Dietary Fat Consumption and Exercise as Strategies to Mitigate Tumorigenesis in Breast Cancer

by

Barbora Hucik

A Thesis
presented to
The University of Guelph

In partial fulfillment for the requirements for the degree of
Master of Science
in
Human Health and Nutritional Sciences

Guelph, Ontario, Canada

© Barbora Hucik, September 2017
Lifestyle habits such as regular exercise or the consumption of a healthy balanced diet may prevent up to 30-50% of breast cancer (BC) cases. Dietary fats are of specific interest, as research provides strong evidence regarding the association of different dietary fats and BC. Exercise also exerts protective effects in BC, yet the combination of various dietary fats and exercise has not been well-studied. Dietary fats exert anti-tumorigenic effects in BC via the incorporation of fatty acids into tumor tissue plasma membrane. This study shows that dietary fat and exercise independently modulate mammary tumor outcomes. Tumor volume and multiplicity was decreased in mice fed n-3 PUFA. Exercise inhibited tumor onset and development in n-6 PUFA-fed mice. Diet and exercise interventions should be investigated further as a means to improve mammary tumor outcomes in BC.
Acknowledgements

First and foremost, I would like to thank my family for supporting me throughout my studies. Daddy, you are the hardest working man I know and the greatest role model. Mama, thank you for always being there, whether I need to call you 54298 times a day or drive over for dinner and wine on a weekday. I love you both more than you know! Andrea, my favourite (and only) sister- you’ve accomplished so much already even though you’re the baby of the family. I hate admitting this but you inspire me (sometimes).

Second, to my advisors Drs. Lindsay Robinson and David Ma, thank you for your continued support and guidance throughout the past few years. Without your encouragement I would not be pursuing academia further, and I am so grateful I have had this opportunity. To Dr. Graham Holloway, thank you for your assistance as a committee member. Your constant feedback has challenged my thinking and improved me as a researcher.

To Lyn Hillyer, the lab would fall apart without you. Thank you for letting me pester you with millions and millions of questions and your never-ending patience. Mitch, it has been so rewarding to watch you grow as a student and a scientist. I am so grateful for all of your help with this project. I am looking forward to seeing all of the amazing things you are going to do!

To my labmates, as well as my peers in the department- my experience would not have been nearly as enjoyable without y’all. I am so lucky to be able to come into work and be surrounded by such friendly and helpful people every day.

My thesis work was funded by the Cancer Research Society.
Table of Contents

Abstract.........................................................................................................................................ii
Acknowledgements .......................................................................................................................iii
Table of Contents ..........................................................................................................................iv
List of Tables ....................................................................................................................................vi
List of Figures ....................................................................................................................................vii
List of Abbreviations ........................................................................................................................viii

Chapter 1: The Influence of Dietary Fat and Exercise on Breast Cancer Risk ............ 1

1.1 Introduction.............................................................................................................................. 2
1.2 Dietary fat & breast cancer ...................................................................................................... 3
1.2.1 – n-3 & n-6 PUFA .............................................................................................................. 3
1.2.2 – n-3 PUFA & breast cancer ............................................................................................ 6
1.2.3 – Monounsaturated fatty acids & breast cancer ............................................................... 8
1.2.4 – Saturated fatty acids & breast cancer .......................................................................... 9
1.3 Lifelong exposure to dietary fat.............................................................................................. 10
1.4 Fatty acid incorporation into tissue plasma membrane ....................................................... 11
1.5 Exercise & breast cancer ........................................................................................................ 12
1.6 Immune function and inflammation in breast cancer ........................................................ 13
1.7 The role of exercise in modulating inflammation ................................................................. 15
1.8 Dietary fat consumption & exercise as a lifestyle strategy to reduce breast cancer risk ................................................................................................................................. 16
1.9 Conclusion .............................................................................................................................. 17

Chapter 2: Thesis Rationale, Objectives and Hypotheses .............................................. 18

2.1 Rationale ............................................................................................................................... 19
Chapter 3: Examining the role of dietary fat, exercise and inflammation in breast cancer

3.1 Abstract ......................................................................................................... 23
3.2 Introduction .................................................................................................. 23
3.3 Materials & Methods .................................................................................. 26
3.4 Results .......................................................................................................... 33
3.5 Discussion .................................................................................................... 52
3.6 & Future Directions ..................................................................................... 60
3.7 Conclusion ................................................................................................... 61

References ........................................................................................................ 63

Appendix .......................................................................................................... 73
List of Tables

Table 3.1 Fatty acid composition of diets ................................................................. 28
Table 3.2 Average tumor latency .................................................................................. 35
Table 3.3 Composition of fatty acids in SM fraction of mammary tumor phospholipids ... 48
Table 3.4 Composition of fatty acids in PC fraction of mammary tumor phospholipids .... 49
Table 3.5 Composition of fatty acids in PS fraction of mammary tumor phospholipids .... 50
Table 3.6 Composition of fatty acids in PE fraction of mammary tumor phospholipids.... 51
List of Figures

Figure 1.1 n-6 vs n-3 PUFA conversion pathways ......................................................... 5

Figure 3.1 Puberty onset .................................................................................................... 34

Figure 3.2 Tumor latency ................................................................................................ 36

Figure 3.3 Percentage of mice without tumors over time. .............................................. 37

Figure 3.4 Average tumor volume over time compared by dietary group....................... 39

Figure 3.5 Average tumor volume over time within dietary group ................................. 41

Figure 3.6 Average tumor multiplicity over time compared by dietary group ............. 43

Figure 3.7 Average tumor multiplicity over time within dietary group ......................... 44
List of Abbreviations

AA: arachidonic acid

ALα: α-linolenic acid

ANOVA: analysis of variance

ANSA: 8-Anilino-1-naphthalenesulfonic acid ammonium

BC: breast cancer

BF₃-MeOH: boron-trifluoride-methanol

COX-2: cyclooxygenase-2

CRP: C-reactive protein

DHA: docosahexaenoic acid

DPA: docosapentaenoic acid

EPA: eicosapentaenoic acid

FFA: free fatty acid

HER-2: human epidermal growth factor receptor-2

IL: interleukin

KCl: potassium chloride

LA: linoleic acid
MAPK: mitogen-activated protein kinase

MCP: monocyte chemoattractant protein

MMTV: mouse mammary tumor virus

MUFA: monounsaturated fatty acid

OA: oleic acid

PA: palmitic acid

PC: phosphatidylcholine

PE: phosphatidylethanolamine

PGE2: prostaglandin E2

PS: phosphatidylserine

PUFA: polyunsaturated fatty acid

SFA: saturated fatty acid

SM: sphingomyelin

TLC: thin-layer chromatography

TNFa: tumor necrosis factor-alpha
CHAPTER 1

The Influence of Dietary Fat and Exercise on Breast Cancer Risk
1.1 Introduction

Breast cancer (BC) is among the top three most commonly diagnosed cancers and is the second leading cause of death in North American women (1). Healthy lifestyle habits can prevent up to 30-50% of BC cases (2). In 2002, 20% of cancer diagnoses were thought to be a direct result of excess body weight caused by sedentary behaviour (3). Therefore, exercise and diet are identified as major modifiable risk factors.

Epidemiological evidence shows an association between dietary fat consumption and BC risk. For example, Asian populations consuming diets rich in long chain n-3 polyunsaturated fatty acids (PUFA) found in fatty fish, or those following a Mediterranean diet rich in monounsaturated fatty acids (MUFA) found in olive oil, have a decreased risk of developing BC (4, 5). In contrast, Western populations consuming diets high in n-6 PUFA, found in vegetable oils, and saturated fatty acids (SFA) found in meat and dairy products, have an increased risk of BC (6, 7). Timing of exposure to dietary fats has shown to exert differing effects on BC risk and development (8,9). Maternal diet as well as pre-pubertal exposure to dietary fats throughout critical points of mammary gland development may have effects on BC risk later in life (8, 9). Therefore, it is important to examine lifelong exposure to dietary fat to determine long-term effects on BC risk, beginning in utero.

Additionally, exercise has been shown to mitigate tumorigenesis, and evidence demonstrates it is an important therapeutic strategy in all stages of BC, including prevention, treatment and post-treatment care (10). Engaging in even recreational levels of exercise may still provide protective benefits against BC (11). However, there is limited research on the specific mechanisms through which exercise exerts anti-tumorigenic effects.
Taken together, existing evidence shows that implementation of exercise programs, or the consumption of specific dietary fats may influence BC development. However, the potential synergistic or additive effects of exercise in combination with the consumption of various dietary fats have not yet been researched. As both dietary fats and exercise can modulate tumorigenesis independently (12-17), in combination they may exert the strongest effect in modulating BC development. Together they represent a possible lifestyle strategy to attenuate BC development.

1.2 Dietary fat and breast cancer

1.2.1 n-3 and n-6 PUFA, sources and metabolism

The fatty acids linoleic acid (18:2 n-6, LA) and α-linolenic acid (ALA, 18:3n3) (14) are considered to be essential to human health as they cannot be produced endogenously in humans and must be obtained in the diet (18). The typical Western diet is rich in n-6 PUFA, including LA, which can be obtained through the consumption of corn and safflower oils, as well as arachidonic acid (20:4 n-6, AA), found in animal-derived sources or synthesized from LA. ALA is a plant-derived n-3 PUFA, found in flax, canola and soy, and is a long chain (LC) n-3 PUFA precursor. ALA can be converted to the LC n-3 PUFAs eicosapentaenoic acid (20:5n3, EPA) and docosapentaenoic acid (22:6n3, DHA). Although EPA and DHA are not considered to be essential fatty acids as they can be synthesized endogenously, total whole body-conversion in humans is less than 5% (19), and therefore should be consumed in the diet to obtain adequate amounts.

In comparison to n-6 PUFA, LC n-3 PUFA are consumed in extremely low amounts in the average Western diet (7, 8). This low intake of LC n-3 PUFA, including EPA and DHA, has
been found to contribute to many health issues and diseases (20). EPA and DHA are derived from marine sources such as fatty fish and other seafood. A typical Japanese diet contains 1-2% of energy as LC n-3 PUFA and the Greenland Inuit consume up to 2.4-6.3% of energy from n-3 PUFA (22). Although Dieticians of Canada recommend a daily intake of EPA and DHA of approximately 300-450 in order to follow a healthy diet based on Health Canada's Canadian Nutrient File (23), the average North American consumes only 0.1-0.2 g per day (24). The typical Western diet, with an excessive intake of n-6 PUFA compared to n-3 PUFA (16:1 ratio), has been associated with increased occurrence of BC (7).
Figure 1.1 n-6 vs n-3 PUFA conversion pathways. Linoleic acid (LA) and α-linolenic acid (ALA) are essential fatty acids that must be derived from dietary sources as they cannot be produced endogenously by humans.
1.2.2 n-3 PUFA & breast cancer

Epidemiology studies have revealed an association between diet and the prevalence of disease in a population; specifically, between the amount of n-3 PUFA consumed through the diet, and the occurrence of BC (4, 25). Asian populations consuming diets rich in LC n-3 PUFA, including EPA and DHA, have a reduced incidence of BC (4). However, Western populations with a high intake of n-6 PUFA, such as LA, and a lower intake of LC n-3 PUFA than their Asian counterparts, displayed a higher incidence of BC (25). Although epidemiological evidence shows and association between increased dietary n-3 PUFA content and decreased BC occurrence in humans, more conclusive findings have been found in animal studies, especially regarding the dose-dependent effects by which both plant- and marine-derived n-3 PUFA exert anti-tumorigenic properties (12, 26). The mechanisms by which n-3 PUFA exert such anti-cancer effects, especially within the tumor microenvironment itself, are still under study and have the potential to provide important insight into dietary strategies for BC.

Previous research has demonstrated that the consumption of n-3 PUFA has anti-tumorigenic effects (12). For example, mice expressing mouse mammary tumor virus (MMTV) displayed a reduction in tumor volume (~30%), as well as increased levels of LC n-3 PUFA detected in phospholipids (12). n-3 PUFA can affect cellular signaling by incorporating into the structure of lipid rafts (27). An increased presence of lipid rafts is observed in BC and is involved in signaling events that contribute to tumor cell proliferation and the promotion of tumor survival (28). However, LC n-3 PUFA can reduce these tumor-promoting effects of lipid rafts by incorporating into their structure, and DHA particularly has an effect on down-regulating the expression of onco-proteins through modulating apoptosis, which is inhibited in cancer,
therefore promoting tumor cell survival (29). Therefore, inhibiting signaling cascades which promote tumor growth may reduce tumor size and number.

