef] [PubMed]
84. Bosire, C.; Stampfer, M.J.; Subar, A.F.; Park, Y.; Kirkpatrick, S.I.; Chiuve, S.E.; Hollenbeck, A.R.; Reedy, J. Index-based dietary patterns and the risk of prostate cancer in the NIH-AARP diet and health study. *Am. J. Epidemiol.* **2013**, *177*, 504–513. [CrossRef] [PubMed]
85. Mitrou, P.N.; Kipnis, V.; Thiébaut, A.C.; Reedy, J.; Subar, A.F.; Wirfält, E.; Flood, A.; Mouw, T.; Hollenbeck, A.R.; Leitzmann, M.F.; *et al.* Mediterranean dietary pattern and prediction of all-cause mortality in a US population: results from the NIH-AARP Diet and Health Study. *Arch. Intern. Med.* **2007**, *167*, 2461–2468. [CrossRef] [PubMed]
86. Schwingshackl, L.; Hoffmann, G. Adherence to Mediterranean diet and risk of cancer: A systematic review and meta-analysis of observational studies. *Int. J. Cancer* **2014**, *135*, 1884–1897. [CrossRef] [PubMed]
87. Grosso, G.; Biondi, A.; Galvano, F.; Mistretta, A.; Marventano, S.; Buscemi, S.; Drago, F.; Basile, F. Factors associated with colorectal cancer in the context of the Mediterranean diet: A case-control study. *Nutr. Cancer* **2014**, *66*, 558–565. [CrossRef] [PubMed]

88. Agnoli, C.; Grioni, S.; Sieri, S.; Palli, D.; Masala, G.; Sacerdote, C.; Vineis, P.; Tumino, R.; Giurdanella, M.C.; Pala, V.; *et al.* Italian Mediterranean Index and risk of colorectal cancer in the Italian section of the EPIC cohort. *Int. J. Cancer* **2013**, *132*, 1404–1411. [CrossRef] [PubMed]
89. Reedy, J.; Mitrou, P.N.; Krebs-Smith, S.M.; Wirfält, E.; Flood, A.; Kipnis, V.; Leitzmann, M.; Mouw, T.; Hollenbeck, A.; Schatzkin, A.; *et al.* Index-based dietary patterns and risk of colorectal cancer: the NIH-AARP Diet and Health Study. *Am. J. Epidemiol.* **2008**, *168*, 38–48. [CrossRef] [PubMed]
90. Kenfield, S.A.; DuPre, N.; Richman, E.L.; Stampfer, M.J.; Chan, J.M.; Giovannucci, E.L. Mediterranean diet and prostate cancer risk and mortality in the health professionals follow-up study. *Eur. Urol.* **2014**, *65*, 887–894. [CrossRef] [PubMed]
91. Filomeno, M.; Bosetti, C.; Garavello, W.; Levi, F.; Galeone, C.; Negri, E.; la Vecchia, C. The role of a Mediterranean diet on the risk of oral and pharyngeal cancer. *Br. J. Cancer* **2014**, *111*, 981–986. [CrossRef] [PubMed]
92. Bosetti, C.; Gallus, S.; Trichopoulou, A.; Talamini, R.; Franceschi, S.; Negri, E.; la Vecchia, C. Influence of the Mediterranean diet on the risk of cancers of the upper aerodigestive tract. *Cancer Epidemiol. Biomarkers Prev.* **2003**, *12*, 1091–1094. [PubMed]
93. Samoli, E.; Lagiou, A.; Nikolopoulos, E.; Lagogiannis, G.; Barbouni, A.; Lefantzis, D.; Trichopoulos, D.; Brennan, P.; Lagiou, P. Mediterranean diet and upper aerodigestive tract cancer: the Greek segment of the Alcohol-Related Cancers and Genetic Susceptibility in Europe study. *Br. J. Nutr.* **2010**, *104*, 1369–1374. [CrossRef] [PubMed]
94. Mourouti, N.; Papavagelis, C.; Plytzanopoulou, P.; Kontogianni, M.; Vassilakou, T.; Malamos, N.; Linos, A.; Panagiotakos, D. Dietary patterns and breast cancer: A case-control study in women. *Eur. J. Nutr.* **2014**, in press.
95. De la Torre-Carbot, K.; Chávez-Servín, J.L.; Jaúregui, O.; Castellote, A.I.; Lamuela-Raventós, R.M.; Nurmi, T.; Poulsen, H.E.; Gaddi, A.V.; Kaikkonen, J.; Zunft, H.F.; *et al.* Elevated circulating LDL phenol levels in men who consumed virgin rather than refined olive oil are associated with less oxidation of plasma LDL. *J. Nutr.* **2010**, *140*, 501–508. [CrossRef] [PubMed]
96. Camargo, A.; Delgado-Lista, J.; Garcia-Rios, A.; Cruz-Teno, C.; Yubero-Serrano, E.M.; Perez-Martinez, P.; Gutierrez-Mariscal, F.M.; Lora-Aguilar, P.; Rodriguez-Cantalejo, F.; Fuentes-Jimenez, F.; *et al.* Expression of proinflammatory, proatherogenic genes is reduced by the Mediterranean diet in elderly people. *Br. J. Nutr.* **2012**, *108*, 500–508. [CrossRef] [PubMed]
97. Aggarwal, B.B.; Shisodia, S. Suppression of the nuclear factor-kappaB activation pathway by spice-derived phytochemicals: reasoning for seasoning. *Ann. N.Y. Acad. Sci.* **2004**, *1030*, 434–441. [CrossRef] [PubMed]
98. Santangelo, C.; Varì, R.; Scazzocchio, B.; Di Benedetto, R.; Filesi, C.; Masella, R. Polyphenols, intracellular signalling and inflammation. *Ann. Ist. Super Sanita* **2007**, *43*, 394–405. [PubMed]
99. Laughton, M.J.; Evans, P.J.; Moroney, M.A.; Hoult, J.R.; Halliwell, B. Inhibition of mammalian 5-lipoxygenase and cyclo-oxygenase by flavonoids and phenolic dietary additives. Relationship to antioxidant activity and to iron ion-reducing ability. *Biochem. Pharmacol.* **1991**, *42*, 1673–1681. [CrossRef] [PubMed]
100. Ferrándiz, M.L.; Alcaraz, M.J. Anti-inflammatory activity and inhibition of arachidonic acid metabolism by flavonoids. *Agents Actions* **1991**, *32*, 283–288. [CrossRef] [PubMed]
101. Kim, H.P.; Mani, I.; Iversen, L.; Ziboh, V.A. Effects of naturally-occurring flavonoids and biflavonoids on epidermal cyclooxygenase and lipoxygenase from guinea-pigs. *Prostaglandins Leukot. Essent. Fatty Acids* **1998**, *58*, 17–24. [CrossRef] [PubMed]
102. Chondrogianni, N.; Kapeta, S.; Chinou, I.; Vassilatou, K.; Papassideri, I.; Gonos, E.S. Anti-ageing and rejuvenating effects of quercetin. *Exp. Gerontol.* **2010**, *45*, 763–771. [CrossRef] [PubMed]
103. Scoditti, E.; Calabriso, N.; Massaro, M.; Pellegrino, M.; Storelli, C.; Martines, G.; de Caterina, R.; Carluccio, M.A. Mediterranean diet polyphenols reduce inflammatory angiogenesis through MMP-9 and COX-2 inhibition in human vascular endothelial cells: A potentially protective mechanism in atherosclerotic vascular disease and cancer. *Arch. Biochem. Biophys.* **2012**, *527*, 81–89. [CrossRef] [PubMed]
104. Liang, Y.C.; Huang, Y.T.; Tsai, S.H.; Lin-Shiau, S.Y.; Chen, C.F.; Lin, J.K. Suppression of inducible cyclooxygenase and inducible nitric oxide synthase by apigenin and related flavonoids in mouse macrophages. *Carcinogenesis* **1999**, *20*, 1945–1952. [CrossRef] [PubMed]

105. Kim, H.K.; Cheon, B.S.; Kim, Y.H.; Kim, S.Y.; Kim, H.P. Effects of naturally occurring flavonoids on nitric oxide production in the macrophage cell line RAW 264.7 and their structure-activity relationships. *Biochem. Pharmacol.* **1999**, *58*, 759–765. [CrossRef] [PubMed]
106. Chen, Y.C.; Shen, S.C.; Lee, W.R.; Hou, W.C.; Yang, L.L.; Lee, T.J. Inhibition of nitric oxide synthase inhibitors and lipopolysaccharide induced inducible NOS and cyclooxygenase-2 gene expressions by rutin, quercetin, and quercetin pentaacetate in RAW 264.7 macrophages. *J. Cell Biochem.* **2001**, *82*, 537–548. [CrossRef] [PubMed]
107. Miles, E.A.; Zoubouli, P.; Calder, P.C. Differential anti-inflammatory effects of phenolic compounds from extra virgin olive oil identified in human whole blood cultures. *Nutrition* **2005**, *21*, 389–394. [CrossRef] [PubMed]
108. Calixto, J.B.; Campos, M.M.; Otuki, M.F.; Santos, A.R. Anti-inflammatory compounds of plant origin. Part II. modulation of pro-inflammatory cytokines, chemokines and adhesion molecules. *Plant. Med.* **2004**, *70*, 93–103. [CrossRef]
109. Comalada, M.; Ballester, I.; Bailón, E.; Sierra, S.; Xaus, J.; Gálvez, J.; de Medina, F.S.; Zarzuelo, A. Inhibition of pro-inflammatory markers in primary bone marrow-derived mouse macrophages by naturally occurring flavonoids: Analysis of the structure-activity relationship. *Biochem. Pharmacol.* **2006**, *72*, 1010–1021. [CrossRef] [PubMed]
110. Blonska, M.; Czuba, Z.P.; Krol, W. Effect of flavone derivatives on interleukin-1beta (IL-1beta) mRNA expression and IL-1beta protein synthesis in stimulated RAW 264.7 macrophages. *Scand. J. Immunol.* **2003**, *57*, 162–166. [CrossRef] [PubMed]
111. Sharma, V.; Mishra, M.; Ghosh, S.; Tewari, R.; Basu, A.; Seth, P.; Sen, E. Modulation of interleukin-1beta mediated inflammatory response in human astrocytes by flavonoids: Implications in neuroprotection. *Brain Res. Bull.* **2007**, *73*, 55–63. [CrossRef] [PubMed]
112. Min, Y.D.; Choi, C.H.; Bark, H.; Son, H.Y.; Park, H.H.; Lee, S.; Park, J.W.; Park, E.K.; Shin, H.I.; Kim, S.H. Quercetin inhibits expression of inflammatory cytokines through attenuation of NF-kappaB and p38 MAPK in HMC-1 human mast cell line. *Inflamm. Res.* **2007**, *5*, 210–215. [CrossRef]
113. Rahman, I.; Biswas, S.K.; Kirkham, P.A. Regulation of inflammation and redox signaling by dietary polyphenols. *Biochem. Pharmacol.* **2006**, *72*, 1439–1452. [CrossRef] [PubMed]
114. Benvenuto, M.; Fantini, M.; Masuelli, L.; de Smaele, E.; Zazzeroni, F.; Tresoldi, I.; Calabrese, G.; Galvano, F.; Modesti, A.; Bei, R. Inhibition of ErbB receptors, Hedgehog and NF-kappaB signaling by polyphenols in cancer. *Front. Biosci. (Landmark Ed.)* **2013**, *18*, 1290–1310. [CrossRef]
115. Chen, C.C.; Chow, M.P.; Huang, W.C.; Lin, Y.C.; Chang, Y.J. Flavonoids inhibit tumor necrosis factor-alpha-induced up-regulation of intercellular adhesion molecule-1 (ICAM-1) in respiratory epithelial cells through activator protein-1 and nuclear factor-kappaB: Structure-activity relationships. *Mol. Pharmacol.* **2004**, *66*, 683–693. [CrossRef] [PubMed]
116. Huang, S.M.; Wu, C.H.; Yen, G.C. Effects of flavonoids on the expression of the pro-inflammatory response in human monocytes induced by ligation of the receptor for AGEs. *Mol. Nutr. Food. Res.* **2006**, *50*, 1129–1139. [CrossRef] [PubMed]
117. Pallauf, K.; Giller, K.; Huebbe, P.; Rimbach, G. Nutrition and healthy ageing: Calorie restriction or polyphenol-rich "MediterrAsian" diet? *Oxid. Med. Cell. Longev.* **2013**, *2013*, 707421. [PubMed]
118. Bruning, A. Inhibition of mTOR signaling by quercetin in cancer treatment and prevention. *Anticancer Agents Med. Chem.* **2013**, *13*, 1025–1031. [CrossRef] [PubMed]
119. Galli, M.; van Gool, F.; Leo, O. Sirtuins and inflammation: friends or foes? *Biochem. Pharmacol.* **2011**, *81*, 569–576. [CrossRef] [PubMed]
120. Horio, Y.; Hayashi, T.; Kuno, A.; Kunimoto, R. Cellular and molecular effects of sirtuins in health and disease. *Clin. Sci. (Lond)* **2011**, *121*, 191–203. [CrossRef]
121. Yeung, F.; Hoberg, J.E.; Ramsey, C.S.; Keller, M.D.; Jones, D.R.; Frye, R.A.; Mayo, M.W. Modulation of NF-kappaB-dependent transcription and cell survival by the SIRT1 deacetylase. *EMBO J.* **2004**, *23*, 2369–2380. [CrossRef] [PubMed]
122. Kawahara, T.L.; Michishita, E.; Adler, A.S.; Damian, M.; Berber, E.; Lin, M.; McCord, R.A.; Ongaigui, K.C.; Boxer, L.D.; Chang, H.Y.; *et al.* SIRT6 links histone H3 lysine 9 deacetylation to NF-kappaB-dependent gene expression and organismal life span. *Cell* **2009**, *136*, 62–74. [CrossRef] [PubMed]

