# VITAMIN D AND MAMMOGRAPHIC DENSITY IN POSTMENOPAUSAL WOMEN: A COHORT STUDY NESTED WITHIN A CHEMOPREVENTION TRIAL

by

Melanie Walker

A Thesis Submitted to the Graduate Program in Epidemiology (Department of Public Health)

In conformity with the requirements for the degree of Doctor of Philosophy

Queen's University

Kingston, Ontario, Canada

(March, 2014)

# Abstract

**Background:** Vitamin D may be important in the causal pathway to breast cancer (BC) by influencing mammographic breast density (MD). However, previous study results in postmenopausal women are inconsistent. Study objectives were to prospectively examine the relationship between biomarkers of vitamin D (25-OH-D) and percent MD in postmenopausal women at northern latitudes. Potential effect modification by exemestane therapy, calcium or genetic polymorphisms in the vitamin D pathway was also examined.

**Methods:** This study evaluated a sub-cohort of postmenopausal women at elevated BC risk who participated in the NCIC Clinical Trials Group placebo-controlled MAP.3 trial with exemestane. Levels of 25-OH-D were measured using LC-MS/MS from serum samples collected at baseline and year 1, averaged and adjusted for month of collection. Baseline and follow-up (≥ 3 year) percent MD was centrally assessed from film and digital mammograms with Cumulus software. Multivariable linear regression was used to estimate the effect of 25-OH-D on log transformed percent MD at follow-up and on the change in percent MD from baseline. Percent MD was also dichotomized and multivariable logistic regression was used to evaluate 25-OH-D levels between 1) women with lower (<25%) compared with higher (≥25%) percent MD and 2) women with a decrease compared with no change or an increase in percent MD over time.

**Results:** Percent MD was measured for 568 participants with a follow-up mammogram and for 388 participants with a baseline mammogram in the same format as the follow-up. The geometric mean percent MD of the follow-up mammograms was 4.3% and few women (13.4%) had percent MD ≥ 25%. The unadjusted mean 25-OH-D concentration was 36.5 ng/mL (SD=10.6) based on pooled baseline and year one samples. After controlling for age, month of sampling and potential confounders, 25-OH-D was not predictive of log transformed percent MD at follow-up ($p$=0.36) or with annual mean changes from baseline ($p$=0.33). Similarly, results from the logistic

regression analyses were not statistically significant and no interactions with exemestane, calcium or genetic polymorphisms were detected.

**Conclusion:** No association was observed between vitamin D levels and percent MD at ≥3 year follow-up or change in percent MD from baseline.

# Acknowledgements

It is not often in life that one gets the opportunity to formally thank and acknowledge the many people who travelled this *long* PhD journey with me. Therefore, I will use this space wisely. First, I would like to thank the NCIC Clinical Trials Group for their support - both during my previous work with them and in making the transition to full-time student. It has been a privilege to conduct my PhD epidemiological research within the context of their largest chemoprevention trial to date. Second, I would like to thank my primary thesis supervisor Dr. Will King for his mentorship, methodological expertise and patience for the duration of this project. All of my epidemiological training, at both the Masters and PhD level, has been under Dr. King's guidance for which I am grateful and well prepared for my career. Thank you also to my secondary supervisors, Drs. Harriet Richardson and Ralph Meyer, who have provided valuable feedback throughout the course of this project and have made this a stimulating and memorable experience.

I would also like to thank several key collaborators without whom this research would not have been possible including Drs. Glen Jones, Martin Kaufmann and Doris Jabs. I would be remiss to not acknowledge the hard work and contributions in the coordination of this project of Nicki Kwon, Celine Simmer, Adam Lewis, Marie Pitcher, Doug Parfett and Mary Halligan. Thank you also to other members of the research team including Dr. Paul Goss and Dr. Dongsheng Tu.

I would like to thank the Department of Public Health Sciences for their dedication in making our training comprehensive and valuable and I am grateful to the faculty and students for their insight, comradery and competitiveness that helped make this a fulfilling and thought-provoking experience. I also extend my gratitude to Queen's University for several awards that I was fortunate to receive during my training including the Queen Elizabeth II Scholarship in Science and Technology, R.S. McLaughlin Fellowship and Ontario Graduate Scholarship – Science and

Technology and to the NCIC Clinical Trials Group for supporting me through the AstraZeneca Breast Fellowship.

I would also like to acknowledge and thank the Canadian Breast Cancer Foundation for fully funding this research project. They recognize the importance of identifying modifiable risk factors for breast cancer in an effort to reduce and prevent the occurrence of this disease which affects so many. To that regard, a sincere thanks to the clinical trials centres that contributed data to this research project and, most importantly, to the selfless participants that extended their time, courage and trust to participate in this study.

Last, but certainly not least, I am *very* grateful to my family and friends for their love, encouragement and patience throughout this project. I am extremely appreciative to my husband, Craig, for his unwavering support, sympathetic ear and humour - all of which I was in high demand of during the last five years. Thanks also to our beautiful son, Jacob, for his incessant happiness and ability to interject fun and laughter into my life. Finally, a big thanks to my mom and dad who have been supportive and encouraging and have provided tremendous help along the way. And, today, I'm finally happy to answer "yes, I am done the thesis!"