A related mechanism by which n-3 PUFA reduce mammary tumor occurrence and development may be through a reduction in inflammatory mediators and immune responses within the mammary tumor microenvironment, given that n-3 PUFA have well-established anti-inflammatory activity and can attenuate the pro-inflammatory effects of n-6 PUFA (30). Many cancers express increased levels of pro-inflammatory eicosanoids such as prostaglandin E2 (PGE₂) (activated by the cyclooxygenase-2 (COX-2) induced signal transduction pathway) derived from AA, which may in turn suppress immune responses that are involved in inhibiting tumor growth and development (31). When n-3 PUFA are present, they are preferentially used in the production of eicosanoids by cyclooxygenases resulting in the production of downstream metabolites that stimulate less inflammation than those synthesized from n-6 PUFA. Subsequently, mammary tumor development could potentially be impeded by a mechanism through which n-3 PUFA cause a reduction in the level of prostaglandins in the mammary tissue microenvironment.

Not only do n-3 PUFA attenuate inflammation directly through the inhibition of pro-inflammatory cytokines (30, 32), they have also been associated with increased production of anti-inflammatory cytokines, such as interleukin-10 (IL-10) (32). IL-10 has demonstrated anti-tumorigenic properties in rodent models (33). It acts to reduce pro-metastasis activity of the tumor by inhibiting angiogenesis and tumor growth through the inhibition of various inflammatory cytokines, including IL-6, tumor necrosis factor α (TNFα), IL-1β, and monocyte chemoattractant protein-1 (MCP-1) (33).
1.2.3 - Monounsaturated fatty acids & breast cancer

Mediterranean countries have reduced rates of cardiovascular diseases and cancers (5). To some extent this is associated with dietary patterns, as the Mediterranean diet contains foods such as fish, nuts, fruit and vegetables, and olive oil. Therefore, MUFA such as oleic acid (18:1n9, OA), which are abundant in olive oil, are of particular interest when studying the potential beneficial effects of dietary fat. Epidemiological evidence regarding the benefits of the Mediterranean diet specifically in BC is scarce and in some cases conflicting. OA has reported anti-proliferative effects in vitro through the modulation of oncogenes (34). Although all olive oils contain substantial amounts of OA, extra-virgin olive oil also contains polyphenols and lignans which are also thought to have anti-carcinogenic properties (35). For example, oleocanthal, a naturally occurring secoiridoid polyphenol found in olive oil, was found to have a dose-dependent inhibitory effect on the growth of several human BC cell lines (MDA-MB-231, MCF-7, BT-474) through the regulation of the c-Met signalling pathway (36). As a result, the potential protective effects of olive oil cannot be solely attributed to MUFA content. The Primary Prevention of Cardiovascular Disease with a Mediterranean Diet (PREDIMED) study, a large-scale intervention study analyzing the effects of the Mediterranean diet on various health benefits, found a 62% reduction in BC risk in participants supplementing their diet with extra-virgin olive oil compared to the control group (5). However, these protective benefits required a high intake of extra-virgin olive oil equal to 15% or more of total energy intake (5). Additionally, a recent meta-analysis regarding the relationship between dietary fats and BC risk found no effect of total MUFA consumption in case-control studies (6). Cohort studies showed almost a 2-fold increase in BC risk when comparing the highest and lowest levels of MUFA (6).
Specifically, OA was found to significantly increase BC risk in cohort studies, although no association between other specific MUFA and BC risk were found (6).

1.2.4 - Saturated fatty acids & breast cancer

Less is known regarding specific effects of SFA on BC, as there is only a weak association between SFA consumption and BC risk (6, 13, 14). A recent case-cohort analysis found that BC occurrence was increased in women with a greater percent incorporation of palmitic acid (16:0, PA) into plasma phospholipids (15). Dietary fat may exert different effects on mammary tissue dependent on age, as only younger women had an increased risk of BC associated with SFA consumption (15). Observations also vary based on the source of SFA, as dairy consumption exert different effects compared to red meat consumption. Total dairy consumption, not including milk, is associated with a reduced risk of breast cancer (37); however, increased intake of red meat is associated with an increased risk of breast cancer (38). However, this may not be a direct reflection of intake as SFA can be endogenously synthesized from acetyl CoA, or used to produce OA through elongation and desaturation (39). A systematic review found that the consumption of SFA was associated with increased or no C-reactive protein, a marker of inflammation, and significant associations with IL-6 but not TNFα or other inflammatory mediators (40). Further research is required as current evidence regarding the inflammatory effects of SFA is inconclusive and it is unknown whether this could contribute to increased BC risk.
1.3 Lifelong exposure to dietary fat

Recent evidence suggests that the timing and duration of exposure to dietary fat may impact BC risk, beginning as early as in utero (8, 9). Changes to diet early in life may influence BC risk, supporting the notion of implementing preventative strategies during early life and throughout the entire lifespan. Of particular interest is the effect of maternal diet and pre-pubertal exposure to dietary fats during the critical points of mammary gland development, such as production of terminal end buds and ductal branching during puberty (41), as it may affect the risk of developing BC in later life. Studies show that a maternal diet high in the n-6 PUFA LA in a corn oil-based diet increased the occurrence of mammary tumors in female offspring, associated with altered mammary gland development and earlier onset of puberty as a result of increased serum estradiol (8). Dietary fat and estradiol modulate the development of epithelial cell structures in mammary glands, and increased estrogen promotes the malignant transformation of these epithelial structures during puberty as they are not yet differentiated and have a high capacity for proliferation (42). In contrast, previous research shows that LC n-3 PUFA consumption, influences mammary gland development and may attenuate proliferative markers such as cyclin D1 (43, 44). Additionally, pre-pubertal exposure to diets supplemented in DHA increased mammary gland differentiation and decreased mammary tumor incidence, although high doses of 20 mg of DHA compared to 10 mg DHA had adverse effects on BC development (45). Evidence regarding the effect of SFA on mammary gland development is conflicting. One study found that lauric acid, a 12-carbon SFA stimulated normal mammary gland development in vivo (46), while stearic acid, an 18-carbon SFA suppressed mammary gland development both in vivo and in vitro through the same pathway (41). Another study found
that exposure to high levels of lard *in utero* actually decreased BC occurrence later in life in offspring, an effect that was attributed to the modulation of mammary gland development by diet (47). Although there is no doubt that exposure to dietary fat through maternal diet or early in life plays a role in mammary gland development, less is known regarding dose-dependent effects of specific fatty acids on mammary tumorigenesis later in life as a result.

### 1.4 Fatty acid incorporation into tissue plasma membrane

Membrane fatty acid composition exerts modulatory effects on cell signalling through the modulation of lipid rafts within plasma membranes. Lipid rafts serve as microdomains and play a role in facilitating downstream signalling effects (48) including pro-tumorigenic pathways promoting cell proliferation and survival (28). Dietary fats integrate into the plasma membrane glycerophospholipids. Typically, the sn-1 position on the glycerol backbone contains a SFA, and the sn-2 position is occupied by an n-6 PUFA (49). Lipid rafts are compartmentalized from the actual cell membrane and are rich in sphingolipids and cholesterol, as well as SFA side chains (50). The presence of cholesterol and SFA allow lipid rafts to be packed tightly, resulting in a highly organized structure. G proteins, growth factor receptors, mitogen activated protein kinase (MAPK) and protein kinase C are all examples of signal-inducing proteins found in lipid rafts (50). n-3 PUFA have a documented effect on lipid raft composition. n-3 PUFAs have a less organized and compact structure compared to SFA due to the presence of double bonds; therefore, incorporation of n-3 PUFA alters the organization of lipid rafts and therefore has the potential to alter lipid raft-modulated signalling (51, 52, 53). DHA treatment was found modulate levels of oleic acid in lipid rafts of T cells, therefore n-3 PUFAs can modulate SFA and MUFA composition of lipid rafts although specific mechanisms are unknown (54). Specifically, in
HER2+ BC, DHA was found to inhibit HER-2-specific signalling through the disruption of lipid rafts in human mammary epithelial cells (27). The modulation of lipid rafts by n-3 PUFA could potentially be used as an anti-tumorigenic therapy to disrupt cancer-promoting signalling pathways (27).

1.5 Exercise & breast cancer

Research provides evidence that exercise has a significant impact on reducing the risk of BC. Physical activity plays an important role in mediating tumorigenesis and exerts its effects at all stages of BC, including prevention, treatment and post-treatment during recovery and aftercare. Women who are physically active and engage in moderate to vigorous-intensity aerobic exercise, experience a 20-30% reduction in BC risk compared to their sedentary counterparts (55). This decrease in risk corresponds to the increase in exercise dose (55). Likewise, previous research using mouse models supports the benefits of long term exercise training in BC (56).

Exercise in early life can also attenuate tumor progression later in life, and represents an important preventative strategy in reducing BC occurrence in more susceptible populations. One study observed a reduction in burden with increased doses of voluntary wheel running before the development of tumors (57). Previous research also shows that these anti-tumorigenic effects of exercise are also seen in the earlier stages of BC development (57). Lifetime physical activity even at a recreational level (jogging, walking, cycling, swimming, dance) decreased the risk of developing breast carcinomas by 35% compared to women that reported no physical activity (58). Although all types of physical activity exert protective effects against BC development, a meta-analysis found that recreational activity reduced BC risk by 21%, a slightly stronger effect
compared to moderate aerobic exercise such as walking and cycling, which reduced risk by 18% (59). However, current US guidelines recommend 4-7 hours of moderate to vigorous-intensity activity per week to have an effect on modulating BC (60). A greater benefit of both rigorous recreational activity or vigorous aerobic exercise (which increases heart rate, respiration, sweating and fatigue) is observed for post-menopausal women after the age of 50, compared to physical activity performed earlier in life (59). In this population, implementation of exercise even later in life attenuates BC (59). Approximately 80% of women with BC are over fifty years of age, and the tumors that developed in older, postmenopausal women have distinct phenotypic characteristics compared to those of younger women due to the change in estrogen levels post-menopause (61). Increased aromatization of androgens in post-menopausal women could also contribute to higher circulating levels of estrogen (62). Increased levels of estrogen are associated with increased inflammation as elevated aromatase expression is associated with increased NFκB binding activity (62), therefore exercise training could also be used to target hormone-driven inflammation.

It is also noteworthy that results from several studies have demonstrated that exercise may promote carcinogenesis and increase tumor incidence or reduced tumor latency in mouse models of BC (63, 64), although specific mechanisms were not investigated. Further research with a consistent model expressing similar characteristics of human BC is needed to come to a conclusion regarding the anti-tumorigenic effects of exercise.

**1.6 Immune function and inflammation in breast cancer**

The tumor microenvironment is regulated and influenced by various growth factors, cytokines, hormones, angiogenic factors and immune cells. Increased expression of pro-
inflammatory cytokines (IL-6, TNFα, IL-1β, MCP-1) resulting in the activation of the transcription factor NFκB within the tumor microenvironment may promote tumorigenesis (65). Therefore, targeting inflammation may be a potential therapeutic target in treating BC as IL-10, an anti-inflammatory cytokine, may inhibit various inflammatory cytokines and reduce pro-metastatic tumor activity (66). However, very few studies have explored specific inflammatory mechanisms modulated by exercise in mitigating tumorigenesis.

In a regulated environment, different components of the immune system respond to inflammatory stimuli in the body, with different subsets of T cells participating in signalling or modulating an immune response. For example, in human tumors, macrophages infiltrate the tumor microenvironment, and are polarized to an inflammatory M1 macrophage phenotype, resulting in the activation of natural killer (NK) cell and ultimately the development of T helper 1 (Th1) immunity (67). However, this response is dysregulated within the tumor microenvironment and chronic activation of immune cells may actually exert pro-tumorigenic effects (68). A study using a chemically-induced BC model showed that exercise promoted M1 polarization of macrophages due to increased expression of IFN-γ, TNFα and IL-12, while sedentary mice favoured a shift towards M2-polarized macrophages (69). One study showed that 3 weeks of treadmill running increased the production of IFN-γ and TNFα, in BALB/c mice, which is also indicative of a shift to an M1 macrophage phenotype suggesting an enhanced innate immune response, although this study did not use a cancer model (70). Six weeks of forced swimming in male mice injected with tumor cells decreased tumor size, as well as macrophages and neutrophils (16). Another study demonstrated that only 2 weeks of treadmill running in BALB/c mice injected with tumor cells decreased inflammation within the tumor,
specifically through a reduction in macrophages (17). Neutrophils and macrophages stimulate the production of inflammatory cytokines, including IFN-γ, IL-6 and TNFα (69). Ultimately, prolonged bouts of unresolved inflammation lead to a cancer-promoting environment, and promotes the progression of tumors through the inhibition of the antitumor immune response (68). Exercise may have the potential to mitigate tumorigenesis through modulating macrophage infiltration, modulating the resulting inflammatory response. However, the link between exercise, immune function and inflammation specifically in BC requires further study.