123. Alhazzazi, T.Y.; Kamarajan, P.; Verdin, E.; Kapila, Y.L. Sirtuin-3 (SIRT3) and the Hallmarks of Cancer. *Genes Cancer* **2013**, *4*, 164–171. [CrossRef] [PubMed]
124. Van Gool, F.; Galli, M.; Gueydan, C.; Kruys, V.; Prevot, P.P.; Bedalov, A.; Mostoslavsky, R.; Alt, F.W.; de Smedt, T.; Leo, O. Intracellular NAD levels regulate tumor necrosis factor protein synthesis in a sirtuin-dependent manner. *Nat. Med.* **2009**, *15*, 206–210. [CrossRef] [PubMed]
125. Parbin, S.; Kar, S.; Shilpi, A.; Sengupta, D.; Deb, M.; Rath, S.K.; Patra, S.K. Histone deacetylases: A saga of perturbed acetylation homeostasis in cancer. *J. Histochem. Cytochem.* **2014**, *62*, 11–33. [CrossRef] [PubMed]
126. Song, N.Y.; Surh, Y.J. Janus-faced role of SIRT1 in tumorigenesis. *Ann. N. Y. Acad. Sci.* **2012**, *1271*, 10–19. [CrossRef] [PubMed]
127. Bayram, B.; Ozcelik, B.; Grimm, S.; Roeder, T.; Schrader, C.; Ernst, I.M.; Wagner, A.E.; Grune, T.; Frank, J.; Rimbach, G. A diet rich in olive oil phenolics in the heart of SAMP8 mice by induction of Nrf2-dependent gene expression. *Rejuvenation Res.* **2012**, *15*, 71–81. [CrossRef] [PubMed]
128. Menendez, J.A.; Joven, J.; Aragonès, G.; Barrajón-Catalán, E.; Beltrán-Debón, R.; Borrás-Linares, I.; Camps, J.; Corominas-Faja, B.; Cufí, S.; Fernández-Arroyo, S.; *et al.* Xenohormetic and anti-aging activity of secoiridoid polyphenols present in extra virgin olive oil: A new family of gerosuppressant agents. *Cell Cycle* **2013**, *12*, 555–578. [CrossRef] [PubMed]
129. Popat, R.; Plesner, T.; Davies, F.; Cook, G.; Cook, M.; Elliott, P.; Jacobson, E.; Gumbleton, T.; Oakervee, H.; Cavenagh, J. A phase 2 study of SRT501 (resveratrol) with bortezomib for patients with relapsed and or refractory multiple myeloma. *Br. J. Haematol.* **2013**, *160*, 714–717. [CrossRef] [PubMed]
130. Bohn, T. Dietary factors affecting polyphenol bioavailability. *Nutr. Rev.* **2014**, *72*, 429–452. [CrossRef] [PubMed]
131. Holzapfel, N.P.; Holzapfel, B.M.; Champ, S.; Feldthusen, J.; Clements, J.; Hutmacher, D.W. The potential role of lycopene for the prevention and therapy of prostate cancer: from molecular mechanisms to clinical evidence. *Int. J. Mol. Sci.* **2013**, *14*, 14620–14646. [CrossRef] [PubMed]
132. Bjelakovic, G.; Nikolova, D.; Gluud, C. Antioxidant supplements and mortality. *Curr. Opin. Clin. Nutr. Metab. Care* **2014**, *17*, 40–44. [PubMed]
133. Ma, Y.; Hébert, J.R.; Li, W.; Bertone-Johnson, E.R.; Olendzki, B.; Pagoto, S.L.; Tinker, L.; Rosal, M.C.; Ockene, I.S.; Ockene, J.K.; *et al.* Association between dietary fiber and markers of systemic inflammation in the Women's Health Initiative Observational Study. *Nutrition* **2008**, *24*, 941–949. [CrossRef] [PubMed]
134. Ma, Y.; Griffith, J.A.; Chasan-Taber, L.; Olendzki, B.C.; Jackson, E.; Stanek, E.J., 3rd; Li, W.; Pagoto, S.L.; Hafner, A.R.; Ockene, I.S. Association between dietary fiber and serum C-reactive protein. *Am. J. Clin. Nutr.* **2006**, *83*, 760–766. [PubMed]
135. Chuang, S.C.; Vermeulen, R.; Sharabiani, M.T.; Sacerdote, C.; Fatemeh, S.H.; Berrino, F.; Krogh, V.; Palli, D.; Panico, S.; Tumino, R.; *et al.* The intake of grain fibers modulates cytokine levels in blood. *Biomarkers* **2011**, *16*, 504–510. [CrossRef] [PubMed]
136. Reddy, B.S.; Hirose, Y.; Cohen, L.A.; Simi, B.; Cooma, I.; Rao, C.V. Preventive potential of wheat bran fractions against experimental colon carcinogenesis: implications for human colon cancer prevention. *Cancer Res.* **2000**, *60*, 4792–4797. [PubMed]
137. Guigoz, Y.; Rochat, F.; Perruisseau-Carrier, G.; Rochat, I.; Schiffrin, E.J. Effects of oligosaccharide on the faecal flora and nonspecific immune system in elderly people. *Nutr. Res.* **2002**, *22*, 13–25. [CrossRef]
138. Kaczmarczyk, M.M.; Miller, M.J.; Freund, G.G. The health benefits of dietary fiber: Beyond the usual suspects of type 2 diabetes mellitus, cardiovascular disease and colon cancer. *Metabolism* **2012**, *61*, 1058–1066. [CrossRef] [PubMed]
139. Durko, L.; Malecka-Panas, E. Lifestyle Modifications and Colorectal Cancer. *Curr. Colorectal. Cancer Rep.* **2014**, *10*, 45–54. [CrossRef] [PubMed]
140. Toner, C.D. Communicating clinical research to reduce cancer risk through diet: Walnuts as a case example. *Nutr. Res. Pract.* **2014**, *8*, 347–351. [CrossRef] [PubMed]
141. Urpi-Sarda, M.; Casas, R.; Chiva-Blanch, G.; Romero-Mamani, E.S.; Valderas-Martínez, P.; Salas-Salvadó, J.; Covas, M.I.; Toledo, E.; Andres-Lacueva, C.; Llorach, R.; *et al.* The Mediterranean diet pattern and its main components are associated with lower plasma concentrations of tumor necrosis factor receptor 60 in patients at high risk for cardiovascular disease. *J. Nutr.* **2012**, *142*, 1019–1025. [CrossRef] [PubMed]

142. Patterson, R.E.; Flatt, S.W.; Newman, V.A.; Natarajan, L.; Rock, C.L.; Thomson, C.A.; Caan, B.J.; Parker, B.A.; Pierce, J.P. Marine fatty acid intake is associated with breast cancer prognosis. *J. Nutr.* **2011**, *141*, 201–206. [CrossRef] [PubMed]
143. Chapkin, R.S.; Kim, W.; Lupton, J.R.; McMurray, D.N. Dietary docosahexaenoic and eicosapentaenoic acid: emerging mediators of inflammation. *Prostaglandins Leukot. Essent. Fatty Acids* **2009**, *81*, 187–191. [CrossRef] [PubMed]
144. Santarelli, R.L.; Pierre, F.; Corpet, D.E. Processed meat and colorectal cancer: A review of epidemiologic and experimental evidence. *Nutr. Cancer* **2008**, *60*, 131–144. [CrossRef] [PubMed]
145. Myles, I.A. Fast food fever: reviewing the impacts of the Western diet on immunity. *Nutr. J.* **2014**, *13*, 61. [CrossRef] [PubMed]
146. Olivieri, F.; Spazzafumo, L.; Santini, G.; Lazzarini, R.; Albertini, M.C.; Rippo, M.R.; Galeazzi, R.; Abbatecola, A.M.; Marcheselli, F.; Monti, D.; *et al.* Age-related differences in the expression of circulating microRNAs: miR-21 as a new circulating marker of inflammaging. *Mech. Ageing Dev.* **2012**, *133*, 675–685. [CrossRef] [PubMed]
147. Esquela-Kerscher, A.; Slack, F.J. Oncomirs—microRNAs with a role in cancer. *Nat. Rev. Cancer* **2006**, *6*, 259–269. [CrossRef] [PubMed]
148. Winter, J.; Jung, S.; Keller, S.; Gregory, R.I.; Diederichs, S. Many roads to maturity: microRNA biogenesis pathways and their regulation. *Nat. Cell Biol.* **2009**, *11*, 228–234. [CrossRef] [PubMed]
149. Tzur, G.; Israel, A.; Levy, A.; Benjamin, H.; Meiri, E.; Shufaro, Y.; Meir, K.; Khvalevsky, E.; Spector, Y.; Rojansky, N.; *et al.* Comprehensive gene and microRNA expression profiling reveals a role for microRNAs in human liver development. *PLoS ONE* **2009**, *4*, e7511. [CrossRef] [PubMed]
150. Hussain, S.P.; Harris, C.C. Inflammation and cancer: an ancient link with novel potentials. *Int. J. Cancer* **2007**, *121*, 2373–2380. [CrossRef] [PubMed]
151. Olivieri, F.; Rippo, M.R.; Monsurrò, V.; Salvioli, S.; Capri, M.; Procopio, A.D.; Franceschi, C. MicroRNAs linking inflamm-aging, cellular senescence and cancer. *Ageing Res. Rev.* **2013**, *12*, 1056–1068. [CrossRef] [PubMed]
152. Lu, J.; Getz, G.; Miska, E.A.; Alvarez-Saavedra, E.; Lamb, J.; Peck, D.; Sweet-Cordero, A.; Ebert, B.L.; Mak, R.H.; Ferrando, A.A.; *et al.* MicroRNA expression profiles classify human cancers. *Nature* **2005**, *435*, 834–838. [CrossRef] [PubMed]
153. Piepoli, A.; Tavano, F.; Copetti, M.; Mazza, T.; Palumbo, O.; Panza, A.; di Mola, F.F.; Pazienza, V.; Mazzoccoli, G.; Biscaglia, G.; *et al.* miRNA expression profiles identify drivers in colorectal and pancreatic cancers. *PLoS ONE* **2012**, *7*, e33663. [CrossRef] [PubMed]
154. Lu, Y.; Govindan, R.; Wang, L.; Liu, P.Y.; Goodgame, B.; Wen, W.; Sezhiyan, A.; Pfeifer, J.; Li, Y.F.; Hua, X.; *et al.* MicroRNA profiling and prediction of recurrence/relapse-free survival in stage I lung cancer. *Carcinogenesis* **2012**, *33*, 1046–1054. [CrossRef] [PubMed]
155. Zhang, Y.; Li, M.; Wang, H.; Fisher, W.E.; Lin, P.H.; Yao, Q.; Chen, C. Profiling of 95 microRNAs in pancreatic cancer cell lines and surgical specimens by real-time PCR analysis. *World. J. Surg.* **2009**, *33*, 698–709. [CrossRef] [PubMed]
156. Fenech, M.; El-Sohemy, A.; Cahill, L.; Ferguson, L.R.; French, T.A.; Tai, E.S.; Milner, J.; Koh, W.P.; Xie, L.; Zucker, M.; *et al.* Nutrigenetics and nutrigenomics: Viewpoints on the current status and applications in nutrition research and practice. *J. Nutrigenet. Nutrigenomics* **2011**, *4*, 69–89. [CrossRef] [PubMed]
157. Chang, W.L.; Chapkin, R.S.; Lupton, J.R. Fish oil blocks azoxymethane-induced rat colon tumorigenesis by increasing cell differentiation and apoptosis rather than decreasing cell proliferation. *J. Nutr.* **1998**, *128*, 491–497. [PubMed]
158. Davidson, L.A.; Wang, N.; Ivanov, I.; Goldsby, J.; Lupton, J.R.; Chapkin, R.S. Identification of actively translated mRNA transcripts in a rat model of early-stage colon carcinogenesis. *Cancer Prev. Res.* **2009**, *2*, 984–994. [CrossRef]
159. Kachroo, P.; Ivanov, I.; Davidson, L.A.; Chowdhary, B.P.; Lupton, J.R.; Chapkin, R.S. Classification of diet-modulated gene signatures at the colon cancer initiation and progression stages. *Dig. Dis. Sci.* **2011**, *5*, 2595–2604. [CrossRef]
160. Turk, H.F.; Barhoumi, R.; Chapkin, R.S. Alteration of EGFR spatiotemporal dynamics suppresses signal transduction. *PLoS ONE* **2012**, *7*, e39682. [CrossRef] [PubMed]

161. Dimri, M.; Bommi, P.V.; Sahasrabuddhe, A.A.; Khandekar, J.D.; Dimri, G.P. Dietary omega-3 polyunsaturated fatty acids suppress expression of EZH2 in breast cancer cells. *Carcinogenesis* **2010**, *31*, 489–495. [CrossRef] [PubMed]
162. Leaver, H.A.; Wharton, S.B.; Bell, H.S.; Leaver-Yap, I.M.; Whittle, I.R. Highly unsaturated fatty acid induced tumour regression in glioma pharmacodynamics and bioavailability of gamma linolenic acid in an implantation glioma model: effects on tumour biomass, apoptosis and neuronal tissue histology. *Prostaglandins Leukot. Essent. Fatty Acids* **2002**, *67*, 283–292. [CrossRef] [PubMed]
163. Reddy, B.S.; Burill, C.; Rigotty, J. Effect of diets high in omega-3 and omega-6 fatty acids on initiation and postinitiation stages of colon carcinogenesis. *Cancer Res* **1991**, *51*, 487–491. [PubMed]
164. Whelan, J.; McEntee, M.F. Dietary (n-6) PUFA and intestinal tumorigenesis. *J Nutr.* **2004**, *134*, 3421–3426.
165. Vinciguerra, M.; Sgroi, A.; Veyrat-Durebex, C.; Rubbia-Brandt, L.; Buhler, L.H.; Foti, M. Unsaturated fatty acids inhibit the expression of tumor suppressor phosphatase and tensin homolog (PTEN) via microRNA-21 up-regulation in hepatocytes. *Hepatology* **2009**, *49*, 1176–1184. [CrossRef] [PubMed]
166. Hodin, R.A.; Meng, S.; Archer, S.; Tang, R. Cellular growth state differentially regulates enterocyte gene expression in butyrate-treated HT-29 cells. *Cell Growth Differ.* **1996**, *7*, 647–653. [PubMed]
167. Hinnebusch, B.F.; Meng, S.; Wu, J.T.; Archer, S.Y.; Hodin, R.A. The effects of short-chain fatty acids on human colon cancer cell phenotype are associated with histone hyperacetylation. *J. Nutr.* **2002**, *132*, 1012–1017. [PubMed]
168. Chirakkal, H.; Leech, S.H.; Brookes, K.E.; Prais, A.L.; Waby, J.S.; Corfe, B.M. Upregulation of BAK by butyrate in the colon is associated with increased Sp3 binding. *Oncogene* **2006**, *25*, 7192–7200. [CrossRef] [PubMed]
169. Comalada, M.; Bailón, E.; de Haro, O.; Lara-Villoslada, F.; Xaus, J.; Zarzuelo, A.; Gálvez, J. The effects of short-chain fatty acids on colon epithelial proliferation and survival depend on the cellular phenotype. *J. Cancer Res. Clin. Oncol.* **2006**, *132*, 487–497. [CrossRef] [PubMed]
170. Hu, S.; Dong, T.S.; Dalal, S.R.; Wu, F.; Bissonnette, M.; Kwon, J.H.; Chang, E.B. The microbe-derived short chain fatty acid butyrate targets miRNA-dependent p21 gene expression in human colon cancer. *PLoS ONE* **2011**, *6*, e16221. [CrossRef] [PubMed]
171. Shah, M.S.; Schwartz, S.L.; Zhao, C.; Davidson, L.A.; Zhou, B.; Lupton, J.R.; Ivanov, I.; Chapkin, R.S. Integrated microRNA and mRNA expression profiling in a rat colon carcinogenesis model: Effect of a chemo-protective diet. *Physiol. Genomics* **2011**, *43*, 640–654. [CrossRef] [PubMed]
172. Sun, Y.; Zuo, L.; Xu, C.; Shen, T.; Pan, H.; Zhang, Z. Apoptosis and differentiation induced by sodium selenite combined with all-trans retinoic acid (ATRA) in NB4 cells. *Zhonghua Xue Ye Xue Za Zhi* **2002**, *23*, 628–630. [PubMed]
173. Terao, M.; Fratelli, M.; Kurosaki, M.; Zanetti, A.; Guarnaccia, V.; Paroni, G.; Tsykin, A.; Lupi, M.; Gianni, M.; Goodall, G.J.; *et al.* Induction of miR-21 by retinoic acid in estrogen receptor-positive breast carcinoma cells: Biological correlates and molecular targets. *J. Biol. Chem.* **2011**, *286*, 4027–4042. [CrossRef] [PubMed]
174. Wang, X.; Gocek, E.; Liu, C.G.; Studzinski, G.P. MicroRNAs181 regulate the expression of p27Kip1 in human myeloid leukemia cells induced to differentiate by 1,25-dihydroxyvitamin D3. *Cell Cycle* **2009**, *8*, 736–741. [CrossRef] [PubMed]
175. Arts, I.C.; Hollman, P.C. Polyphenols and disease risk in epidemiologic studies. *Am. J. Clin. Nutr.* **2005**, *81*, 317–325.
176. Scalbert, A.; Manach, C.; Morand, C.; Rémésy, C.; Jiménez, L. Dietary polyphenols and the prevention of diseases. *Crit. Rev. Food Sci. Nutr.* **2005**, *45*, 287–306. [CrossRef] [PubMed]
177. Schroeter, H.; Heiss, C.; Balzer, J.; Kleinbongard, P.; Keen, C.L.; Hollenberg, N.K.; Sies, H.; Kwik-Uribe, C.; Schmitz, H.H.; Kelm, M. Epicatechin mediates beneficial effects of flavanol-rich cocoa on vascular function in humans. *Proc. Natl. Acad. Sci. USA* **2006**, *103*, 1024–1029. [CrossRef] [PubMed]
178. Spencer, J.P.; Abd El Mohsen, M.M.; Minihane, A.M.; Mathers, J.C. Biomarkers of the intake of dietary polyphenols: strengths, limitations and application in nutrition research. *Br. J. Nutr.* **2008**, *99*, 12–22. [PubMed]
179. Joven, J.; Espinel, E.; Rull, A.; Aragonès, G.; Rodríguez-Gallego, E.; Camps, J.; Micol, V.; Herranz-López, M.; Menéndez, J.A.; Borrás, I.; *et al.* Plant-derived polyphenols regulate expression of miRNA paralogs miR-103/107 and miR-122 and prevent diet-induced fatty liver disease in hyperlipidemic mice. *Biochim. Biophys. Acta* **2012**, *1820*, 894–899. [CrossRef] [PubMed]