1.7 The role of exercise in modulating inflammation in cancer

Exercise modulates inflammation through the production of cytokines from skeletal muscle. This initial increase in inflammatory cytokines following a bout of exercise, such as IL-6, stimulates the release of anti-inflammatory mediators such as IL-10 and IL-1Ra (71). This anti-inflammatory environment can be maintained with longer periods of exercise (71). However, few human studies have evaluated the effects of exercise on the expression of inflammatory markers in cancer.

Several animal studies have demonstrated the potential for exercise to decrease inflammation. Compared to sedentary controls, BALB/c mice treated with tamoxifen or in an interval exercise training group showed a reduction in tumor size as well as decreased levels of nuclear factor kappa B (NFκB) expression (72). Estrogen can also increase NFκB expression resulting in increases in pro-inflammatory cytokines through estrogen receptor Eα (73). A potential mechanism of action could be through an exercise-mediated decrease in inflammation through a reduction in estrogen, therefore inhibiting tumorigenesis, as exercise was as effective as tamoxifen at reducing serum estradiol and ER-α expression (73). Moderate aerobic exercise
typically decreases the expression of pro-inflammatory cytokines such as TNFα (74). However, following exercise, especially an acute bout of rigorous activity, TNFα levels are initially increased to initiate an immune response to stimulate the repair of damaged muscle fibers (75). Following a prolonged bout of strenuous exercise, circulating levels of IL-6 and the anti-inflammatory cytokine IL-10 are increased (75). Following this initial release of IL-6 from muscle, levels of this cytokine remain elevated for an extended period of time during recovery to promote repair of damaged tissue (76). Although an acute increase in pro-inflammatory cytokines is a normal and necessary response to initiate the tissue repair processes following exercise, less is known regarding the role of this response in reducing chronic inflammation or possible effects on inflammation within the tumor microenvironment in a BC model.

1.8 Dietary fat consumption and exercise as a lifestyle strategy to reduce BC risk

Modifications to diet and exercise regimens have gained recent interest as strategies to mitigate BC risk and recurrence, as healthy lifestyle habits can prevent up to 30-50% of BC cases (2). Although emerging evidence suggests that cancer recurrence may be reduced through a number of lifestyle factors (2), underlying mechanisms still need to be examined. Although it is intuitive that changes in diet and exercise plays a role in modifying BC development and has been reviewed extensively (77-79), there have been no studies examining the effects of dietary fat in combination with exercise on tumor outcomes in a BC model. A potential mechanism by which dietary fat and exercise may modulate tumorigenesis in BC may be through an effect on inflammatory mediators. As inflammation is linked to BC progression (80-85), it presents a potential therapeutic target in BC prevention and treatment. As literature supports the role of dietary fats, such as n-3 PUFA, and exercise in mitigating inflammation independently (86-88),
together they may exert additive or synergistic effects on mitigating tumorigenesis in BC. Therefore, previous research provides a basis to explore the potential beneficial effects of dietary fats and exercise on BC development and mammary tumor outcomes.

1.9 - Conclusion

There has been increased interest in the use of nutrition and exercise strategies in BC. Epidemiological evidence shows that populations consuming diets high in marine-derived n-3 PUFA have a decreased occurrence of BC compared to populations with a high n-6:n-3 PUFA ratio in the diet (4, 7, 25). Other types of dietary fats such as SFA and MUFA also exert various effects on tumorigenesis in BC, although evidence is not conclusive regarding the potential benefits of these fats (13-15, 34, 38). In addition to dietary fat consumption, other lifestyle factors, such as exercise have a potential role in modulating BC risk. There is evidence that aerobic exercise is associated with reduced tumor occurrence in a dose-dependent manner (57); however, the beneficial effects of exercise in BC have not been researched as extensively or in combination with various types of dietary fats. Lifelong implementation of healthy lifestyle habits may play a role in BC prevention, as diet and exercise at critical points of development in utero or throughout puberty may exert effects on BC risk later in life (43, 44, 89). Therefore, further research is required to determine specific effects of dietary fat and exercise on BC outcomes.
CHAPTER 2

Rationale, Hypothesis and Experimental Design
Rationale

Breast cancer (BC) is the second leading cause of death in North American women (1). Although cancer can be caused by many factors including genetic pre-disposition, 30-50% of BCs can be prevented by implementing a healthy lifestyle, including the consumption of healthy foods and exercise (2). Yet, to the best of our knowledge, no study has examined the effects of a wide range of dietary fats and/or exercise on mammary tumor outcomes. More specifically, it is unknown whether the consumption of dietary fats, such as n-3 PUFA, along with the implementation of an exercise protocol has additive or synergistic effects on BC development. Lifestyle interventions represent an important preventative and therapeutic target in mitigating BC occurrence and development. Therefore, the overall research objective of this thesis was to determine the effects of lifelong exposure to various dietary fats, alone or in combination with exercise, on mammary tumor development and progression. To achieve this research objective, the MMTV-neu (ndl) YD5 mouse model was used for all experiments (described below).

Specific Objectives

To determine the effects of dietary fat alone or in combination with exercise on:

1) Mammary tumor development over 20 weeks

2) Fatty acid composition of mammary tumor tissue

Hypothesis

The overall hypothesis of this thesis was that lifelong (including gestation) exposure to marine- and plant- derived n-3 PUFA and MUFA and the implementation of an exercise program will prevent mammary tumor development, while sedentary behaviour and the consumption of n-
6 PUFA and SFA will promote mammary tumor growth. More specifically, it is hypothesized that:

1) Lifelong exposure to n-3 PUFA or MUFA will prevent mammary tumor development through a delay in tumor latency, and a reduction in tumor volume and multiplicity, while lifelong exposure to SFA or n-6 PUFA will promote mammary tumor development through an advance in tumor latency and increase in tumor volume and multiplicity.

2) An exercise intervention consisting of 4 weeks of intense aerobic exercise will prevent mammary tumor development through a delay in tumor latency, and a reduction in tumor volume and multiplicity regardless of diet.

3) Dietary n-3 PUFA combined with an exercise program will have an additive effect on BC prevention and development in comparison to all other diet/exercise combinations.

**Experimental design**

*Mouse model: MMTV-neu (ndl) YD5*

The mouse mammary tumor virus (MMTV) model develops mammary tumors once oncoRNA virus, of the *Retroviridae* family, is activated (90). MMTV is replicated in alveolar epithelial cells of the mammary gland, and is particularly increased by steroid hormones (90). The MMTV inserts bases to induce mutagenesis of DNA or activating transcription of nearby oncogenes, resulting in the development of malignant mammary gland tumors (90).

50% of MMTV-*neu* (ndl)-YD5 mice develop mammary tumors by approximately 102 days of age and are useful in studying breast cancer due to rapid proliferation and early tumor
onset relative to other models (91). The mouse *neu* gene is representative of the human *Her2* gene. Overexpression of human epithelial growth factor receptor-2 (HER-2) gene in human breast cancer results in a highly aggressive form of breast cancer and is associated with poor prognosis and metastasis.

*Diets and exercise*

Heterozygous MMTV-*neu* (ndl)-YD5 males were crossed with wild-type female FVB fat-1 mice. Only heteryzgous MMTV-*neu* (ndl)-YD5 female offspring were kept. The offspring were fed one of 5 maternal diets containing 1) 10% safflower oil (n-6 PUFA enriched), 2) 3% menhaden oil + 7% safflower oil (marine-derived n-3 PUFA enriched), 3) 3% flaxseed oil + 7% safflower oil (plant-derivd n-3 PUFA enriched), 4) 10% olive oil (MUFA) and 5) 10% lard (SFA). The 3% menhaden oil diet contains equivalent amounts of EPA and DHA to what is present in traditional Japanese diets, in which 1-2% of daily energy is derived from marine sources of n-3 PUFA (22). Previous research in the lab (26) studying dose-dependent effects of n-3 PUFA has determined that both 10% and 3% menhaden or flaxseed oil has an effect on attenuating tumor development and progression in the MMTV model. Dose-dependent effects of olive oil and lard have not yet been studied in the same capacity. Mice were maintained on diet for a 20- week period and designated as either sedentary controls or were placed on an exercise training program starting at 8 weeks of age for a period of 4 weeks. At week 12 mice were palpated for new tumors, and at detection, tumor dimensions were measured 3 times/week until euthanization at 20 weeks. To determine the effect of dietary fats on tumor fatty acid composition, fatty acid analysis by gas chromatography was performed on mammary tumor tissue in 20 week old mice.
Chapter 3

Exercise and Marine-derived n-3 Polyunsaturated Fatty Acids Independently Attenuate Tumorigenesis in Breast Cancer
3.1 Abstract

There is interest in utilizing lifestyle-based interventions in the prevention of breast cancer (BC). Evidence shows that implementation of exercise programs, as well as the consumption of different dietary fats may exert effects on BC development. Female MMTV-neu (ndl)-YD5 mice were fed (20 weeks) one of the following diets: 1) 10% kcal safflower oil, 2) 3% kcal menhaden oil+7% kcal safflower oil, 3) 3% flaxseed+7% kcal safflower oil, 4) 10% kcal olive oil, or 5) 10% kcal lard (n=8-12/experimental group). At 8 weeks of age, mice remained either sedentary or began an exercise intervention, consisting of five 45-minute treadmill running sessions a week for four weeks. Tumors were palpated and dimensions were measured using a digital caliper 3 times per week until termination at 20 weeks. Tumors were analyzed for fatty acid composition by gas chromatography. Exercise delayed tumor latency and mammary tumor development mice fed safflower oil. Menhaden oil reduced mammary tumor volume and multiplicity. Overall, this research furthers our understanding of lifestyle interventions as a strategy to reduce BC development.

3.2 Introduction

Breast cancer (BC) is among the top three most commonly diagnosed cancers in the world, and is the second leading cause of death in North American women (1). Although commonly cancer is caused by non-modifiable risk factors, such as genetic mutations resulting in uncontrolled growth and proliferation of mutagenic tumor cells, more than a third of breast cancers can be prevented by lifestyle modifications such as good nutritional or exercise habits (2). In fact, only 5-10% of breast cancers occurred as a result of genetic predisposition alone (92). Epidemiological evidence supports the benefits of diet and exercise in reducing breast
cancer risk. Asian populations consuming high amount of n-3 polyunsaturated fatty acids (PUFA) from marine sources such as fish and other seafood prevalent in their diet, or Mediterranean populations with high levels of olive oil-derived monounsaturated fatty acids (MUFA) have a lower incidence of BC compared to their sedentary, n-6 PUFA fatty acid (SFA)-consuming Western counterparts.

Asian populations consuming diets rich in different forms of n-3 PUFA, including eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3), have a reduced incidence of BC (4). However, Western populations with a high intake of n-6 PUFA, such as linoleic acid (LA, 18:2n-6), and lower in n-3 PUFA than their Asian counterparts displayed a higher incidence of BC (25). Asian-American women born in the West have a 60% higher risk of BC (18) partially attributed to the high n-6:n-3 PUFA ratio in the Western diet. N-6 PUFA such as LA and arachidonic acid (AA, 20:4n-6), are thought to be detrimental to health when present in large amounts in the diet. Their negative effect on health may be relative to n-3 PUFA α-linolenic acid (ALA, 18:3n-3) and downstream metabolites, EPA and DHA (25). EPA and DHA are found primarily in marine sources especially in fatty fish such as salmon, while ALA is found in plant sources such as flaxseed oil. Although not as potent as marine- derived n-3 PUFA, previous research (26) shows that ALA from flaxseed oil possesses protective effects in BC .

Monounsaturated fatty acids

Oleic acid (C18:1, OA), a MUFA found in olive oil has also been of recent interest as consumption of the Mediterranean diet is associated with a reduction of risk for various diseases, including cancer. Although the Mediterranean diet is also rich in foods containing polyphenols
and other biologically active compounds derived from fruits, vegetables, seafood and nuts, extra-virgin olive oil is a large component of this diet and is thought to exert independent effects on disease prevention (5, 34, 35). A recent meta-analysis of postmenopausal BC showed that adherence to the Mediterranean diet was significantly associated with a decrease risk in estrogen receptor negative (ER-) BC (93). However, further research is required to assess the specific effects by which oleic acid consumption affects BC.