180. Sun, M.; Estrov, Z.; Ji, Y.; Coombes, K.R.; Harris, D.H.; Kurzrock, R. Curcumin (diferuloylmethane) alters the expression profiles of microRNAs in human pancreatic cancer cells. *Mol. Cancer Ther.* **2008**, *7*, 464–473. [CrossRef] [PubMed]
181. Bao, B.; Ali, S.; Kong, D.; Sarkar, S.H.; Wang, Z.; Banerjee, S.; Aboukameel, A.; Padhye, S.; Philip, P.A.; Sarkar, F.H. Anti-tumor activity of a novel compound-CDF is mediated by regulating miR-21, miR-200, and PTEN in pancreatic cancer. *PLoS ONE* **2011**, *6*, e17850. [CrossRef] [PubMed]
182. Biagi, E.; Candela, M.; Fairweather-Tait, S.; Franceschi, C.; Brigidi, P. Aging of the human metaorganism: the microbial counterpart. *Age (Dordr)* **2012**, *34*, 247–267. [CrossRef]
183. Claesson, M.J.; Cusack, S.; O'Sullivan, O.; Greene-Diniz, R.; de Weerd, H.; Flannery, E.; Marchesi, J.R.; Falush, D.; Dinan, T.; Fitzgerald, G.; *et al.* Composition, variability, and temporal stability of the intestinal microbiota of the elderly. *Proc. Natl. Acad. Sci. USA* **2011**, *108*, 4586–4591. [CrossRef] [PubMed]
184. Eckburg, P.B.; Bik, E.M.; Bernstein, C.N.; Purdom, E.; Dethlefsen, L.; Sargent, M.; Gill, S.R.; Nelson, K.E.; Relman, D.A. Diversity of the human intestinal microbial flora. *Science* **2005**, *308*, 1635–1638. [CrossRef] [PubMed]
185. Candela, M.; Biagi, E.; Brigidi, P.; O'Toole, P.W.; de Vos, W.M. Maintenance of a healthy trajectory of the intestinal microbiome during aging: A dietary approach. *Mech. Ageing Dev.* **2014**, *136–137*, 70–75. [CrossRef] [PubMed]
186. Yatsunenko, T.; Rey, F.E.; Manary, M.J.; Trehan, I.; Dominguez-Bello, M.G.; Contreras, M.; Magris, M.; Hidalgo, G.; Baldassano, R.N.; Anokhin, A.P.; *et al.* Human gut microbiome viewed across age and geography. *Nature* **2012**, *486*, 222–227. [PubMed]
187. Jeffery, I.B.; O'Toole, P.W. Diet-microbiota interactions and their implications for healthy living. *Nutrients* **2013**, *5*, 234–252. [CrossRef] [PubMed]
188. Claesson, M.J.; Jeffery, I.B.; Conde, S.; Power, S.E.; O'Connor, E.M.; Cusack, S.; Harris, H.M.; Coakley, M.; Lakshminarayanan, B.; O'Sullivan, O.; *et al.* Gut microbiota composition correlates with diet and health in the elderly. *Nature* **2012**, *488*, 178–284. [CrossRef] [PubMed]
189. Turnbaugh, P.J.; Ridaura, V.K.; Faith, J.J.; Rey, F.E.; Knight, R.; Gordon, J.I. The effect of diet on the human gut microbiome: A metagenomic analysis in humanized gnotobiotic mice. *Sci. Transl. Med.* **2009**, *1*, 6ra14. [CrossRef] [PubMed]
190. Bäckhed, F.; Ley, R.E.; Sonnenburg, J.L.; Peterson, D.A.; Gordon, J.I. Host-bacterial mutualism in the human intestine. *Science* **2005**, *307*, 1915–1920. [CrossRef] [PubMed]
191. Duncan, S.H.; Belenguer, A.; Holtrop, G.; Johnstone, A.M.; Flint, H.J.; Lobley, G.E. Reduced dietary intake of carbohydrates by obese subjects results in decreased concentrations of butyrate and butyrate-producing bacteria in feces. *Appl. Environ. Microbiol.* **2007**, *73*, 1073–1078. [CrossRef] [PubMed]
192. David, L.A.; Maurice, C.F.; Carmody, R.N.; Gootenberg, D.B.; Button, J.E.; Wolfe, B.E.; Ling, A.V.; Devlin, A.S.; Varma, Y.; Fischbach, M.A.; *et al.* Diet rapidly and reproducibly alters the human gut microbiome. *Nature* **2014**, *505*, 559–563. [CrossRef] [PubMed]
193. Biagi, E.; Nylund, L.; Candela, M.; Ostan, R.; Bucci, L.; Pini, E.; Nikkïla, J.M.; Monti, D.; Satokari, R.; Franceschi, C.; *et al.* Through aging, and beyond: gut microbiota and inflammatory status in seniors and centenarians. *PLoS ONE* **2010**, *5*, e10667. [CrossRef] [PubMed]
194. Boyle, P.; Ferlay, J. Mortality and survival in breast and colorectal cancer. *Nat. Clin. Pract. Oncol.* **2005**, *2*, 424–425. [CrossRef] [PubMed]
195. Mladenova, D.; Kohonen-Corish, M.R. Review: Mouse models of inflammatory bowel disease: insights into the mechanisms of inflammation-associated colorectal cancer. *In Vivo* **2012**, *26*, 627–646. [PubMed]
196. Nikolaki, S.; Tsiamis, G. Microbial diversity in the era of omic technologies. *Biomed. Res. Int.* **2013**, *2013*, 958719. [CrossRef] [PubMed]
197. Tjalsma, H.; Boleij, A.; Marchesi, J.R.; Dutilh, B.E. A bacterial driver-passenger model for colorectal cancer: beyond the usual suspects. *Nat. Rev. Microbiol.* **2012**, *10*, 575–582. [CrossRef] [PubMed]
198. Schwabe, R.F.; Wang, T.C. Cancer. Bacteria deliver a genotoxic hit. *Science* **2012**, *338*, 52–53. [CrossRef] [PubMed]
199. Nicholson, J.K.; Lindon, J.C. Systems biology: Metabonomics. *Nature* **2008**, *455*, 1054–1056. [CrossRef] [PubMed]

200. Wishart, D.S.; Knox, C.; Guo, A.C.; Eisner, R.; Young, N.; Gautam, B.; Hau, D.D.; Psychogios, N.; Dong, E.; Bouatra, S.; *et al.* HMDB: A knowledgebase for the human metabolome. *Nucl. Acids Res.* **2009**, *37*, 603–610. [CrossRef]
201. Fahy, E.; Subramaniam, S.; Murphy, R.C.; Nishijima, M.; Raetz, C.R.; Shimizu, T.; Spener, F.; van Meer, G.; Wakelam, M.J.; Dennis, E.A. Update of the LIPID MAPS comprehensive classification system for lipids. *J. Lipid Res.* **2009**, *50*, 9–14. [CrossRef]
202. Vinayavekhin, N.; Homan, E.; Saghatelian, A. Exploring disease through metabolomics. *ACS Chem. Biol.* **2010**, *5*, 91–103. [CrossRef] [PubMed]
203. Gieger, C.; Geistlinger, L.; Altmaier, E.; Hrabé de Angelis, M.; Kronenberg, F.; Meitinger, T.; Mewes, H.W.; Wichmann, H.-E.; Weinberger, K.M.; Adamski, J.; *et al.* Genetics meets metabolomics: A genome-wide association study of metabolite profiles in human serum. *PLoS Genet* **2008**, *4*, e1000282. [CrossRef] [PubMed]
204. Chadeau-Hyam, M.; Ebbels, T.M.; Brown, I.J.; Chan, Q.; Stamler, J.; Huang, C.C.; Daviglus, M.L.; Ueshima, H.; Zhao, L.; Holmes, E.; *et al.* Metabolic profiling and the metabolome-wide association study: significance level for biomarker identification. *J. Proteome Res.* **2010**, *9*, 4620–4627. [CrossRef] [PubMed]
205. Nicholson, J.K.; Holmes, E.; Elliott, P. The metabolome-wide association study: A new look at human disease risk factors. *J Proteome Res* **2008**, *7*, 3637–3638. [CrossRef] [PubMed]
206. Bictash, M.; Ebbels, T.M.; Chan, Q.; Loo, R.L.; Yap, I.K.; Brown, I.J.; de Iorio, M.; Daviglus, M.L.; Holmes, E.; Stamler, J.; *et al.* Opening up the "Black Box": Metabolic phenotyping and metabolome-wide association studies in epidemiology. *J. Clin. Epidemiol.* **2010**, *63*, 970–979. [CrossRef] [PubMed]
207. Watson, E.; MacNeil, L.T.; Ritter, A.D.; Yilmaz, L.S.; Rosebrock, A.P.; Caudy, A.A.; Walhout, A.J. Interspecies systems biology uncovers metabolites affecting C. elegans gene expression and life history traits. *Cell* **2014**, *156*, 759–770. [CrossRef] [PubMed]
208. Nookaew, I.; Gabrielsson, B.G.; Holmäng, A.; Sandberg, A.S.; Nielsen, J. Identifying molecular effects of diet through systems biology: Influence of herring diet on sterol metabolism and protein turnover in mice. *PLoS ONE* **2010**, *5*, e12361. [CrossRef] [PubMed]
209. Griffin, J.L.; Shockcor, J.P. Metabolic profiles of cancer cells. *Nat. Rev. Cancer* **2004**, *4*, 551–561. [CrossRef] [PubMed]
210. Spratlin, J.L.; Serkova, N.J.; Eckhardt, S.G. Clinical applications of metabolomics in oncology: A review. *Clin. Cancer Res.* **2009**, *15*, 431–440. [CrossRef] [PubMed]
211. Tieri, P.; de la Fuente, A.; Termanini, A.; Franceschi, C. Integrating Omics data for signaling pathways, interactome reconstruction, and functional analysis. *Methods Mol. Biol.* **2011**, *719*, 415–433. [PubMed]
212. Sreekumar, A.; Poisson, L.M.; Rajendiran, T.M.; Khan, A.P.; Cao, Q.; Yu, J.; Laxman, B.; Mehra, R.; Lonigro, R.J.; Li, Y.; *et al.* Metabolomic profiles delineate potential role for sarcosine in prostate cancer progression. *Nature* **2009**, *457*, 910–914. [CrossRef] [PubMed]
213. De Boer, N.K.H.; de Meij, T.G.; Oort, F.A.; Ben Larbi, I.; Mulder, C.J.; van Bodegraven, A.A.; van der Schee, M.P. The Scent of Colorectal Cancer: Detection by Volatile Organic Compound Analysis. *Clin. Gastroenterol. Hepatol.* **2014**, *12*, 1085–1089. [CrossRef] [PubMed]
214. Floegel, A.; Wientzek, A.; Bachlechner, U.; Jacobs, S.; Drogan, D.; Prehn, C.; Adamski, J.; Krumsiek, J.; Schulze, M.B.; Pischon, T.; *et al.* Linking diet, physical activity, cardiorespiratory fitness and obesity to serum metabolite networks: findings from a population-based study. *Int. J. Obes. (Lond)* **2014**, *38*, 1388–1396. [CrossRef]
215. Eurostat. Available online: http://ec.europa.eu/eurostat/help/new-eurostat-website (accessed on 7 April 2015).
216. The European Project NU-AGE (FP7,n° 266486; 2011–2016). Available online: http://www.nu-age.eu (accessed on 7 April 2015).
217. Santoro, A.; Pini, E.; Scurti, M.; Palmas, G.; Berendsen, A.; Brzozowska, A.; Pietruszka, B.; Szczecinska, A.; Cano, N.; Meunier, N.; *et al.* The NU-AGE Consortium. Combating inflammaging through a Mediterranean whole diet approach: the NU-AGE project's conceptual framework and design. *Mech. Ageing Dev.* **2014**, *136–137*, 3–13. [CrossRef] [PubMed]
218. Berendsen, A.; Santoro, A.; Pini, E.; Cevenini Ostan, R.; Pietruszka, B.; Rolf, K.; Cano, N.; Caille, A.; Lyon-Belgy, N.; Fairweather-Tait, S.; *et al.* A randomized trial on the effect of a full dietary intervention on ageing in European elderly people: Design of the NU-AGE dietary intervention study. *Mech. Ageing Dev.* **2013**, *134*, 523–530. [CrossRef] [PubMed]