*Saturated fatty acids*

Although the effects of dietary fats on BC risk have been studied, much less is known regarding the specific effects of SFA consumption as there is only a weak association between SFA consumption and BC risk (6, 13, 14). A recent case-cohort analysis found that BC risk was elevated in women with a greater percent incorporation of palmitic acid (16:0, PA) into plasma phospholipids (15). However, this may not be a direct reflection of intake as SFA can be endogenously synthesized from acetyl CoA, or used to produce oleic acid through elongation and desaturation (39). A systematic review found that the consumption of SFA was associated with increased circulating CRP in plasma, and significant associations with IL-6 but not TNFα or other inflammatory mediators (40). Further research in consistent models is required as current evidence regarding the effects of SFA is inconclusive, and it is unknown whether this could contribute to increased BC risk.

*Exercise*

Regular participation in moderate intensity exercise has also been associated with a decreased risk of cancer development specifically in BC (11). Physically active women engaging
in moderate- to vigorous-intensity exercise experienced a dose-dependent effect in BC risk reduction (55). One study using a transgenic mouse model, voluntary wheel running also induced dose-dependent effects on tumor development, with reduced mammary tumor volume and multiplicity observed in exercise mice compared to their sedentary counterparts (57). However, there is limited research on the specific mechanisms through which exercise exerts anti-tumorigenic effects. Additionally, results from several studies have demonstrated that both voluntary wheel running as well as treadmill running at a speed of 20 m/min may promote the progression of BC (63, 64). Further research investigating dose-dependent effects of exercise on a consistent model is required to elucidate the potential benefits of exercise in BC.

As emerging evidence suggests that cancer recurrence may be reduced through a combination of lifestyle factors, a combination of dietary fats such as n-3 PUFA and exercise training may provide a more protective benefit compared to changes in either diet or exercise alone. To date, there are no studies examining the specific mechanisms by which dietary fat and exercise together modulate BC risk, although they present a potential therapy in BC prevention and treatment. The objective of this study was to determine the effects of dietary fats, alone or in combination with exercise, in mitigating tumorigenesis in a BC model.

3.3 Materials and Methods

Animals and diets

Each harem consisted of one male heterozygous MMTV-neu(ndl)-YD5 mouse and three female FVB Fat-1 wild type mice. The mouse mammary tumor virus MMTV-neu(ndl)-YD5 model was developed to provide a murine equivalent to study HER2+ BC in humans. Due to its
highly aggressive phenotype, this model develops mammary tumors by 100 days of age, providing a relevant model to research dietary and exercise strategies to modulate tumorigenesis in BC (91).

Harems were randomly assigned to one of five diets (Research Diets Inc.): 1) 10% w/w safflower oil, 2) 3% w/w menhaden oil + 7% w/w safflower oil, 3) 3% w/w flaxseed oil + 7% w/w safflower oil, 4) 10% w/w olive oil and 5) 10% w/w lard diets. Mice were provided *ad libitum* access to diet and double-distilled water. Food intake was recorded 3 times per week. Fatty acid composition of diets was confirmed using gas chromatography (Table 1). Macronutrient composition of diets was provided by the manufacturer (Appendix). All experimental procedures were approved by the Animal Care Committee (University of Guelph).
Table 3.1. Fatty acid composition of diets. Fatty acid composition (%) of safflower, flaxseed, menhaden, olive and lard diets. Lipids were extracted from 3 pellets per diet and analyzed by gas chromatography.

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>10% Safflower Oil</th>
<th>3% Menhaden Oil</th>
<th>3% Flaxseed Oil</th>
<th>10% Olive Oil</th>
<th>10% Lard</th>
</tr>
</thead>
<tbody>
<tr>
<td>12:0</td>
<td>0.04</td>
<td>0.08</td>
<td>0.03</td>
<td>0.04</td>
<td>1.49</td>
</tr>
<tr>
<td>12:1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>13:0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>13:1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>14:0</td>
<td>0.24</td>
<td>2.67</td>
<td>0.22</td>
<td>0.16</td>
<td>1.28</td>
</tr>
<tr>
<td>14:1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>15:0</td>
<td>0</td>
<td>0.26</td>
<td>0.03</td>
<td>0</td>
<td>0.11</td>
</tr>
<tr>
<td>15:1c10</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>16:0</td>
<td>6.91</td>
<td>10.11</td>
<td>6.52</td>
<td>14.51</td>
<td>21.27</td>
</tr>
<tr>
<td>16:1c9</td>
<td>0.15</td>
<td>3.99</td>
<td>1.13</td>
<td>1.98</td>
<td>1.67</td>
</tr>
<tr>
<td>17:1c10</td>
<td>0</td>
<td>0.19</td>
<td>0.02</td>
<td>0.13</td>
<td>0.24</td>
</tr>
<tr>
<td>18:0</td>
<td>2.59</td>
<td>2.85</td>
<td>2.89</td>
<td>2.11</td>
<td>11.51</td>
</tr>
<tr>
<td>18:1c9</td>
<td>15.25</td>
<td>13.78</td>
<td>16.59</td>
<td>64.50</td>
<td>35.76</td>
</tr>
<tr>
<td>18:1c11</td>
<td>0.80</td>
<td>1.47</td>
<td>0.78</td>
<td>3.51</td>
<td>2.33</td>
</tr>
<tr>
<td>18:2n6</td>
<td>71.93</td>
<td>51.71</td>
<td>53.81</td>
<td>11.15</td>
<td>20.85</td>
</tr>
<tr>
<td>18:2tt</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>18:3n6</td>
<td>0.11</td>
<td>0.16</td>
<td>0</td>
<td>0</td>
<td>0.11</td>
</tr>
<tr>
<td>19:0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.10</td>
</tr>
<tr>
<td>19:1c7</td>
<td>0</td>
<td>0.18</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>18:3n3</td>
<td>0.31</td>
<td>0.86</td>
<td>17.30</td>
<td>0.66</td>
<td>1.56</td>
</tr>
<tr>
<td>18:4n3</td>
<td>0.13</td>
<td>0.90</td>
<td>0.14</td>
<td>0</td>
<td>0.17</td>
</tr>
<tr>
<td>20:0</td>
<td>0.36</td>
<td>0.36</td>
<td>0.51</td>
<td>0.39</td>
<td>0.22</td>
</tr>
<tr>
<td>20:1c5&amp;8</td>
<td>0.10</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>20:1c11</td>
<td>0.22</td>
<td>0.49</td>
<td>0.34</td>
<td>0.37</td>
<td>0.79</td>
</tr>
<tr>
<td>20:2n6</td>
<td>0.05</td>
<td>0.12</td>
<td>0.09</td>
<td>0</td>
<td>0.84</td>
</tr>
<tr>
<td>20:3n6</td>
<td>0</td>
<td>0.10</td>
<td>0</td>
<td>0</td>
<td>0.17</td>
</tr>
<tr>
<td>20:4n6</td>
<td>0</td>
<td>0.49</td>
<td>0</td>
<td>0</td>
<td>0.37</td>
</tr>
<tr>
<td>20:3n3</td>
<td>0</td>
<td>0.20</td>
<td>0</td>
<td>0</td>
<td>0.25</td>
</tr>
<tr>
<td>20:5n3</td>
<td>0.05</td>
<td>4.20</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>22:0</td>
<td>0.30</td>
<td>0.27</td>
<td>0.28</td>
<td>0.13</td>
<td>0</td>
</tr>
<tr>
<td>22:1n9</td>
<td>0.02</td>
<td>0.09</td>
<td>0.02</td>
<td>0.03</td>
<td>0.02</td>
</tr>
<tr>
<td>22:2n6</td>
<td>0</td>
<td>0.27</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>22:4n6</td>
<td>0.11</td>
<td>0.14</td>
<td>0</td>
<td>0</td>
<td>0.12</td>
</tr>
<tr>
<td>22:3n3</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>
Offspring were weaned and genotyped at three weeks of age as described previously (19). Female transgenic offspring were placed on maternal diets and male offspring were terminated. Mice were housed together in ventilated cages with a maximum number of 4 mice per cage. Sedentary and exercised mice were housed separately. Starting at 3 weeks of age, female mice were checked daily for vaginal opening, a marker of puberty onset. Body weights were recorded weekly.

**Exercise protocol**

Offspring were randomly assigned to an exercise group or remained control for each diet (n=8-12 per group). At 7 weeks of age, animals in the exercise group were acclimated to the treadmill for 15 minutes a day for 3 days a week. During acclimation, the treadmill was not turned on. The exercise intervention began at 8 weeks of age for a period of four weeks, and consisted of five 45-minute exercise sessions a week. The treadmill was set to a 5° incline and speed was set to 10 m/min at week 8 and increased by 2 m/min up to 16 m/min at week 12. Mice exercising within this range of speed are at 74-80% maximal oxygen consumption (VO₂ max) (114). In humans, exercising at 60-84% VO₂ max is considered to be vigorous intensity (115).
**Mammary tumor measurements**

Starting at 10 weeks of age, tumors were palpated and measured using a digital caliper. Tumor measurements were taken 3 times per week when a new tumor was detected until termination at 20 weeks of age. Tumor volume was calculated using \( V = \frac{\text{length} \times (\text{width}^2)}{2} \).

**Euthanization and tissue collection**

Mice were terminated at 20 weeks of age by CO\(_2\) asphyxiation. If tumor dimensions exceeded 17mm in length or width, or had tumors more than 5000 mm\(^3\), mice were terminated prior to the 20-week endpoint. Two mice fed the olive oil diet were terminated early due to tumors under the jaw that prevented animals from eating. The vagina was flushed with 30 μL of phosphate buffer solution. The solution was observed under a microscope (Nikon Eclipse TS100) on a glass slide to determine stage of estrous cycle. Mice were terminated if in proestrus, estrus, or metestrus stages of the estrous cycle. If in the diestrus stage, termination was delayed for a maximum of 2 days to control for hormonal fluctuations and subsequent effects on markers of proliferation.

At time of termination, blood was collected by cardiac puncture. After 20 minutes of clot time, blood was spun by centrifugation (10,000 x g for 5 minutes) to separate red blood cells from plasma. The mouse pelt with mammary gland and tumors attached was removed for measurements of final tumor dimensions, and tumors were later removed and weighed. One tumor and uninvolved MG was set aside to be fixed in formaldehyde for future mounting, staining and immunohistochemistry analysis. Remaining tissues (mammary glands, muscle,
visceral adipose, vagina, uterus, liver, spleen, kidneys, lungs, heart, brain and eyes) were collected, snap-frozen in liquid nitrogen and stored in a -80°C freezer for future analysis.

**Fatty acid analysis**

Lipid composition of tumor tissue was determined by gas chromatography as described previously (12).

Lipid extraction: 0.1g of tissue was homogenized in 1.0 mL of 0.1M KCl and 2 mL of 2:1 chloroform:methanol. The homogenate was further rinsed with another 2 mL of 2:1 chloroform:methanol. The samples were vortexed and flushed with nitrogen before being stored at 4°C overnight. The following day the samples were centrifuged at 1460 rpm for 10 minutes to separate phases. The lower chloroform layer was dried down under nitrogen and reconstituted in 300 µL chloroform to 10 mg/ml.

Phospholipid class TLC: Lipid samples extracted from tumor tissue were spotted on an activated H-plate (EMD Chemicals, #5721-7). TLC solvent (30 ml chloroform, 9 ml methanol, 25 ml 2-propanol, 6 ml 0.25M KCl and 18 ml trimethylamine) was prepared at the time of the experiment. The plate was placed in solvent for ~1.5 hours, after which the plate was removed from solvent and sprayed with 0.1% (w/v) ANSA (Fluka, #GA12046). Phospholipid bands were visualized under UV light. Sphingomyelin (SM), phosphatidylcholine (PC), phosphatidylserine (PS) and phosphatidylthanolamine (PE) bands were collected for analysis. A standard (C17:0 FFA) was added to each sample.

Methylation: Hexane and 14% BF3-MeOH (Sigma, N1252) were added to each sample and methylated at 100°C for 90 minutes. To stop methylation 2 ml ddH2O was added to each.
sample. Samples were then centrifuged at 1460 rpm for 10 minutes. The hexane layer was collected, dried down under nitrogen, and reconstituted in 500 μL of hexane. Samples were analyzed for fatty acid composition using gas chromatography (Agilent Technologies 6890). Fatty acid composition was expressed as a percentage of total fatty acids.

**Statistical analysis**

A one-way analysis of variance (ANOVA) was used to determine differences in puberty onset, tumor onset, final tumor weight and volume and fatty acids composition of tumors between experimental groups. If significant (p< 0.05) differences were found, Tukey’s multiple comparison test was conducted. A Shapiro-Wilk test was used to test normality. Variables were log or inverse-log transformed if data were not normally distributed. A Log-Tank test was used to determine differences in the percent of tumor-free mice between experimental groups. A repeated-measures test was used to determine differences in tumor volume and multiplicity over a period of 20 weeks between experimental groups. As there were no significant differences in tumor volume and multiplicity between sedentary and exercising mice within dietary groups, data was pooled by diet. Tumor weight, volume and multiplicity in situ measured at euthanization were analyzed by two-way ANOVA.

As there was no significant difference in fatty acid composition of tumors between sedentary and exercise mice within dietary groups as determined by a two-way ANOVA, data was pooled by dietary group and fatty acid composition of tumors was analyzed using a one-way ANOVA followed by a Tukey’s Studentized Range test if justified by statistical significance.

All analyses were performed using SASv9.1.
3.4 Results

Body weight, food intake and puberty onset

There were no significant differences between dietary groups, or between exercising and sedentary mice within each dietary group in weekly food intake; however, body weights were lower in exercising mice fed lard (p=0.0006), olive (p=0.0024) and menhaden oil (p=0.0002) compared to sedentary mice but not compared to mice fed safflower (p=0.4876) or flaxseed oil (p=0.2101) (3-20 weeks of age) (Appendix). However, food intake was higher in exercising mice fed safflower (p=0.0452) or flaxseed oil (p=0.0372), but not in mice fed lard (p=0.1524), olive (p=0.0517) or menhaden oil (p=0.6046). (Appendix). Puberty onset was assessed by age at vaginal opening. The onset of puberty was delayed (p<0.0001) in mice fed menhaden oil or safflower oil compared to mice fed lard. Additionally, mice fed a menhaden oil diet showed a delay (p<0.0001) delay in puberty onset compared to mice fed olive oil, flaxseed oil and lard.
Figure 3.1 Puberty onset. Puberty onset as assessed by age in days at vaginal opening in mice fed either a 10% w/w safflower oil diet (n=15), 3% w/w menhaden oil and 7% w/w safflower oil diet (n=16), 3% w/w flaxseed oil and 7% w/w safflower oil diet (n=25), 10% w/w lard diet, or 10% w/w olive oil diet (n=23).

Spleen weights

Spleen weights were recorded at time of euthanization as increased spleen weights have been associated with an increased in inflammatory markers and tumor burden in a study examining the role of exercise on inflammation in BC (94). There were no differences (p=0.5034) between dietary groups, or between exercised and sedentary mice within each dietary group (Appendix).
Primary tumor outcomes (Tumor onset, volume, multiplicity, survival curve)

*Tumor latency and tumor-free status*

Average tumor latency was extended (p<0.0001) in exercised mice fed a safflower oil diet (106.4±16.2 days) compared to sedentary mice (90.3±6.1 days). However, there was no significant difference between sedentary and exercised mice within other dietary groups (Table 3.2.2). Tumor-free status followed the same trend. At $T_{50}$ (point at which 50% of mice are tumor-free), exercise delayed (p=0.0112) the development of tumors in mice fed a safflower oil diet (104 days) by ~59% compared to sedentary mice (43 days). No differences were observed between sedentary and exercised mice fed lard (p=0.8319), olive oil (p=0.6451), flaxseed oil (p=0.1244) or menhaden oil (p=0.6851). (Figure 3.3).

**Table 3.2 Average tumor latency.** The comparison of average tumor latency in sedentary or exercising mice fed either a 10% w/w safflower oil diet (n=21), 10% w/w lard diet (n=21), 10% w/w olive oil diet (n=16), 3% w/w flaxseed oil and 7% w/w safflower oil diet (n=19) or 3% w/w menhaden oil and 7% w/w safflower oil diet (n=18). Data was analyzed using a t-test. Values are displayed as mean ± standard deviation.

<table>
<thead>
<tr>
<th>Diet</th>
<th>Average tumor latency (days) in SED mice</th>
<th>Average tumor latency (days) in EX mice</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Safflower</td>
<td>106.4±16.2 days</td>
<td>90.3±6.1</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Olive</td>
<td>109.2±6.6</td>
<td>111.3±5.9</td>
<td>0.46</td>
</tr>
<tr>
<td>Lard</td>
<td>104.8±13.8</td>
<td>105.6±9.3</td>
<td>0.87</td>
</tr>
<tr>
<td>Flax</td>
<td>108.4±9.6</td>
<td>100.9±7.2</td>
<td>0.33</td>
</tr>
<tr>
<td>Menhaden</td>
<td>101.5±8.2</td>
<td>101.1±11.2</td>
<td>0.99</td>
</tr>
</tbody>
</table>
**Figure 3.2 Tumor latency.** Tumor latency in sedentary or exercising mice fed either a 10% w/w safflower oil diet (n=21), 10% w/w lard diet (n=21), 10% w/w olive oil diet (n=16), 3% w/w flaxseed oil and 7% w/w safflower oil diet (n=19) or 3% w/w menhaden oil and 7% w/w safflower oil diet (n=18). Error bars represent standard deviation. All data were analyzed by two-way ANOVA (p<0.05). Significant differences between sedentary and exercising mice within dietary groups are denoted with an asterisk (*).
Figure 3.3 Percentage of mice without tumors over time. The percentage of tumor-free mice over time in sedentary or exercising mice fed either a (A) 10% w/w safflower oil diet (n=21), (B) 10% w/w lard diet (n=21), (C) 10% w/w olive oil diet (n=16), (D) 3% w/w flaxseed oil and 7% w/w safflower oil diet (n=19) or (E) 3% w/w menhaden oil and 7% w/w safflower oil diet (n=18). Data were analyzed using a Log-Rank test (p<0.05). Significant differences are denoted by an asterisk (*).
Tumor volume

Menhaden oil reduced (p=0.0334) tumor volume compared to safflower oil, but not compared to lard (p=0.4986), olive oil (p=0.7785) or flaxseed oil (p=0.9367). Additionally, lard (p=0.022), olive oil (p=0.0232) and flaxseed oil (0.0202) reduced tumor volume compared to safflower oil. No significant differences were observed between sedentary and exercising mice within each dietary group.

Figure 3.4 Average tumor volume over time compared by dietary group. The comparison of average tumor volume over time in mice fed either a 10% w/w safflower oil diet (n=21), 3% w/w menhaden oil and 7% w/w safflower oil diet (n=18), 3% w/w flaxseed oil and 7% w/w safflower oil (n=19), 10% w/w olive oil diet (n=16) or10% w/w lard diet (n=21). Tumor measurements
were recorded 3 times/week. Data were analyzed by repeated measures ANOVA (p<0.05). Lines not sharing a common letter are significantly different from one another.
A

Tumor volume (mm³)

Age (weeks)

Sedentary

Exercise

B

C

Tumor volume (mm³)

Age (weeks)

Sedentary

Exercise
Figure 3.5 Average tumor volume over time within dietary groups. The comparison of average tumor volume over time in sedentary or exercising mice fed either a 10% w/w safflower oil diet (n=21), 3% w/w menhaden oil and 7% w/w safflower oil diet (n=18), 3% w/w flaxseed oil and 7% w/w safflower oil (n=19), 10% w/w olive oil diet (n=16) or 10% w/w lard diet (n=21). Tumor measurements were recorded 3 times/week. Data were analyzed by repeated measures ANOVA (p<0.05).
**Tumor multiplicity**

Menhaden oil reduced tumor multiplicity compared to safflower oil (p=0.0078), lard (p<0.0001) and flaxseed oil (o<0.0001), but not compared to olive oil (p=0.0734). Olive oil reduced tumor multiplicity compared to lard (p=0.0079) and flaxseed oil (p=0.0029), but not compared to safflower oil (p=0.2343). No significant differences in tumor multiplicity were observed between sedentary and exercising mice within each dietary group.

![Figure 3.6 Average tumor multiplicity over time compared by dietary group.](image)

The comparison of average number of tumors over time in mice fed either a 10% w/w safflower oil diet (n=21), 3% w/w menhaden oil and 7% w/w safflower oil diet (n=18), 3% w/w flaxseed oil and 7% w/w safflower oil (n=19), 10% w/w olive oil diet (n=16) or10% w/w lard diet (n=21). Tumor measurements were recorded 3 times/week. Data were analyzed by repeated measures ANOVA (p<0.05). Lines not sharing a common letter are significantly different from one another.
Figure 3.7 Average tumor multiplicity over time within dietary groups The comparison of average number of tumors over time in sedentary or exercising mice fed either a 10% w/w safflower oil diet (n=21), 3% w/w menhaden oil and 7% w/w safflower oil diet (n=18), 3% w/w flaxseed oil and 7% w/w safflower oil (n=19), 10% w/w olive oil diet (n=16) or 10% w/w lard diet (n=21). Tumor measurements were recorded 3 times/week. Data were analyzed by repeated measures ANOVA (p<0.05).
**In situ tumor measurements**

*In situ* tumor weights, volume and multiplicity were recorded at euthanization. Menhaden oil decreased (p=0.0022) tumor weight compared to olive and lard diets as determined by a two-way ANOVA; however there were no differences in tumor volume due to exercise (p=0.0700) or diet*exercise interaction (0.4676) (Appendix). There was a significant diet*exercise interaction effect on tumor volume (p=0.0427) but no differences due to diet (p=0.5726) or exercise (p=0.00753) alone as determined by a two-way ANOVA (Appendix). There were no differences (p=0.8595) in tumor multiplicity between experimental groups as determined by a two-way ANOVA.

**Fatty acid composition of tumor phospholipids**

Phospholipid analysis of mammary tumors was conducted to determine if the observed effects on primary tumor outcomes could be attributed to the incorporation of dietary fats into the target tissue. Dietary fat significantly affected the fatty acid composition of phospholipid fractions in mammary tumors. The percent composition (including all fatty acids) is reported in Tables 3.3-3.6.

In the SM fraction (Table 3.3), the mammary tumors of mice fed menhaden or flaxseed oil were composed of higher (p=0.0246) quantities of LA (18:2n-6) compared to mice fed lard, but not mice fed safflower or olive oil. Mice fed olive oil had higher (p=0.0009) quantities of OA (18:1) compared to all other diets, and significantly higher quantities in lard compared to safflower, menhaden or flaxseed oil-fed mice.
In the PC fraction (Table 3.4), the mammary tumors of mice fed olive oil or lard had higher (p=0.0008) amounts of OA (18:1) in mammary tumors compared to menhaden, safflower and flaxseed oil-fed mice. Mice fed flaxseed or menhaden oil had higher (p=0.0007) amounts of LA (18:2n-6) present in tumors compared to lard and olive oil-fed mice, but not safflower. EPA (20:5n-3) was increased (p=0.0001) in mice fed menhaden compared to olive oil, and DHA (22:6n-3) was increased (p=0.0129) in mice fed menhaden oil compared to all diets.

In the PS fraction (Table 3.5), the mammary tumors of mice fed menhaden, lard or olive oil were higher (p=0.0008) in OA (18:1) compared to mice fed safflower oil, but not flaxseed oil.

In the PE fraction (Table 3.6), the mammary tumors of mice fed olive oil were composed of higher (p=0.0008) quantities of OA (18:1) compared to all diets. Mammary tumors of mice fed lard had significantly higher amounts of OA (18:1) (p=0.0008) compared to mice fed safflower, menhaden or flaxseed oil. LA (18:2n-6) was increased (p=0.0007) in flax compared to lard and olive oil, but not menhaden or safflower oils. DPA was significantly increased in mammary tumors of mice fed safflower oil compared to all other diets. DHA (22:6n-3) was increased (p=0.0129) in mammary tumors of mice fed menhaden oil compared to all other diets.
Table 3.3 Composition of fatty acids in SM fraction of mammary tumor phospholipids. Values are expressed as % composition. Letter within a row indicate significant differences between dietary groups (n=6/dietary group) for the particular fatty acid. Dietary groups sharing the same letters showed no significant differences in fatty acid composition. Values are displayed as mean ± standard deviation. Data were analyzed using a one-way ANOVA (p<0.05).