219. Calçada, D.; Vianello, D.; Giampieri, E.; Sala, C.; Castellani, G.; de Graaf, A.; Kremer, B.; van Ommen, B.; Feskens, E.; Santoro, A.; *et al.* The role of low-grade inflammation and metabolic flexibility in aging and nutritional modulation thereof: a systems biology approach. *Mech. Ageing Dev.* **2014**, *136–137*, 138–147. [CrossRef] [PubMed]
220. Mediterranean diet and inflammaging in the elderly: The European project NU-AGE. *Mech. Ageing Dev.* **2014**, *136–137*. [CrossRef] [PubMed]
221. Hughes, V. Microbiome: Cultural differences. *Nature* **2012**, *492*, S14–S15. [CrossRef] [PubMed]

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NF-κBp65 and Expression of Its Pro-Inflammatory Target Genes Are Upregulated in the Subcutaneous Adipose Tissue of Cachectic Cancer Patients

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Abstract: Cancer cachexia, of which the most notable symptom is severe and rapid weight loss, is present in the majority of patients with advanced cancer. Inflammatory mediators play an important role in the development of cachexia, envisaged as a chronic inflammatory syndrome. The white adipose tissue (WAT) is one of the first compartments affected in cancer cachexia and suffers a high rate of lipolysis. It secretes several cytokines capable of directly regulating intermediate metabolism. A common pathway in the regulation of the expression of pro-inflammatory cytokines in WAT is the activation of the nuclear transcription factor kappa-B (NF-κB). We have examined the gene expression of the subunits NF-κBp65 and NF-κBp50, as well as NF-κBp65 and NF-κBp50 binding, the gene expression of pro-inflammatory mediators under NF-κB control (IL-1β, IL-6, INF-γ, TNF-α, MCP-1), and its inhibitory protein, nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha (IκB-α). The observational study involved 35 patients (control group, n = 12 and cancer group, n = 23, further divided into cachectic and non-cachectic). NF-κBp65 and its target genes expression (TNF-α, IL-1β, MCP-1 and IκB-α) were significantly higher in cachectic cancer patients. Moreover, NF-κBp65 gene expression correlated positively with the expression of its target genes. The results strongly suggest that the NF-κB pathway plays a role in the promotion of WAT inflammation during cachexia.

Keywords: cancer cachexia; inflammation; white adipose tissue; NF-κB; IκB

1. Introduction

Cancer cachexia is mainly characterized by involuntary weight loss. This syndrome is present in around fifty percent of all cancer patients and may be found in more than two thirds of those in the advanced stage of the disease [1]. It represents the direct cause of at least twenty to forty percent of all deaths associated with cancer [2]. The etiology of cachexia is extremely complex and the syndrome compromises survival and quality of life [3]. It is currently accepted that systemic inflammation plays a major role in the plethora of alterations that characterize cachexia [4]. Indeed, high concentration of inflammatory cytokines is reported in the plasma and tissues of both animal models and patients [5]. Yet, one question remains unclear: What elements trigger and maintain cachexia-related systemic inflammation? We have previously provided evidence that the white adipose tissue is a potential contributor to the maintenance of systemic inflammation in cachexia, as it secretes several inflammatory cytokines and adipokines [4,6,7]. These factors directly regulate several functions related with metabolism, body composition, activity of the complement system and vascular homeostasis [8]. Among these adipose-derived factors, several pro-inflammatory and anti-inflammatory mediators are described, including tumor necrosis factor alpha (TNF-α), interleukin 1 beta (IL-1β), interleukin-6 (IL-6) and monocyte chemoattractant protein 1 (MCP-1) [9]. Therefore, besides being profoundly affected by cachexia [10], the adipose tissue may play an important role in its etiology.

A central step in the control of the cellular expression of pro-inflammatory cytokines is the activation in cells of the nuclear transcription factor kappa B (NF-κB), which induces the transcription of most genes related with inflammation, including the so called 'classic cachectic cytokines' TNF-α, IL-6, IL-1β, interferon gamma (INF-γ) and of chemokines such as MCP-1. This pathway also induces nitric oxide synthase (iNOS), as well as the expression of adhesion molecules [11]. A large body of evidence indicates a link between inflammation promoted by the activation of this transcription factor and cancer (with regard to tumor progression) and it has been shown that inhibition of NF-κB activation markedly affects cachexia [12]. Thus, NF-κB is considered a target for cancer treatment [13]. Furthermore, extensive lipolysis in white adipose tissue seems to be related with TNF-α action through the activation of the NF-κB signaling pathway, as demonstrated in cultured adipocytes [14].

The functional form of the molecule of NF-κB consists of dimers (homo-or heterodimers) [14], Different combinations of NF-κB subunits present different functions in the regulation of the immune response. The transcription of pro-inflammatory genes in the NF-κB classical signaling pathway is regulated by the heterodimer NF-κBp65-p50, while the homodimer NF-κBp50-p50 has been described as anti-inflammatory, repressing the expression of several pro-inflammatory molecules due to the absence of its COOH-terminal transactivation domain [15]. The most studied heterodimers are NF-κBp65/NF-κBp50 (NF-κBp65-p50), and NF-κBp52/RelB (NF-κBp52-RelB) [12]. The vast majority of the studies on inflammation focus on the heterodimer NF-κBp65-p50, due to its unequivocal inflammatory function. Given the potential role of the white adipose tissue to maintain systemic inflammation in cancer cachexia, and the high circulating levels of cytokines that are characteristic of the syndrome, we performed an observational study in which we examined, for the first time, the correlation of the dimer NF-κBp65-p50 with the induction of gene expression of pro-inflammatory cytokines and chemokines in the subcutaneous adipose tissue of cachectic cancer patients, as compared with non-cachectic.

2. Experimental Section

2.1. Patient Recruitment

Patients (n = 35) were recruited between July 2011 and January 2013 at the University Hospital of the University of São Paulo. The recruitment was conducted by the hospital personnel and consisted in selecting patients engaged in the treatment of hernia (control group (N), n = 12) and cancer, further divided in non-cachectic (T), n = 11 and cachectic [16], n = 12. The project was approved by the

University of São Paulo Biomedical Sciences Institute Ethics Committee (1004/CEP), and by the University Hospital Ethics Committee (CEP-HU/USP: 752/07). The inclusion criteria were: not having received prior anticancer or anti-inflammatory treatment, and willingness to participate. The exclusion criteria were: liver failure, renal failure, AIDS, inflammatory diseases of the bowel and autoimmune disorders. After the selection, anthropometric measurements were obtained (height, weight) and the patients were interviewed with a quality of life questionnaire validated for Portuguese (EORTC QLQ-C30) [17,18], which addresses three clusters that compose quality of life: Functionality (physical, cognitive, emotional and social), Symptomatic (fatigue, pain, nausea and vomiting) and Global health. The cancer patients groups division was based on "Cachexia a new definition" [16], in which cachexia is diagnosed in patients with involuntary weight loss of at least 5% in the past 12 months or BMI <20 kg/m^2, plus at least three of the five following criteria: decreased muscle strength, fatigue, anorexia, low fat-free mass index and abnormal biochemistry (increased circulated inflammatory markers as IL-6 >4.0 pg/mL or C-Reactive Protein (CRP) >5.0 mg/L, anemia (Hb < 12 g/dL) or low serum albumin (<3.2 g/dL). The non-cachectic cancer group was composed of patients under cancer treatment that did not fulfill the mentioned criteria. A full written consent form was obtained from each patient.

2.2. Clinical and Biochemical Parameters Assessment

Height and weight were determined and approximately 10mL of blood collected on the interview day previous to surgery. The samples were then centrifuged and serum was collected and frozen at −80 °C for further analysis. The serum measurements (CRP, Albumin) were performed with the commercial kit (Turbiquest plus (Cat# 331) ultrasensitive CRP and Albumin (Cat#19)) from Labstest, Lagoa Santa, MG, Brazil. Haemoglobin measurements were performed by the University Hospital laboratory (Cidade universitária, São Paulo, Brazil).

2.3. Adipose Tissue Biopsies

Approximately one gram of subcutaneous white adipose tissue was collected during surgery. Tissue samples were rapidly divided in two tubes: The first with 1 mL of Trizol® for subsequent total RNA extraction and Quantitative real-time PCR (qPCR) experiments, and the second with 20 mL of PBS 1 X with 5% of phosphatase inhibitor for subsequent ELISA binding assay experiments. This procedure presented a minimal degree of risk, and did not interfere with the standard surgery procedure or with anesthesia.

2.4. Gene Expression

Total RNA was isolated using the Trizol® Reagent according to the manufacturer's instructions. Total RNA concentrations were quantified using the Biomate 3 spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA). Complementary DNA synthesis was carried out using the high capacity cDNA reverse transcription kit (Life Technologies, Grand Island, NY, USA), which consisted of an assay mix containing 1 μg total RNA, 2 μL 10× RT Buffer, 0.8 μL 25× dNTP mix (100 mM), 2 μL 10× Random primers, 1 μL MultiScribe™ Reverse Transcriptase and 4.2 μL of nuclease-free water in a final volume of 20 μL. The thermal cycler conditions were: 25 °C for 10 min, then 37 °C for 120 min followed by 85 °C for 5 min. Then, 20 ng of cDNA were mixed with 2× SYBR Green fast PCR master mix—and primers (Table 1) (Life Technologies, Grand Island, NY, USA)—in a final volume of 10 μL for qPCR, performed in the Quantstudio 12K Real Time Systems (Life Technologies, Grand Island, NY, USA).The mRNA levels were determined by the comparative Ct method. For each sample, a ΔCt value was obtained by subtracting RPL-27 values from those of the gene of interest. The average ΔCt value of the control group was then, subtracted from the sample to derive a $-\Delta\Delta Ct$ value. The expression of each gene was evaluated by $2^{-\Delta\Delta Ct}$, according to Livak *et al*. 2001 [19].

Table 1. Primer sequences used in the qPCR experiments.

Gene	Sense (5′–3′)	Antisense (5′–3′)
RPL-27 (NM_000988.3)	CCGAAATGGGCAAGTTCAT	CCATCATCAATGTTCTTCACGA
NF-κBp65 (NM_021975.3)	CCTGGAGCAGGCTATCAGTC	ATGGGATGAGAAAGGACAGG
NF-κBp50 (NM_003998.3)	CATCCCATGGTGGACTACCT	TGGGTCCAGCAGTTACAGTG
IκB-α (NM_020529.2)	CTCCGAGACTTTCGAGGAAATAC	GCCATTGTAGTTGGTAGCCTTCA
IL-1β (NM_000576.2)	AGCCAATCTTCATTGCTCAAGT	AGTCATCCTCATTGCCACTGT
IL-6 (NM_000600.3)	CAGCCCTGAGAAAGGAGACAT	AGCCATCTTTGGAAGGTTCA
TNF-α (NM_000594.3)	CTCTCTCCCCTGGAAAGGAC	ATCACTCCAAAGTGCAGCAG
INF-γ (NM_000619.2)	TGGAAAGAGGAGAGTGACAGAA	TGGAAAGAGGAGAGTGACAGAA
MCP-1 (NM_002982.3)	TCAGCCAGATGCAATCAATG	ACACTTGCTGCTGGTGATTCT

2.5. NF-κB Binding Assay

Subcutaneous adipose tissue protein extraction was carried out employing the Active Motif® Nuclear extract kit (Active Motif®, Carlsbad, CA, USA) according to the manufacturer's protocol. Total protein was assessed with the commercial Pierce BCA protein assay kit (Life Technologies, Grand Island, NY, USA), according to the manufacturer's protocol. Western blot was performed to verify the efficacy of nuclear protein extraction as described below:

Samples were boiled at 95 °C for 5 min in SDS-mercaptoethanol sample buffer. Then, were centrifuged for 5 min at 12,000× *g*. Equal amounts of protein (20 μg per sample) were separated in the NuPAGE® Novex® 4%–12% Bis-Tris protein gel (Catalog # NP00336BOX) (Life Technologies, Grand Island, NY, USA) and then transferred to a PVDF membrane. After blocking with 5% non-fat milk in Tris buffered saline Tween 20 (TBS-Tween 0.1%) for 1 h at room temperature, membranes were washed three times with TBS-Tween 0.1% for 10 min and then incubated overnight with primary antibodies at 4 °C. The Primary antibodies against Lamin A 1:500 (Catalog # sc-20680; Lamin A antibody H-102) and β-Tubulin 1:1000 (Catalog # sc-9104 β-Tubulin antibody H-235) were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). After three washes on the next day, the membranes were incubated with anti-rabbit IgG secondary antibody (1:5000) for two hours. The membranes were then incubated with ECL-Plus chemiluminescent detection HRP reagents (Bio-rad, Hercules, California, USA). Immunoreactive bands were visualized using the ImageQuant LAS 4000 (GE, Fairfield, CT, USA).

The binding assay was performed employing the NF-κB Family Transam transcription factor assay kit® (Active Motif®, Carlsbad, CA, USA), according to the manufacturer's protocol which consisted of a NF-κB binding sequence (5′-GGGACTTTCC-3′) immobilized in each of the 96-well plate used in the assay. The protein extract (20 μg) was then pipetted in each well and the binding process occurred. After binding, antibodies against NF-κBp65 and NF-κBp50 were pipetted, followed by the secondary antibodies and a developing solution. Absorbance was then measured and compared between the groups.

2.6. Statistical Analysis

General characteristics (Table 2), Biochemical parameters results are expressed as means ± SD (Figure 1), Quality of life Score (Figure 2) and gene expression (Table 3 and Figure 3) data are expressed as means ± SE.. Binding assay results are expressed as means ± SE of percentage of the control group value (Figure A2). Statistical significance was determined either by ANOVA non-parametric analysis (Kruskal-Wallis test with Dunn's post-test), for those parameters that did not present equal variances, or ANOVA one-way with Tukey's post-test, for the parameters that showed equal variance, as assessed by the Bartlett's test. $p < 0.05$ was considered statistically significant. Spearman's correlation analysis was then performed between paired samples. All statistics analyses were performed with the Graphpad Prism software (version 5.0).

Table 2. General characteristics of patients in each group.

	N	T	TC	*p*
n	12	11	12	
Male/Female (*n*)	9/3	7/4	6/6	
Age (years)	62.00 ± 2.51	58.64 ± 4.04	60.42 ± 2.93	0.7609
Height (m)	1.65 ± 0.03	1.64 ± 0.02	1.64 ± 0.02	0.9909
Previous body mass (Kg)	75.48 ± 4.86	75.64 ± 4.532	74.44 ± 2.665	0.9728
Current body mass (Kg)	75.48 ± 4.86	67.83 ± 3.87	64.45 ± 2.98	0.1432
Δ Body mass (%)	0.00 ± 0.00	9.36 ± 3.27 *	13.58 ± 1.75 *	**0.0005**
BMI (kg/m^2)	27.76 ± 1.40	25.31 ± 1.58	23.89 ± 1.16	0.1573
Tumor stage				
I	-	18.2%	0%	-
IIA/IIB/IIC	-	27.3%	25%	-
IIIA/IIIB/IIIC	-	45.4%	33.3%	-
IVA/IVB	-	9.1%	41.7%	-
Primary tumour site				
Colon and rectum	-	72.7%	58.3%	-
Stomach	-	18.2%	41.7%	-
Other	-	9.1%	0%	-

Data expressed as mean ± standard error. Δ: Difference between self-declared previous body mass and current body mass. *: Significant difference *versus* N.

Table 3. Subcutaneous adipose tissue NF-κB signaling pathway proteins and pro-inflammatory mediators under NF-κB control gene expression.

Gene Expression	Statistical Analysis	Significance
(A) NF-κBp65	$p = 0.0147$	TC *vs.* T; TC *vs.* N
(B) NF-κBp50	$p = 0.1719$	—
(C) IL-6	$p = 0.1458$	—
(D) IL-1β	$p = 0.0049$	TC *vs.* T
(E) TNF-α	$p = 0.0201$	TC *vs.* N
(F) INF-γ	$p = 0.2255$	—
(G) MCP-1	$p = 0.0033$	TC *vs.* T; TC *vs.* N
(H) IkB-α	$p = 0.0019$	TC *vs.* T; TC *vs.* N

(**A**) Gene expression analysis of NF-κBp65 showed higher values ($p = 0.0147$) in cachectic cancer patients compared to controls; (**B**) Gene expression of NF-κBp50 protein showed no differences among the patients ($p = 0.1719$); (**C**) IL-6 gene expression showed no differences among the patients ($p = 0.1458$); (**D**) IL-1β gene expression was higher in cachectic cancer patients ($p = 0.049$) compared to non-cachectic patients; (**E**) TNF-α gene expression was higher in cachectic cancer patients ($p = 0.0201$) compared to the control group; (**F**) INF-γ gene expression showed no differences among the groups ($p = 0.2255$); (**G**) MCP-1 gene expression was higher in cachectic cancer patients ($p = 0.0033$), compared to controls; (**H**) The inhibitory protein IκB-α gene expression was higher in cachectic cancer patients ($p = 0.0019$), compared to controls.

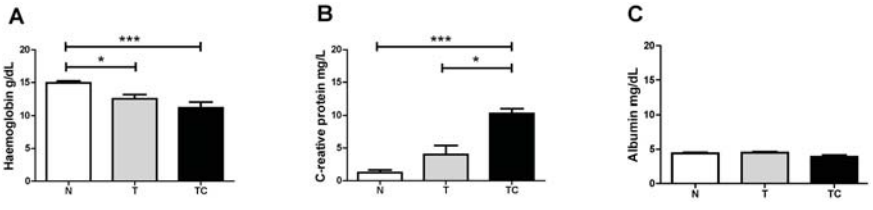

Figure 1. Serum Hemoglobin (**A**) C-Reactive Protein (**B**) and Albumin (**C**) concentration. Data expressed as mean ± standard error; *: $p < 0.05$; ***: $p < 0.001$.

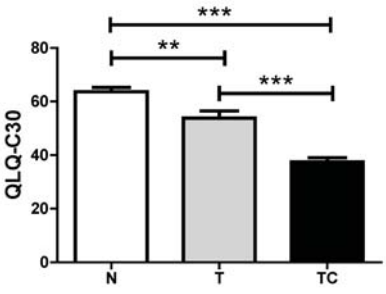

Figure 2. Quality of life Score. Data expressed as mean ± standard error; **: $p < 0.01$; ***: $p < 0.001$.

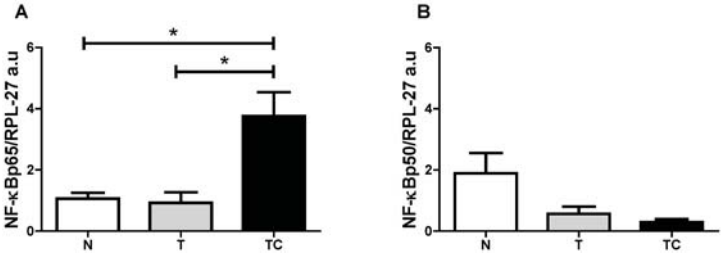

Figure 3. Subcutaneous adipose tissue NF-κBp65 and NF-κBp50 gene expression. (**A**) NF-κBp65/RPL-27 gene expression; (**B**) NF-κBp50/RPL-27 gene expression. Data expressed as mean ± standard error; *: $p < 0.05$.

3. Results

3.1. Clinical Findings

Baseline characteristics of the patients are shown in Table 2. The subjects in the three groups were of similar height, weight and body mass index (BMI). Non-cachectic and cachectic cancer patients showed a significant difference in serum hemoglobin ($p < 0.001$), compared with the control group. TCC also presented significantly higher CRP serum levels ($p < 0.001$) compared with the other groups. We did not evaluate lean body mass among the groups, although groups were matched by BMI.

3.2. Quality of Life Analysis

The three parameters that compose the criteria for global quality of life analysis showed cachexia to negatively influence these parameters ($p < 0.001$). Non-cachectic cancer patients also demonstrated a reduction in quality of life compared with the control group ($p < 0.001$), as shown in Figure 2.

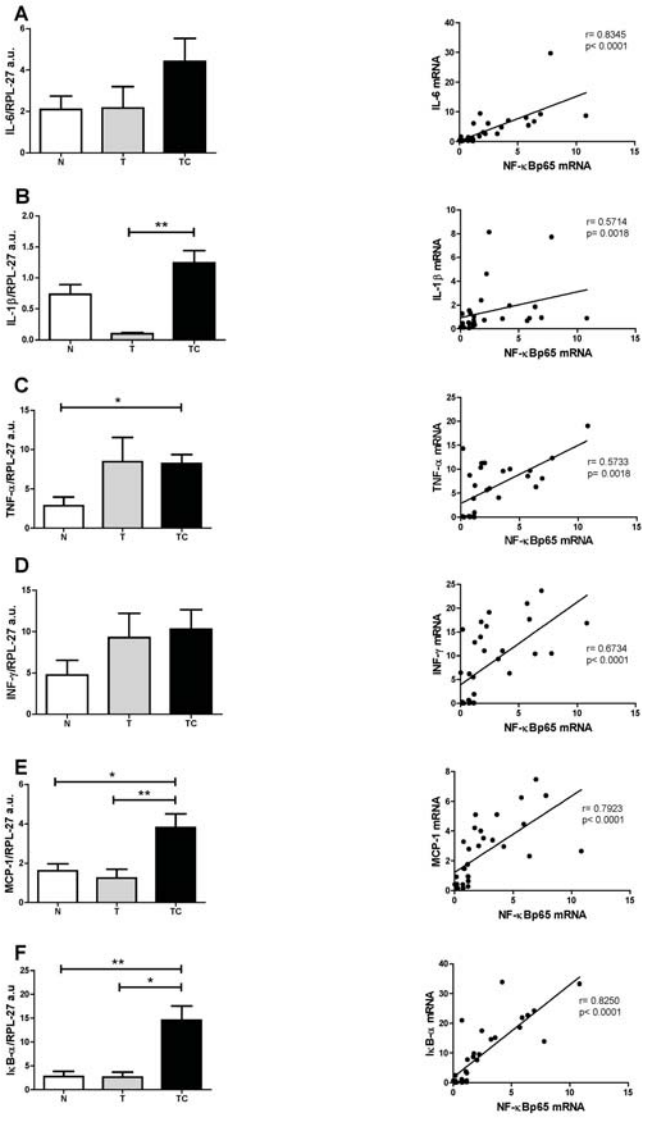

Figure 4. Subcutaneous adipose tissue gene expression and Spearman's correlation with NF-κBp65. (**A**) IL-6; (**B**) IL1-β; (**C**) TNF-α; (**D**) INF-γ; (**E**) MCP-1; (**F**) IκB-α. Data expressed as mean ± standard error. *: $p < 0.05$; **: $p < 0.01$.