<table>
<thead>
<tr>
<th>Fatty Acids</th>
<th>Tumor fatty acid composition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10% Safflower</td>
</tr>
<tr>
<td>12:0</td>
<td>0.0±0.0</td>
</tr>
<tr>
<td>14:0</td>
<td>1.7±0.5</td>
</tr>
<tr>
<td>15:0</td>
<td>0.2±0.4</td>
</tr>
<tr>
<td>16:0</td>
<td>37.5±2.6</td>
</tr>
<tr>
<td>18:0</td>
<td>9.1±2.3</td>
</tr>
<tr>
<td>20:0</td>
<td>0.5±0.6</td>
</tr>
<tr>
<td>22:0</td>
<td>0.3±1.3</td>
</tr>
<tr>
<td>24:0</td>
<td>3.6±1.1</td>
</tr>
<tr>
<td>Total SFA</td>
<td>52.9</td>
</tr>
<tr>
<td>16:1c-9</td>
<td>2.5±0.6</td>
</tr>
<tr>
<td>18:1c-9</td>
<td>8.2±1.5 b</td>
</tr>
<tr>
<td>22:1n-9</td>
<td>3.0±1.8</td>
</tr>
<tr>
<td>24:1</td>
<td>15.4±5.3</td>
</tr>
<tr>
<td>Total MUFA</td>
<td>29.1</td>
</tr>
<tr>
<td>18:2n-6</td>
<td>6.8±3.1 ab</td>
</tr>
<tr>
<td>18:3n-3</td>
<td>0.0±0.0</td>
</tr>
<tr>
<td>20:2n-6</td>
<td>0.1±0.2</td>
</tr>
<tr>
<td>20:3n-6</td>
<td>0.6±1.0 b</td>
</tr>
<tr>
<td>20:4n-6</td>
<td>3.6±2.3</td>
</tr>
<tr>
<td>20:3n-3</td>
<td>0.0±0.0</td>
</tr>
<tr>
<td>20:5n-3</td>
<td>0.0±0.0</td>
</tr>
<tr>
<td>22:2n-6</td>
<td>0.1±0.2 a</td>
</tr>
<tr>
<td>22:4n-6</td>
<td>0.1±0.3</td>
</tr>
<tr>
<td>22:3n-3</td>
<td>0.0±0.0</td>
</tr>
<tr>
<td>22:5n-6</td>
<td>0.1±0.4</td>
</tr>
<tr>
<td>22:5n-3</td>
<td>0.0±0.0</td>
</tr>
<tr>
<td>22:6n-3</td>
<td>0.0±0.0</td>
</tr>
<tr>
<td>Total PUFA</td>
<td>11.4</td>
</tr>
</tbody>
</table>
Table 3.4 Composition of fatty acids in PC fraction of mammary tumor phospholipids. Values are expressed as % composition. Letter within a row indicate significant differences between dietary groups (n=6/dietary group) for the particular fatty acid. Dietary groups sharing the same letters showed no significant differences in fatty acid composition. Values are displayed as mean ± standard deviation. Data were analyzed using a one-way ANOVA (p<0.05).

<table>
<thead>
<tr>
<th>Fatty Acids</th>
<th>Tumor fatty acid composition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PC</td>
</tr>
<tr>
<td></td>
<td>10% Safflower</td>
</tr>
<tr>
<td>12:0</td>
<td>0.0±0.0</td>
</tr>
<tr>
<td>14:0</td>
<td>1.1±0.3</td>
</tr>
<tr>
<td>15:0</td>
<td>0.2±0.0</td>
</tr>
<tr>
<td>16:0</td>
<td>29.8±2.8</td>
</tr>
<tr>
<td>18:0</td>
<td>8.4±1.1</td>
</tr>
<tr>
<td>20:0</td>
<td>0.1±0.1</td>
</tr>
<tr>
<td>22:0</td>
<td>0.1±0.1</td>
</tr>
<tr>
<td>24:0</td>
<td>1.0±0.7 B</td>
</tr>
<tr>
<td>Total SFA</td>
<td>40.7</td>
</tr>
<tr>
<td>16:1c-9</td>
<td>2.9±0.8</td>
</tr>
<tr>
<td>18:1c-9</td>
<td>13.2±1.1 C</td>
</tr>
<tr>
<td>22:1n-9</td>
<td>0.5±0.1</td>
</tr>
<tr>
<td>24:1</td>
<td>0.5±0.2</td>
</tr>
<tr>
<td>Total MUFA</td>
<td>17.1</td>
</tr>
<tr>
<td>18:2n-6</td>
<td>10.9±4.8 AB</td>
</tr>
<tr>
<td>18:3n-3</td>
<td>0.2±0.2 A</td>
</tr>
<tr>
<td>20:2n-6</td>
<td>0.3±0.1 A</td>
</tr>
<tr>
<td>20:3n-6</td>
<td>3.4±1.4 AB</td>
</tr>
<tr>
<td>20:4n-6</td>
<td>14.2±4.0</td>
</tr>
<tr>
<td>20:3n-3</td>
<td>0.1±0.1 B</td>
</tr>
<tr>
<td>20:5n-3</td>
<td>0.5±0.7 AB</td>
</tr>
<tr>
<td>22:2n-6</td>
<td>0.3±0.1 A</td>
</tr>
<tr>
<td>22:4n-6</td>
<td>1.2±0.2 A</td>
</tr>
<tr>
<td>22:3n-3</td>
<td>0.1±0.1</td>
</tr>
<tr>
<td>22:5n-6</td>
<td>1.1±0.4 A</td>
</tr>
<tr>
<td>22:5n-3</td>
<td>0.6±0.5</td>
</tr>
<tr>
<td>22:6n-3</td>
<td>1.0±0.7 B</td>
</tr>
<tr>
<td>Total PUFA</td>
<td>33.9</td>
</tr>
</tbody>
</table>
**Table 3.5** Composition of fatty acids in PS fraction of mammary tumor phospholipids. Values are expressed as % composition. Letter within a row indicate significant differences between dietary groups (n=6/dietary group) for the particular fatty acid. Dietary groups sharing the same letters showed no significant differences in fatty acid composition. Values are displayed as mean ± standard deviation. Data were analyzed using a one-way ANOVA (p<0.05).

<table>
<thead>
<tr>
<th>Fatty Acids</th>
<th>10% Safflower</th>
<th>10% Lard</th>
<th>10% Olive</th>
<th>3% Flaxseed</th>
<th>3% Menhaden</th>
</tr>
</thead>
<tbody>
<tr>
<td>12:0</td>
<td>0.0±0.0</td>
<td>0.0±0.0</td>
<td>0.0±0.0</td>
<td>0.0±0.0</td>
<td>0.0±0.0</td>
</tr>
<tr>
<td>14:0</td>
<td>0.0±0.0</td>
<td>0.0±0.0</td>
<td>0.0±0.0</td>
<td>0.0±0.0</td>
<td>0.0±0.0</td>
</tr>
<tr>
<td>15:0</td>
<td>0.0±0.0</td>
<td>0.0±0.0</td>
<td>0.0±0.0</td>
<td>0.0±0.0</td>
<td>0.0±0.0</td>
</tr>
<tr>
<td>16:0</td>
<td>0.9±0.5</td>
<td>1.1±0.2</td>
<td>0.8±0.4</td>
<td>1.3±1.2</td>
<td>1.4±1.0</td>
</tr>
<tr>
<td>18:0</td>
<td>13.6±6.8</td>
<td>21.8±1.8</td>
<td>20.7±3.3</td>
<td>24.6±21.4</td>
<td>16.4±3.2</td>
</tr>
<tr>
<td>20:0</td>
<td>0.0±0.0</td>
<td>0.0±0.0</td>
<td>0.0±0.0</td>
<td>0.0±0.0</td>
<td>0.0±0.0</td>
</tr>
<tr>
<td>22:0</td>
<td>1.5±0.8</td>
<td>2.0±0.3</td>
<td>2.5±1.1</td>
<td>0.9±0.6</td>
<td>1.4±0.6</td>
</tr>
<tr>
<td>24:0</td>
<td>1.4±0.3^B</td>
<td>3.4±0.5^A</td>
<td>4.5±0.6^A</td>
<td>4.1±1.9^A</td>
<td>1.7±2.8^B</td>
</tr>
<tr>
<td>Total SFA</td>
<td>17.4</td>
<td>28.3</td>
<td>28.5</td>
<td>30.9</td>
<td>20.9</td>
</tr>
<tr>
<td>16:1c-9</td>
<td>0.0±0.0</td>
<td>0.0±0.0</td>
<td>0.0±0.0</td>
<td>0.0±0.0</td>
<td>0.0±0.0</td>
</tr>
<tr>
<td>18:1c-9</td>
<td>2.8±1.5^B</td>
<td>4.6±0.8^A</td>
<td>4.1±1.1^A</td>
<td>3.5±1.3^AB</td>
<td>4.2±1.6^A</td>
</tr>
<tr>
<td>22:1n-9</td>
<td>0.0±0.0</td>
<td>0.0±0.0</td>
<td>0.0±0.0</td>
<td>0.0±0.0</td>
<td>0.0±0.0</td>
</tr>
<tr>
<td>24:1</td>
<td>0.2±0.4</td>
<td>1.4±0.2</td>
<td>2.5±0.2</td>
<td>0.7±0.4</td>
<td>0.7±0.6</td>
</tr>
<tr>
<td>Total MUFA</td>
<td>3.0</td>
<td>6.0</td>
<td>6.6</td>
<td>4.2</td>
<td>4.9</td>
</tr>
<tr>
<td>18:2n-6</td>
<td>0.0±0.0</td>
<td>0.0±0.0</td>
<td>0.0±0.0</td>
<td>0.0±0.0</td>
<td>0.0±0.0</td>
</tr>
<tr>
<td>18:3n-3</td>
<td>0.0±0.0</td>
<td>0.0±0.0</td>
<td>0.0±0.0</td>
<td>0.0±0.0</td>
<td>0.0±0.0</td>
</tr>
<tr>
<td>20:2n-6</td>
<td>0.0±0.0</td>
<td>0.0±0.0</td>
<td>0.0±0.0</td>
<td>0.0±0.0</td>
<td>0.0±0.0</td>
</tr>
<tr>
<td>20:3n-6</td>
<td>0.0±0.0</td>
<td>0.0±0.0</td>
<td>0.0±0.0</td>
<td>0.0±0.0</td>
<td>0.0±0.0</td>
</tr>
<tr>
<td>20:4n-6</td>
<td>0.0±0.0</td>
<td>0.0±0.0</td>
<td>0.0±0.0</td>
<td>0.0±0.0</td>
<td>0.0±0.0</td>
</tr>
<tr>
<td>20:3n-3</td>
<td>0.0±0.0</td>
<td>0.1±0.3</td>
<td>0.0±0.0</td>
<td>0.2±0.3</td>
<td>0.3±0.4</td>
</tr>
<tr>
<td>20:5n-3</td>
<td>0.6±0.4</td>
<td>0.4±0.3</td>
<td>0.7±0.4</td>
<td>0.4±0.3</td>
<td>0.6±0.5</td>
</tr>
<tr>
<td>22:2n-6</td>
<td>1.2±0.1</td>
<td>1.9±0.5</td>
<td>3.0±1.2</td>
<td>1.0±0.5</td>
<td>0.5±0.8</td>
</tr>
<tr>
<td>22:4n-6</td>
<td>0.0±0.0^B</td>
<td>0.0±0.0^B</td>
<td>0.0±0.0^B</td>
<td>0.0±0.0^B</td>
<td>1.5±1.9^A</td>
</tr>
<tr>
<td>22:3n-3</td>
<td>0.0±0.0</td>
<td>1.3±0.3</td>
<td>2.3±1.0</td>
<td>0.2±0.4</td>
<td>0.2±0.6</td>
</tr>
<tr>
<td>22:5n-6</td>
<td>0.0±0.0</td>
<td>1.0±0.5</td>
<td>2.8±1.0</td>
<td>1.6±0.7</td>
<td>0.0±0.0</td>
</tr>
<tr>
<td>22:5n-3</td>
<td>6.6±2.6</td>
<td>1.0±0.4</td>
<td>1.8±0.7</td>
<td>0.7±0.4</td>
<td>7.7±5.0</td>
</tr>
<tr>
<td>22:6n-3</td>
<td>0.2±0.4</td>
<td>1.4±0.2</td>
<td>2.5±0.2</td>
<td>0.7±0.4</td>
<td>0.7±0.6</td>
</tr>
<tr>
<td>Total PUFA</td>
<td>8.6</td>
<td>7.1</td>
<td>13.1</td>
<td>4.8</td>
<td>11.5</td>
</tr>
</tbody>
</table>

Data were analyzed using a one-way ANOVA (p<0.05).
Table 3.6 Composition of fatty acids in PE fraction of mammary tumor phospholipids. Values are expressed as % composition. Letter within a row indicate significant differences between dietary groups (n=6/dietary group) for the particular fatty acid. Dietary groups sharing the same letters showed no significant differences in fatty acid composition. Values are displayed as mean ± standard deviation. Data were analyzed using a one-way ANOVA (p<0.05).

<table>
<thead>
<tr>
<th>Fatty Acids</th>
<th>Tumor fatty acid composition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10% Safflower</td>
</tr>
<tr>
<td>12:0</td>
<td>0.0±0.0</td>
</tr>
<tr>
<td>14:0</td>
<td>0.3±0.1</td>
</tr>
<tr>
<td>16:0</td>
<td>6.6±0.5</td>
</tr>
<tr>
<td>18:0</td>
<td>14.4±2.9</td>
</tr>
<tr>
<td>20:0</td>
<td>0.0±0.0</td>
</tr>
<tr>
<td>22:0</td>
<td>0.0±0.0</td>
</tr>
<tr>
<td>24:0</td>
<td>0.0±0.0</td>
</tr>
<tr>
<td>Total SFA</td>
<td>21.5</td>
</tr>
<tr>
<td>16:1c-9</td>
<td>1.8±0.3</td>
</tr>
<tr>
<td>18:1n-9</td>
<td>19.9±1.2</td>
</tr>
<tr>
<td>22:1n-9</td>
<td>0.1±0.1</td>
</tr>
<tr>
<td>24:1</td>
<td>0.3±0.5</td>
</tr>
<tr>
<td>Total MUFA</td>
<td>22.1</td>
</tr>
<tr>
<td>18:2n-6</td>
<td>9.5±0.9</td>
</tr>
<tr>
<td>18:3n-3</td>
<td>0.3±0.3</td>
</tr>
<tr>
<td>20:2n-6</td>
<td>0.0±0.0</td>
</tr>
<tr>
<td>20:3n-6</td>
<td>4.2±0.6</td>
</tr>
<tr>
<td>20:4n-6</td>
<td>17.8±8.6</td>
</tr>
<tr>
<td>20:3n-3</td>
<td>0.0±0.0</td>
</tr>
<tr>
<td>20:5n-3</td>
<td>0.9±0.2</td>
</tr>
<tr>
<td>22:2n-6</td>
<td>0.0±0.0</td>
</tr>
<tr>
<td>22:4n-6</td>
<td>2.6±3.5</td>
</tr>
<tr>
<td>22:3n-3</td>
<td>0.0±0.0</td>
</tr>
<tr>
<td>22:5n-6</td>
<td>0.4±0.1</td>
</tr>
<tr>
<td>22:5n-3</td>
<td>5.8±9.5</td>
</tr>
<tr>
<td>22:6n-3</td>
<td>7.6±2.4</td>
</tr>
<tr>
<td>Total PUFA</td>
<td>49.1</td>
</tr>
</tbody>
</table>
3.5 Discussion

The present study is the first to compare the effects of a wide range of dietary fats in combination with exercise on mammary tumor development. Although there is both epidemiological and experimental evidence supporting the beneficial effects of various dietary fats or exercise in BC (13, 14, 34, 37, 38, 43, 57, 58) there is still a gap in research involving the combined effects of specific dietary fats and exercise on BC development. Using the MMTV-neu (ndl)YD5 transgenic mouse model, the present study demonstrated that exercise attenuated the development of mammary tumors in mice being fed an n-6 PUFA rich diet. Additionally, marine-derived n-3 PUFA decreased tumor volume compared to n-6 PUFA, and decreased tumor multiplicity compared to all diets. Therefore, lifelong exposure to dietary fat can have an effect on mammary tumor outcomes.

Food intake and body weight

With the exception of mice fed safflower or flaxseed oil, body weight in exercising mice was decreased compared to sedentary mice; however, food intake was increased in exercising mice fed safflower or flaxseed oil. This is consistent with research regarding energy expenditure, which demonstrates that individuals with a decreased caloric intake or those exercising weigh less than sedentary counterparts (116). Given that exercising mice fed safflower oil had delayed mammary tumor development in the absence of a change in body weight, our data support the notion that the benefits of exercise in BC can occur independently of decreased body weight. Although a mechanism for the anti-tumor effects of exercise in the absence of a change in body weight has not been established, it is speculated that exercise may decrease estrogen levels (117, 118), which are associated with tumorigenesis in BC (62). One systematic review found that the
effects of exercise on body weight were less common compared to a change in body composition in BC survivors; therefore body composition may be a more useful marker in determining the effects of exercise in BC (119). Overall, research regarding the role of exercise in modulating body weight and body composition in BC is sparse and warrants further study.

**Role of dietary fat in puberty onset**

The present study provides evidence that the onset of puberty is significantly delayed in mice fed a menhaden oil diet compared to olive, flax and lard diets, as well as in mice fed a safflower oil diet compared to lard (Fig. 3.1). Earlier puberty onset has been linked to an increased risk of developing BC (95). Therefore, a diet high in n-3 PUFA may reduce BC occurrence through a delay in puberty onset. A possible mechanism for improved tumor outcomes as a result of n-3 PUFA consumption could be due to reduced levels of estrogen through a decrease in PGE2, which stimulates the expression of aromatase, an enzyme which converts androgens to estrogens (96). Increased estrogen levels are associated with earlier onset of puberty, specifically age of menarche in females (95). Serum estradiol was not assessed. Further research is required to assess the role of dietary fats on estrogen on puberty onset, and the relationship to mammary tumor development.

**Role of dietary fat in modulating mammary tumor outcomes**

This study demonstrates that lifelong exposure to dietary fat modulates tumorigenesis in BC. Most notably, menhaden oil reduced tumor volume (Fig. 3.4) and multiplicity (Fig. 3.5) compared to safflower oil, as demonstrated by previous research (26). Surprisingly, olive oil and lard exerted protective benefits compared to the safflower group. Experimental findings show
that a diet high in booth ALA and LC n-3 PUFA attenuates tumorigenesis in BC, inhibiting BC growth, angiogenesis and metastasis, compared to diets high in n-6 PUFA (26, 97-102). Although it was hypothesized that n-3 PUFA (marine or plant derived) would decrease mammary tumorigenesis compared to SFA, there have not been any animal studies comparing the role of n-6 PUFA, n-3 PUFA, MUFA and SFA on BC outcomes in a single animal study, therefore relative effects of these dietary fats on tumorigenesis have not yet been established. OA, a MUFA found in olive oil, was found to inhibit tumor cell growth in a MCF-7 BC cell line (103), and also had anti-proliferative effects in vitro through the modulation of cancer-promoting oncogenes (34). OA also improved chemosensitivity to paclitaxil, a therapeutic drug used to treat BC, therefore enhancing cytotoxity in a MCF-7 BC cell line (34). In humans, the Primary Prevention of Cardiovascular Disease with a Mediterranean Diet (PREDIMED) study, a large-scale intervention study analyzing the effects of the Mediterranean diet on various health benefits, found a 62% reduction in BC risk in participants supplementing their diet with extra-virgin olive oil compared to the control group (5). Although traditionally viewed as being detrimental to health, SFA may not necessarily exert pro-tumorigenic effects as there is only a weak association between SFA consumption and BC risk (6, 13, 14). The association between SFA consumption and BC risk may be dependent on timing and duration of exposure, as one study found that younger women had an increased risk of BC associated with SFA consumption, while older women actually had a reduced risk (15). Research examining the effects of specific SFA found that lauric acid, a 12-carbon SFA stimulated normal mammary gland development in vivo (41), while stearic acid, an 18-carbon SFA suppressed mammary gland development both in vivo and in vitro through the inhibition of the PI3K/Akt signalling pathway (41). Additionally,
exposure to high levels of lard in utero actually decreased BC risk later in life in offspring, an
effect that was attributed to the modulation of mammary gland development by diet (46).

Dietary fat may exert different effects on mammary tissue dependent on age, as younger
women had an increased risk of BC associated with SFA consumption, while older women
actually had a reduced risk (15). Observations also vary based on the source of SFA, as dairy
consumption exert different effects compared to red meat consumption. Total dairy consumption,
not including milk, is associated with a reduced risk of breast cancer (37); however, increased
intake of red meat is associated with an increased risk of breast cancer (38).

Although it is unequivocal that exposure to dietary fat through maternal diet or early in
life plays a role in mammary gland development, less is known regarding dose-dependent effects
of specific fatty acids or combinations of fatty acids on BC occurrence and mammary
tumorigenesis later in life.

**Role of exercise in mammary tumor development**

Exercise significantly extended tumor latency compared to sedentary mice in only the
safflower oil group. There was approximately a 59% difference in the age in days at which 50%
of mice are tumor free, with exercise exerting protective effects against tumor development.
Surprisingly, this observation was not reflected in tumor volume and multiplicity data for the
safflower oil group. This study demonstrates that although exercise may exert beneficial effects
on tumor onset and progression, it did not have any effects on tumor volume and multiplicity
after onset. Perhaps the mouse model used in the study was too aggressive for exercise to
attenuate tumorigenesis after onset of tumors (91).
There was also no significant difference in tumor development over time between exercising and sedentary groups within the menhaden, flaxseed, olive oil and lard diets. Although literature shows a positive effect of moderate exercise in BC in human studies (10, 104), the exercise protocol used in the present study may not have exerted any effects on tumor outcomes with the exception of mice fed the safflower oil diet. A case-control study examining the effects of animal versus plant fat consumption on BC risk found that animal fat intake (high in SFA) increased risk independent of physical activity, while the plant fat consumption was negatively associated with BC risk in sedentary women (104), although specific mechanisms were not investigated. It is possible that the n-3 PUFA fed mice showed no additional benefit of exercise as the anti-tumorigenic benefits of the diet may have reached a threshold in mitigating tumorigenesis which was not further improved with exercise. In future studies, a lifelong moderate exercise protocol beginning earlier than 8 weeks of age, rather than shorter term intervention involving rigorous exercise training, would be more relevant to human activity, thus more accurately representing a whole lifestyle change. Timing of exercise may also play a role in modulating BC risk. Physically active women experience a dose dependent 20-30% reduction in BC risk compared to sedentary counterparts (55). Exercise in early life has preventative affects on BC risk in later life, and has protective effects at earlier stages of BC development (62). In post-menopausal women, a population more susceptible to BC due to hormonal changes, exercise training reduced tumor volume and multiplicity (62). A speculated mechanism could be due to a regulation in inflammation as a result of the effects of exercise on estrogen levels (62). Increased levels of estrogen are associated with increased inflammation as elevated aromatase expression is associated with increased NFκB binding activity (62), therefore exercise training could also be used to target hormone-driven inflammation.
Additionally, the MMTV-*neu*(ndl)YD5 model used in this study may be too aggressive for any beneficial effects of exercise to be observed; therefore, future studies can utilize the less aggressive MMTV-*neu* or MMTV-*neu* YB mouse model to assess potential benefits of exercise on BC risk.

**Tumor fatty acid composition and tumorigenesis**

Evidence suggests that membrane fatty acid composition of tissues can affect cellular signalling through the modification of lipid rafts. Lipid rafts in the cell membrane of a cell act as microdomains to facilitate signalling events (48), particularly those involved in pro-tumorigenic activities promoting tumor cell proliferation and survival (20). Lipid rafts are compartmentalized from the actual cell membrane and are rich in sphingolipids and cholesterol, as well as SFA side chains. The presence of cholesterol and SFA allow lipid rafts to be packed tightly, resulting in a highly organized structure. G proteins, growth factor receptors, mitogen activated protein kinase (MAPK) and protein kinase C are all examples of signal-inducing proteins found in lipid rafts (28).

Significant increases in OA were observed in mice fed an olive oil or lard diet in PC and PE fractions compared to safflower, menhaden and flax diets. OA was also significantly increased in lard compared to all other diets in the PC and PE fractions. However, this may be due to the fact that SFA can be converted to OA through elongation and desaturation reactions (29). This evidence suggests that SFA and MUFA may exert pro-tumorigenic effects as they regulate signalling through incorporation into lipid rafts of tumor tissue.
Analysis of mammary tumors showed significant increases in n-3 PUFA, specifically DHA, in mice fed a menhaden oil diet in the PC and PE fractions, as well as DPA in the PE fraction, suggesting that EPA may be incorporated initially and further elongated. As the menhaden oil diet contained 7% safflower oil, which is rich in n-6 PUFA, the presence of DPA and DHA in tumor tissue indicates that n-3 PUFA are preferentially used as a substrate. This is important in eicosanoid synthesis, as when they are derived from AA are thought to be promote inflammation and subsequent tumorigenesis, but have protective effects when derived from EPA and DHA (21, 31, 105). n-3 PUFA have a documented effect on lipid raft composition and may inhibit pro-tumorigenic signalling pathways. As they are highly unorganized compared to SFA, incorporation of n-3 PUFA alters the organization of lipid rafts and therefore has the potential to alter lipid raft-modulated signalling. One study found that DHA treatment was found modulate levels of oleic acid in lipid rafts of T cells, therefore n-3 PUFAs can modulate SFA and MUFA composition of lipid rafts although the mechanism is still unclear (54). Specifically in HER2+ BC, DHA reduced HER-2 signalling through the disruption of lipid rafts. The modulation of lipid rafts by n-3 PUFA could potentially be used as an anti-tumorigenic therapy to disrupt cancer-promoting signalling pathways (27).