3.3. Gene Expression

Groups: Control (N), non-cachectic cancer patients (T) and cachectic cancer patients [16].

4. Discussion

Systemic inflammation is a central feature of cancer cachexia [16,20–22]. Circulating pro-inflammatory mediators such as Il-6, TNF-α and acute phase proteins (CRP) are upregulated in cachectic patients and correlate positively with weight loss and poor prognosis. In this study,

CRP concentration was significantly higher in cachectic cancer patients. This acute-phase protein has been described as a marker of systemic inflammation and is also considered as part of cachexia diagnostic criteria [16]. Our results reinforce the importance of circulatory pro-inflammatory mediators as cachectic markers and corroborate previous studies with cancer patients [23]. The search for non-invasive cachexia markers is mandatory, and would warrant earlier intervention, thus preventing the onset of the symptoms and adverse prognosis. In this study, quality of life was evaluated by the application of the QLQ-C30 questionnaire. Patients reported diminished quality of life, which compromises the treatment adherence and survival. The three clusters analyzed in this questionnaire: Functionality (physical, cognitive, emotional and social), Symptomatic (fatigue, pain, nausea and vomiting) and Global health were all impaired in the cachectic cancer patients. Amongst the symptomatic cluster, fatigue is the main declared symptom and is usually present in more than 75% of cachectic patients [24]. It is accepted that systemic inflammation markedly contributes to the worsening of cachexia prognosis. Several peripheral organs suffer the consequences of inflammation triggered by the high circulatory level of pro-inflammatory mediators, that, in turn, induce in peripheral and central organs the activation of the inflammatory signaling pathways, such as the NF-κB pathway [25]. The NF-κB/Rel family of proteins, described first by Sen and Baltimore in 1986 [26] consists of transcription factors intensely studied due to the major implication as key mediators of a wide variety of cellular responses associated mainly with inflammation, infection and apoptosis. These include the stimulation of the expression of pro-inflammatory mediators such as TNF-α, IL-6, INF-δ, IL-1β, of chemokines such as MCP-1, and of reactive oxygen species (ROS) [12,13,27]. The relationship of the NF-κB signaling pathway with poor prognosis of cancer cachexia has already been extensively studied in the muscle, where increased NF-κB signaling has been described in patients [28]. Similarly, in patients under treatment for lung cancer, circulating pro-inflammatory mediators were associated with the activation of the NF-κB signaling pathway in the muscle [29]. Moreover, the importance of this transcription factor was confirmed by pharmacological inhibition, which was demonstrated to be an effective tool to reduce muscle proteolysis and consequently, atrophy [30,31]. Besides the muscle, other organs such as the liver, the brain, the gut and the adipose tissue are affected by cachexia. Adipose tissue metabolism is impaired and extensive lipolysis is observed [32]. WAT actively contributes to the inflammatory state in cachexia by actively secreting pro-inflammatory mediators [4,6,33]. Despite being recognized as an active player in cachexia, no information is available in the literature about the role of NF-κB in the development and maintenance of local inflammation of the adipose tissue. In the present study we demonstrate for the first time, that gene expression of NF-κBp65, which is a subunit of the one of the most important transcription factors that induce pro-inflammatory mediator gene expression, is upregulated in the subcutaneous adipose tissue of cachectic cancer patients, compared with controls. This is a strong indication of the role of NF-κBp65 in the promotion of inflammation in the subcutaneous adipose tissue in cachectic cancer patients. To confirm such participation of NF-κBp65 in the regulation of the adipose tissue inflammation, an assay was performed to evaluate NF-κBp65 and NF-κBp50 binding capacity to its promoter region of the DNA. This assay may be envisaged as a 'snapshot' of the cellular nucleus subunits NF-κBp65 and NF-κBp50 capacity of action, at the moment of tissue collection. Previous experiments of our group showed that the NF-κBp65 binding to its promoter region is higher, but not statistically significant among groups, while NF-κBp50 did not differ among groups (Figure A1). This assay, nevertheless, does not provide optimal evidence of total binding rate, as NF-κB dimer activity presents a fast up-regulation in the nucleus and then decays rapidly, migrating back to the cytoplasm, where it is again sequestered by its inhibitory protein, IκB-α. This is described as a rapid and dose-dependent response, that involves phosphorylation and subsequently proteasome degradation of the NF-κB inhibitory protein, IκB-α, which leaves the transactivation domain of the NF-κB dimer free to translocate to the nucleus and exert its functions as a transcription factor [34]. This rapid and transient stimulus, however, is sufficient to alter gene expression and cause prolonged changes in the NF-κB target proteins mRNA levels [35]. This led us to hypothesize that actually the best experiment to examine NF-κBp65 activity would

rather consist of sequential evaluation of binding at different collection times; what is, unfortunately not possible in a human study due to ethical limitations. An alternative, however, would be the analysis of the expression of its target genes. Thus, we proceeded with the gene expression analysis of the NF-κBp65 pro-inflammatory target genes by qPCR, having found that IL-1β, TNF-α and MCP-1 expression were upregulated in cachectic cancer patients compared with controls. This is a strong indication that the local subcutaneous adipose tissue inflammation described in cachectic cancer patients may be mediated by the increased expression and activity of NF-κBp65. Considering that several inflammatory signaling pathways work in concert in promoting inflammation and, in order to verify the specific relationship between NF-κBp65 and the induction of pro-inflammatory genes expression, we performed Spearman's correlation tests. The results show that all genes described as NF-κBp65 targets present a positive correlation with the expression of NF-κBp65 in the patients' subcutaneous adipose tissue, including its inhibitor IκB-α, which is significantly more expressed in cachectic cancer patients, as compared with controls (Figure 4). These data strongly corroborate the NF-κB target gene expression results obtained in the study. Furthermore, NF-κBp65 is the most active protein able to induce IκB-α expression [36] and the accumulation of newly synthesized IκB-α is described as a pivotal factor in the termination of NF-κB activity and shuttling NF-κB complexes from the nucleus back to the cytoplasm [36]. Therefore, parallelism between NF-κBp65 and IκB-α, in a feedback mechanism was expected. This result was a confirmation of previous studies that pointed out a role of the NF-κB signaling pathway in the promotion of an inflammatory state related with cachexia. Despite these very encouraging results, other signaling pathways such as the signal transducer and activator of transcription 3 (STAT3), the c-Jun N-terminal kinase (JNK), p38 and AP-1 are also of interest in WAT inflammation. While in this study we focused solely on the NF-κB signaling pathway, these other pro-inflammatory pathways may not be disregarded, as they are known to participate actively as co-players of the worsened prognosis in cachexia [37–39]. The limitations of the study should be acknowledged. There was no measurement of the patient's lean body mass, although the patients and groups presented similar BMI. Owing to human tissue sample implicit variation, some of the analyses were not performed with the total number of patients formerly enrolled as some samples fell out of the detection range of assays.

5. Conclusions

NF-κB classical signaling pathway protein NF-κBp65 gene expression is increased in the subcutaneous white adipose tissue of cachectic cancer patients. Its target genes IL-1β, TNF-α, MCP-1 and IκB-α are also up-regulated. NF-κBp65 gene expression was positively correlated with all the targets genes analysed in this study. We strongly suggest a role for the NF-κB classical pathway in the inflammation of WAT in cachectic cancer patients.

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Author Contributions: Rodolfo Gonzalez Camargo together with Marilia Seelaender and Miguel Batista conceptualized the study and drafted the initial manuscript; Daniela Mendes dos Reis Riccardi, Henrique Ribeiro and Luiz Carnevali together with Rodolfo Gonzalez Camargo carried out the quantitative real-time PCR experiments and data analyses; Lucas Enjiu, Emidio Marques de Matos-Neto and Rodrigo Xavier da Neves together with Rodolfo Gonzalez Camargo carried out the NF-κB binding assay and also data analysis; Paulo Sérgio Martins de Alcântara, Linda Maximiano and José Otoch, Raquel Galvão Figuerêdo, Joanna Darck Carola Correia Lima and Daniela Mendes dos Reis Riccardi together with Rodolfo Gonzalez Camargo carried out the patients recruitment, interview, tissue collection, serum and data analysis. Gerhard Püschel participated in the supervision of the project.

Conflicts of Interest: The authors declare no conflicts of interest.

Appendix A

NF-κB Binding Assay

Groups: Control (N), non-cachectic cancer patients (T) and cachectic cancer patients [16].

In order to assure that only nuclear proteins were used in the assay, first a Western blot assay with antibodies against a specific nuclear protein (Lamin A) cytoplasmic protein (β-Tubulin) in two different samples (sample 1 and 2) was performed. The cytoplasmic fraction was contaminated with nuclear proteins, but the nuclear fraction, which was employed in the assay, was free from cytoplasmic protein. The binding assay revealed no difference between the groups in the two NF-κB subunits: NF-κBp65 ($p = 0.2874$) and NF-κBp50 ($p = 0.1469$).

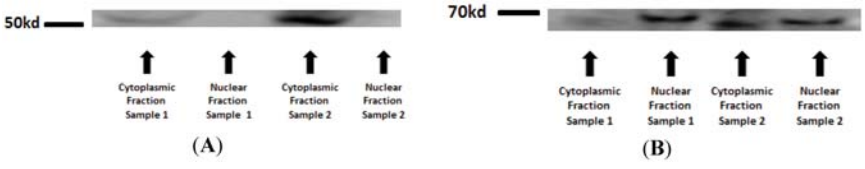

Figure A1. Western blot for nuclear and cytoplasmic protein extraction purity confirmation: β-Tubulin—51kd (**A**) and Lamin A—69 kd (**B**).

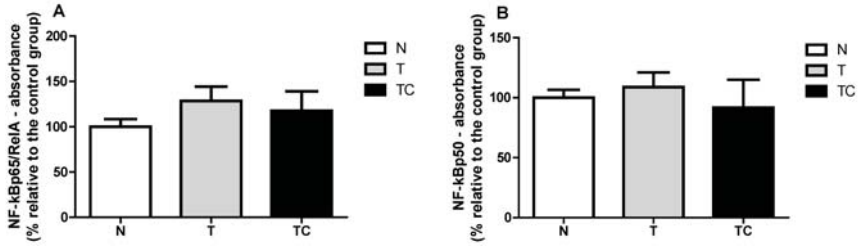

Figure A2. NF-κBp65 binding to the DNA NF-κB promoter region (**A**) and NF-κBp50 binding to the DNA NF-κB promoter region (**B**).

References

1. Fearon, K.C.; Moses, A.G. Cancer cachexia. *Int. J. Cardiol.* **2002**, *85*, 73–81. [CrossRef]
2. Clay, W.D. Balancing the scales: A common-sense look at global nutrition problems and what can be done about them. *World Rev. Nutr. Diet.* **2008**, *98*, 179–197. [PubMed]
3. Barber, M.D.; Ross, J.A.; Fearon, K.C. Cancer cachexia. *Surg. Oncol.* **1999**, *8*, 133–141. [CrossRef]
4. Lira, F.S.; Rosa, J.C.; Zanchi, N.E.; Yamashita, A.S.; Lopes, R.D.; Lopes, A.C.; Batista, M.L., Jr.; Seelaender, M. Regulation of inflammation in the adipose tissue in cancer cachexia: Effect of exercise. *Cell Biochem. Funct.* **2009**, *27*, 71–75. [CrossRef] [PubMed]
5. Pajak, B.; Orzechowska, S.; Pijet, B.; Pijet, M.; Pogorzelska, A.; Gajkowska, B.; Orzechowski, A. Crossroads of cytokine signaling—The chase to stop muscle cachexia. *J. Physiol. Pharmacol.* **2008**, *59* (Suppl. 9), 251–264. [PubMed]
6. Machado, A.P.; Costa Rosa, L.F.; Seelaender, M.C. Adipose tissue in Walker 256 tumour-induced cachexia: Possible association between decreased leptin concentration and mononuclear cell infiltration. *Cell Tissue Res.* **2004**, *318*, 503–514. [CrossRef] [PubMed]
7. Batista, M.L., Jr.; Peres, S.B.; McDonald, M.E.; Alcantara, P.S.; Olivan, M.; Otoch, J.P.; Farmer, S.R.; Seelaender, M. Adipose tissue inflammation and cancer cachexia: Possible role of nuclear transcription factors. *Cytokine* **2012**, *57*, 9–16. [CrossRef] [PubMed]

8. Booth, A.; Magnuson, A.; Fouts, J.; Foster, M. Adipose tissue, obesity and adipokines: Role in cancer promotion. *Horm. Mol. Biol. Clin. Investig.* **2015**, *21*, 57–74. [CrossRef] [PubMed]
9. Trayhurn, P.; Wood, I.S. Adipokines: Inflammation and the pleiotropic role of white adipose tissue. *Br. J. Nutr.* **2004**, *92*, 347–355. [CrossRef] [PubMed]
10. Arner, P. Medicine. Lipases in cachexia. *Science* **2011**, *333*, 163–164. [CrossRef] [PubMed]
11. Barnes, P.J.; Karin, M. Nuclear factor-kappaB: A pivotal transcription factor in chronic inflammatory diseases. *N. Engl. J. Med.* **1997**, *336*, 1066–1071. [PubMed]
12. Fraser, C.C. Exploring the positive and negative consequences of NF-kappaB inhibition for the treatment of human disease. *Cell Cycle* **2006**, *5*, 1160–1163. [CrossRef] [PubMed]
13. Garg, A.; Aggarwal, B.B. Nuclear transcription factor-kappaB as a target for cancer drug development. *Leukemia* **2002**, *16*, 1053–1068. [CrossRef] [PubMed]
14. Laurencikiene, J.; van Harmelen, V.; Arvidsson Nordstrom, E.; Dicker, A.; Blomqvist, L.; Naslund, E.; Langin, D.; Arner, P.; Ryden, M. NF-kappaB is important for TNF-alpha-induced lipolysis in human adipocytes. *J. Lipid Res.* **2007**, *48*, 1069–1077. [CrossRef] [PubMed]
15. Saccani, A.; Schioppa, T.; Porta, C.; Biswas, S.K.; Nebuloni, M.; Vago, L.; Bottazzi, B.; Sica, A.; Colombo, M.P.; Mantovani, A. p50 nuclear factor-kappaB overexpression in tumor-associated macrophages inhibits M1 inflammatory responses and antitumor resistance. *Cancer Res.* **2006**, *66*, 11432–11440. [CrossRef] [PubMed]
16. Evans, W.J.; Morley, J.E.; Argiles, J.; Bales, C.; Baracos, V.; Guttridge, D.; Jatoi, A.; Lochs, H.; Kalantar-Zadeh, K.; Mantovani, G.; *et al.* Cachexia: A new definition. *Clin. Nutr.* **2008**, *27*, 793–799. [CrossRef] [PubMed]
17. Aaronson, N.K.; Ahmedzai, S.; Bergman, B.; Bullinger, M.; Cull, A.; Duez, N.J.; Filiberti, A.; Flechtner, H.; Fleishman, S.B.; Haes, J.C.J.M.; *et al.* The European Organization for Research and Treatment of Cancer QLQ-C30: A Quality-of-Life Instrument for Use in International Clinical Trials in Oncology. *J. Natl. Inst.* **1986**, *85*, 365–376. [CrossRef]
18. Pais-Ribeiro, J.; Pinto, C.; Santos, C. Validation studyof the portuguese version of the QLC-C30-V.3. *Psicol. Saúde Doenças* **2008**, *9*, 89–102.
19. Livak, K.J.; Schmittgen, T.D. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* **2001**, *25*, 402–408. [CrossRef] [PubMed]
20. Richards, C.H.; Roxburgh, C.S.D.; McMillan, M.T.; Isswiasi, S.; Robertson, E.G.; Guthrie, G.K.; Horgan, P.G.; McMillan, D.C. The Relationships between Body Composition and the Systemic Inflammatory Response in Patients with Primary Operable Colorectal Cancer. *PLoS ONE* **2012**, *7*, e41883. [CrossRef] [PubMed]
21. Fearon, K.; Strasser, F.; Anker, S.D.; Bosaeus, I.; Bruera, E.; Fainsinger, R.L.; Jatoi, A.; Loprinzi, C.; MacDonald, N.; Mantovani, G.; *et al.* Definition and classification of cancer cachexia: An international consensus. *Lancet Oncol.* **2011**, *12*, 489–495. [CrossRef]
22. Argiles, J.M.; Moore-Carrasco, R.; Fuster, G.; Busquets, S.; Lopez-Soriano, F.J. Cancer cachexia: The molecular mechanisms. *Int. J. Biochem. Cell Biol.* **2003**, *35*, 405–409. [CrossRef]
23. McMillan, D.C. Systemic inflammation, nutritional status and survival in patients with cancer. *Curr. Opin. Clin. Nutr. Metab.Care* **2009**, *12*, 223–226. [CrossRef] [PubMed]
24. Gupta, S.C.; Kim, J.H.; Kannappan, R.; Reuter, S.; Dougherty, P.M.; Aggarwal, B.B. Role of nuclear factor kappaB-mediated inflammatory pathways in cancer-related symptoms and their regulation by nutritional agents. *Exp. Biol. Med.* **2011**, *236*, 658–671. [CrossRef] [PubMed]
25. Deans, C.; Wigmore, S.J. Systemic inflammation, cachexia and prognosis in patients with cancer. *Curr. Opin. Clin. Nutr. Metab.Care* **2005**, *8*, 265–269. [CrossRef] [PubMed]
26. Sen, R.; Baltimore, D. Inducibility of kappa immunoglobulin enhancer-binding protein Nf-kappa B by a posttranslational mechanism. *Cell* **1986**, *47*, 921–928. [CrossRef]
27. Schwartz, S.A.; Hernandez, A.; Mark Evers, B. The role of NF-kappaB/IkappaB proteins in cancer: Implications for novel treatment strategies. *Surg. Oncol.* **1999**, *8*, 143–153. [CrossRef]
28. Rhoads, M.G.; Kandarian, S.C.; Pacelli, F.; Doglietto, G.B.; Bossola, M. Expression of NF-kappaB and IkappaB proteins in skeletal muscle of gastric cancer patients. *Eur. J. Cancer* **2010**, *46*, 191–197. [CrossRef] [PubMed]
29. Op den Kamp, C.M.; Langen, R.C.; Snepvangers, F.J.; de Theije, C.C.; Schellekens, J.M.; Laugs, F.; Dingemans, A.M.; Schols, A.M. Nuclear transcription factor kappa B activation and protein turnover adaptations in skeletal muscle of patients with progressive stages of lung cancer cachexia. *Am. J. Clin. Nutr.* **2013**, *98*, 738–748. [CrossRef] [PubMed]

30. Cai, D.; Frantz, J.D.; Tawa, N.E., Jr.; Melendez, P.A.; Oh, B.C.; Lidov, H.G.; Hasselgren, P.O.; Frontera, W.R.; Lee, J.; Glass, D.J.; *et al.* IKKbeta/NF-kappaB activation causes severe muscle wasting in mice. *Cell* **2004**, *119*, 285–298. [CrossRef] [PubMed]
31. Zhou, W.; Jiang, Z.W.; Tian, J.; Jiang, J.; Li, N.; Li, J.S. Role of NF-κB and cytokine in experimental cancer cachexia. *World J. Gastroenterol.* **2003**, *9*, 1567–1570. [PubMed]
32. Arner, P.; Langin, D. Lipolysis in lipid turnover, cancer cachexia, and obesity-induced insulin resistance. *Trends Endocrinol. Metab.* **2014**, *25*, 255–262. [CrossRef] [PubMed]
33. Batista, M.L., Jr.; Olivan, M.; Alcantara, P.S.; Sandoval, R.; Peres, S.B.; Neves, R.X.; Silverio, R.; Maximiano, L.F.; Otoch, J.P.; Seelaender, M. Adipose tissue-derived factors as potential biomarkers in Cachectic cancer patients. *Cytokine* **2013**, *61*, 532–539. [CrossRef] [PubMed]
34. Han, Y.; Meng, T.; Murray, N.R.; Fields, A.P.; Brasier, A.R. Interleukin-1-induced nuclear factor-kappaB-IkappaBalpha autoregulatory feedback loop in hepatocytes. A role for protein kinase calpha in post-transcriptional regulation of ikappabalpha resynthesis. *J. Biol. Chem.* **1999**, *274*, 939–947. [CrossRef] [PubMed]
35. Argiles, J.M.; Busquets, S.; Lopez-Soriano, F.J. Anti-inflammatory therapies in cancer cachexia. *Eur. J. Pharmacol.* **2011**, *668* (Suppl. 1), S81–S86. [CrossRef] [PubMed]
36. Le Bail, O.; Schmidt-Ullrich, R.; Israel, A. Promoter analysis of the gene encoding the I kappa B-alpha/MAD3 inhibitor of NF-kappa B: Positive regulation by members of the rel/NF-kappa B family. *EMBO J.* **1993**, *12*, 5043–5049. [PubMed]
37. Narsale, A.A.; Enos, R.T.; Puppa, M.J.; Chatterjee, S.; Murphy, E.A.; Fayad, R.; Pena, M.O.; Durstine, J.L.; Carson, J.A. Liver Inflammation and Metabolic Signaling in ApcMin/+ Mice: The Role of Cachexia Progression. *PLoS ONE* **2015**, *10*, e0119888. [CrossRef] [PubMed]
38. McClung, J.M.; Judge, A.R.; Powers, S.K.; Yan, Z. p38 MAPK links oxidative stress to autophagy-related gene expression in Cachectic muscle wasting. *Am. J. Physiol. Cell Physiol.* **2010**, *298*, C542–C549. [CrossRef] [PubMed]
39. Moore-Carrasco, R.; Garcia-Martinez, C.; Busquets, S.; Ametller, E.; Barreiro, E.; Lopez-Soriano, F.J.; Argiles, J.M. The AP-1/CJUN signaling cascade is involved in muscle differentiation: Implications in muscle wasting during cancer cachexia. *FEBS Lett.* **2006**, *580*, 691–696. [CrossRef] [PubMed]

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