Collectively, these data demonstrate that the consumption of dietary fat affects tumor membrane fatty acid composition; therefore, any effects on tumor outcomes may be attributed to diet.

Relevance of diets in comparison to human intake

All diets in the present study provided mice with 22% of total caloric intake from fat. Previous research has determined dose dependant effects of plant- and marine-derived n-3 PUFA
Therefore, mice were fed a 3% menhaden or 3% flaxseed oil + 7% safflower oil diet, as an anti-tumorigenic response to these quantities was reported. However, dose dependant responses to MUFA or SFA consumption have yet to be determined, therefore mice consuming these diets were fed 10% olive oil or 10% lard, respectively. This level was chosen to compare against previously tested diets containing 10% menhaden and safflower oil (26).

The 3% menhaden oil diet contained physiological relevant amount of EPA and DHA. Based on previous work (26), the average intake of food per mouse was 2g/day and therefore provided mice with 7.8 mg EPA and 6.8 mg DHA per day, or a combined 1.6% total daily energy. A traditional Japanese diet contains 1-2% of daily energy as EPA and DHA, translating to about 3.5 g of EPA and DHA/day based on a recommended 2000 cal/day diet (24).

Mice fed the flax diet consume ~35 mg of ALA/day, representing 3.8% of daily caloric intakes. This is higher than the typical North American diet, which is composed of 1.4 g of ALA based on a recommended 2000 calorie diet (106). ALA serves as a precursor to EPA and DHA, however in humans, whole body conversion of ALA to DHA is less than 5% (19). Remaining ALA is used for the synthesis of MUFA and SFA (107). Therefore, little ALA remains stored in its consumed form and may not be detected in tissue. A previous clinical study demonstrated that ALA had anti-tumorigenic benefits and reduced cell proliferation as well as HER-2 protein expression at high doses of 5.7 g/day in BC patients (108).

The 2015 US Dietary Guidelines Advisory Committee recommends a maximum of 10% in energy intake of SFAs (109). Based on this recommendation, the lard diet contains 9% daily caloric intake of SFA, which is at the upper limit but still physiologically relevant to human consumption.
Recommended MUFA intake is up to 15-20% of total daily energy intake, although levels are not consistently met (110). The olive oil diet contains ~15% of daily caloric intake of MUFA, and therefore represents levels of intake that are physiologically relevant to human consumption.

3.6 Limitations and Future Directions

The MMTV-neu-(ndl)YD5 mouse model used represents human HER-2 positive BC. Over-expression of HER-2 is associated with an invasive and highly metastatic phenotype in BC (111). As a result, the mouse model used may have been too aggressive to observe potential beneficial effects of exercise. As a continuation of this study, a less aggressive mouse model should be used to observe the effects of moderate lifelong exercise on mammary tumor outcomes.

Additionally, the exercise protocol does not accurately represent a realistic method to be used in BC prevention in humans. A moderate lifelong exercise protocol, beginning after weaning and continuing for the duration of the lifespan may be more suitable for BC prevention, and would be more transferable to humans as a preventative measure or potential therapy as literature shows that even lifelong recreational activity exerts positive effects in BC (71). As exercise did not have a significant effect on tumour outcomes within diets with the exception of mice fed safflower oil, this study should have included a Western blot analysis to determine if markers of mitochondrial biogenesis were present in muscle of exercising mice. This would confirm that there were training adaptations in muscle occurred after 4 weeks of treadmill running.
Although both the flaxseed and menhaden oil diets contained doses of oils that were representative of levels of human consumption and have established dose-dependent effects (26), further work looking at dose-dependent effects of MUFA and SFA is required as this study only used diets containing 10% w/w olive oil or lard and the effects these oils would exert in smaller or larger doses on mammary tumor outcomes are unknown.

As increased estrogen is associated with earlier puberty onset, and therefore increased risk of BC, it would be of interest to analyze serum estradiol in the future. Consumption of dietary fats, specifically marine-derived n-3 PUFA, could modulate hormone production and subsequent effects on tumor outcomes in BC (31, 112).

Finally, as both n-3 PUFA and exercise mitigate inflammation (113), a potential mechanism by which these lifestyle factors exert their anti-tumorigenic effects may be through the modulation of inflammatory pathways. Thus, future studies should determine the effects of dietary fats and exercise on gene and protein expression of inflammatory cytokines.

3.7 Conclusion

In conclusion, the present study has shown that diet and exercise independently modulate mammary tumorigenesis. n-3 PUFA consumption mitigates tumorigenesis compared to other dietary fats examined in this study. These effects may be mediated by altering the fatty acid composition of tumor tissues which, in turn, may modulate the expression of proteins within the tumor microenvironment involved in tumor cell proliferation and apoptosis such as Ki67, although further work is needed to confirm this. Exercise also exerted protective effects in the n-6 PUFA-rich safflower oil diet through a delay in tumor latency and delayed mammary tumor
development. The results from this study demonstrate that there are independent effects of dietary fats and exercise on mammary tumor outcomes. Additionally, there are also effects of diet-exercise interactions although mechanisms are unclear and require further research. A potential mechanism causing these effects may be through a reduction in inflammatory cytokines, subsequently exerting effects on proliferative and apoptotic factors involved in cancer cell signalling; however, this requires further study. Overall, these findings support the implementation of lifestyle changes involving diet and exercise in reducing mammary tumor development.
References


18. Anderson BM and Ma DW. Are all n-3 polyunsaturated fatty acids created equal? *Lipids Health Dis* 2009; 8(33).


Figure 1. Food intake measured in sedentary or exercising mice fed either a (A) 10% w/w safflower oil diet (n=21), (B) 10% w/w lard diet (n=21), (C) 10% w/w olive oil diet (n=16), (D) 3% w/w flaxseed oil + 7% w/w safflower oil diet (n=19) or (E) 3% w/w menhaden oil + 7% w/w safflower oil diet (n=18). Data were analyzed by repeated measures ANOVA (p<0.05). Significant differences between sedentary and exercising groups over a period of 20 weeks denoted by an asterisk (*).
A

B

C

Body weight (g)

Age (weeks)

Sedentary

Exercise

Body weight (g)

Age (weeks)

Sedentary

Exercise

Body weight (g)

Age (weeks)

Sedentary

Exercise

Body weight (g)

Age (weeks)

Sedentary

Exercise

*
Figure 2. **Body weights** Body weights measured in sedentary or exercising mice fed either a (A) 10% w/w safflower oil diet (n=21), (B) 10% w/w lard diet (n=21), (C) 10% w/w olive oil diet (n=16), (D) 3% w/w flaxseed oil + 7% w/w safflower oil diet (n=19) or (E) 3% w/w menhaden oil + 7% w/w safflower oil diet (n=18). Body weights were recorded once a week. Data were analyzed by repeated measures ANOVA (p<0.05). Significant differences denoted by an asterisk (*).
Figure 3. Spleen to body weight ratio Spleen weight as a fraction of total body weight in sedentary or exercising mice fed either a (A) 10% w/w safflower oil diet (n=21), (B) 10% w/w lard diet (n=21), (C) 10% w/w olive oil diet (n=16), (D) 3% w/w flaxseed oil + 7% w/w safflower oil diet (n=19) or (E) 3% w/w menhaden oil + 7% w/w safflower oil diet (n=18). Spleen weights were recorded at termination. Error bars represent standard deviation. Data were analyzed by a two way ANOVA (p=0.5034).
Figure 4. Final tumor multiplicity Tumor multiplicity in sedentary or exercising mice fed either a (A) 10% w/w safflower oil diet (n=21), (B) 10% w/w lard diet (n=21), (C) 10% w/w olive oil diet (n=16), (D) 3% w/w flaxseed oil + 7% w/w safflower oil diet (n=19) or (E) 3% w/w menhaden oil + 7% w/w safflower oil diet (n=18). Final tumor multiplicity was recorded at termination. Error bars represent standard deviation. Data were analyzed by a two way ANOVA (p=0.8595).
Figure 5. Final tumor volume Tumor volume in sedentary or exercising mice fed either a (A) 10% w/w safflower oil diet (n=21), (B) 10% w/w lard diet (n=21), (C) 10% w/w olive oil diet (n=16), (D) 3% w/w flaxseed oil + 7% w/w safflower oil diet (n=19) or (E) 3% w/w menhaden oil + 7% w/w safflower oil diet (n=18). Final tumor volume was recorded at termination. Error bars represent standard deviation. Data were analyzed by a two way ANOVA (p<0.05).
Figure 6. Final tumor weight Tumor weight in mice fed either a (A) 10% w/w safflower oil diet (n=21), (B) 10% w/w lard diet (n=21), (C) 10% w/w olive oil diet (n=16), (D) 3% w/w flaxseed oil + 7% w/w safflower oil diet (n=19) or (E) 3% w/w menhaden oil + 7% w/w safflower oil diet (n=18). Final tumor weight was recorded at termination. Data were pooled by diet as there was only a diet effect. Data were analyzed by a two way ANOVA (p<0.05).
Table 1. Macronutrient composition of 10% w/w safflower oil diet (D04092701), 10% w/w lard diet (D16012401), 10% w/w olive oil diet (D16012101), 3% w/w flaxseed oil + 7% w/w safflower oil diet (D04092711N) and 3% w/w menhaden oil + 7% w/w safflower oil diet (D04092703).

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>D19112G</th>
<th>D04092701</th>
<th>D04092703</th>
<th>D04092711N</th>
<th>D16012101</th>
<th>D16012401</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>gm</td>
<td>kcal</td>
<td>gm</td>
<td>kcal</td>
<td>gm</td>
<td>kcal</td>
</tr>
<tr>
<td>Protein</td>
<td>20.3</td>
<td>20.3</td>
<td>21.6</td>
<td>20.3</td>
<td>21.6</td>
<td>20.3</td>
</tr>
<tr>
<td>Carbohydrate</td>
<td>63.9</td>
<td>63.9</td>
<td>59.5</td>
<td>57.9</td>
<td>59.5</td>
<td>57.9</td>
</tr>
<tr>
<td>Fat</td>
<td>7.9</td>
<td>15.8</td>
<td>10.6</td>
<td>21.8</td>
<td>10.6</td>
<td>21.8</td>
</tr>
<tr>
<td>Total</td>
<td>100.0</td>
<td>100.0</td>
<td>100.0</td>
<td>100.0</td>
<td>100.0</td>
<td>100.0</td>
</tr>
<tr>
<td>Ingredient</td>
<td>gm</td>
<td>kcal</td>
<td>gm</td>
<td>kcal</td>
<td>gm</td>
<td>kcal</td>
</tr>
<tr>
<td>Corn Starch</td>
<td>397.4</td>
<td>1589.9</td>
<td>336.7</td>
<td>1347.2</td>
<td>336.7</td>
<td>1347.2</td>
</tr>
<tr>
<td>maltodextrin 100</td>
<td>132.4</td>
<td>526.8</td>
<td>132.5</td>
<td>526.8</td>
<td>132.5</td>
<td>526.8</td>
</tr>
<tr>
<td>Sucrose</td>
<td>100.0</td>
<td>400.0</td>
<td>100.0</td>
<td>400.0</td>
<td>100.0</td>
<td>400.0</td>
</tr>
<tr>
<td>Cellulose, BW200</td>
<td>60.0</td>
<td>60.0</td>
<td>60.0</td>
<td>60.0</td>
<td>50.0</td>
<td>50.0</td>
</tr>
<tr>
<td>Soybean Oil</td>
<td>70.6</td>
<td>60.0</td>
<td>60.0</td>
<td>60.0</td>
<td>60.0</td>
<td>60.0</td>
</tr>
<tr>
<td>Safflower Oil</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Olive Oil</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Lard</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Flaxseed Oil</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Menhaden Oil</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>L-Buthydroquinone</td>
<td>0.014</td>
<td>0.019</td>
<td>0.019</td>
<td>0.019</td>
<td>0.019</td>
<td>0.019</td>
</tr>
<tr>
<td>Mineral Mix S10022G</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Vitamin Mix V150637</td>
<td>10.40</td>
<td>10.40</td>
<td>10.40</td>
<td>10.40</td>
<td>10.40</td>
<td>10.40</td>
</tr>
<tr>
<td>Choline Bitartrate</td>
<td>5.40</td>
<td>4.00</td>
<td>4.00</td>
<td>4.00</td>
<td>4.00</td>
<td>4.00</td>
</tr>
<tr>
<td>FD&amp;C Yellow #5</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>FD&amp;C Red #40</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>FD&amp;C Blue #1</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Total</td>
<td>100.0</td>
<td>400.0</td>
<td>100.0</td>
<td>400.0</td>
<td>100.0</td>
<td>400.0</td>
</tr>
</tbody>
</table>

Formulated by Research Diets, Inc.
April 26, 2016