# Table of Contents

# List of Figures

# List of Tables

# List of Abbreviations

| | |
|---|---|
| $1,25[OH]_2D$ | 1,25-dihydroxyvitamin D |
| 25-OH-D | 25-hydroxyvitamin D |
| AI | Aromatase inhibitor |
| ANOVA | Analysis of variance |
| BC | Breast cancer |
| BIRADS | Breast Imaging Reporting and Data Systems |
| BMI | Body mass index |
| CI | Confidence interval |
| EPT | Estrogen progestin therapy |
| ER | Estrogen receptor |
| HDH | Hotel Dieu Hospital |
| HR | Hazard ratio |
| HRT | Hormone replacement therapy |
| IOM | Institute of Medicine |
| MD | Mammographic density |
| ng/mL | Nanogram per milliliter |
| OC | Oral contraceptive |
| OR | Odds ratio |
| PR | Progesterone receptor |
| QLMP | Queen's Laboratory for Molecular Pathology |
| RCT | Randomized controlled trial |
| REB | Research Ethics Board |
| RR | Relative risk |
| SERM | Selective estrogen-receptor modulator |

| | |
|---|---|
| SNP | Single nucleotide polymorphism |
| UV | Ultraviolet |
| VDR | Vitamin D Receptor |

# Chapter 1

# Introduction

## 1.1 General Introduction

Established risk factors account for only 30 to 40% of incident breast cancer (BC) cases and therefore research is required to identify additional modifiable risk factors [1]. There is evidence to suggest that vitamin D may be important to the etiology of BC. However, this potential relationship has not been firmly established and the examination of higher risk populations and those in geographical locations where a high proportion of the population receives inadequate vitamin D exposure during the winter months is a priority.

This PhD dissertation examines whether circulating blood levels of vitamin D in postmenopausal women at higher risk for BC development are associated with mammographic density (MD), an intermediate endpoint for BC. Study participants enrolled in a large placebo-controlled chemoprevention trial of exemestane, an inhibitor of estrogen, also allows for examination of an interaction between vitamin D and exemestane on breast density which may add to the current understanding of the mechanisms that may lead to reduced breast density and/or BC risk. This study also evaluates potential effect modification on the vitamin D and breast density relationship by calcium and select genetic polymorphisms in the vitamin D pathway which may exacerbate the observed associations. Overall study results will add to the current knowledge regarding vitamin D's role in BC etiology. In addition, results will inform population health stakeholders on the prevalence of vitamin D deficiency in postmenopausal women residing in northern latitudes which may lead to appropriate interventions targeted at high-risk individuals.

## 1.2 Methodological Limitations of Existing Studies

Cancers represent a group of diseases which develop slowly and occur relatively infrequently. Retrospective studies of the vitamin D and BC relationship are limited by exposure misclassification and the potential for information and selection bias, while prospective studies of BC require a large number of subjects followed for decades. These methodological challenges can be overcome to some degree by substituting relevant intermediate endpoints (such as breast density) for cancer outcomes. Molecular epidemiology studies which incorporate valid intermediate endpoints for a vitamin D → BC relationship can provide an understanding of important steps in the carcinogenic pathway. The investigation of markers of intermediate carcinogenic effect offers study advantages in the examination and clarification of exposure-cancer relationships, including: i) a study population of otherwise healthy subjects, ii) an outcome (e.g. intermediate event) which is much more common than a cancer event, iii) a shorter time period between exposure and intermediate event than between exposure and malignancy, and iv) a potentially stronger underlying relationship [2].

## 1.3 Thesis Setting, Purpose and Objectives

### 1.3.1 Thesis Setting

The NCIC Clinical Trials Group conducted a phase III international, multi-centre randomized controlled trial (RCT) comparing exemestane, an aromatase inhibitor (AI), with placebo in postmenopausal women at higher than average risk for BC (MAP.3). Results of the primary objectives of the trial reported that invasive BC was significantly reduced in postmenopausal women who were on exemestane therapy compared with placebo (HR: 0.35; 95% CI: 0.18 - 0.70)[3].

The totality of study results implicating vitamin D in the causal pathway to BC is inconsistent to date, particularly among postmenopausal women. The prospective nature of the underlying RCT, which collected blood samples at the time of randomization, provided an opportunity to conduct a strong observational study eliminating the biases inherent in retrospective evaluations of such an association and substantially reducing the costs and time that would be traditionally required to initiate a prospective cohort. We set out to conduct a nested observational study within the MAP.3 chemoprevention trial utilizing up to 6 year prospective data collection from this trial.

### 1.3.2 Thesis Purpose

The overall purpose of this thesis was to examine the relationship between serum vitamin D (serum 25-OH-D) and follow-up mammographic breast density (MD) in a sub set of women who participated in MAP.3. In addition, this study sought to determine whether serum 25-OH-D was associated with a change in the percentage of MD over time adjusting for the season the sample was drawn and other important covariates. Nesting an observational study within this trial and utilizing up to 6 years of prospective data collection was an efficient, economical and methodologically strong approach to research BC etiology.

### 1.3.3 Thesis Objectives

It is hypothesized that lower baseline levels of serum 25-OH-D, defined as the average between levels at the time of randomization and year 1, will be reflective of usual lifetime exposure and will be associated with higher percent breast density at follow-up. Further, women with lower baseline levels of serum 25-OH-D are postulated to have no or smaller decreases in percent breast density over time compared with women with higher baseline serum 25-OH-D levels.

**Primary Objectives**

1. To examine the relationship between baseline serum 25-OH-D and percent MD at ≥ 3 year follow-up among postmenopausal women.
2. To examine the relationship between baseline serum 25-OH-D and the average change over time (i.e. baseline mammogram – follow-up mammogram / years of follow-up) in percent MD.

**Secondary Objectives**

1. To explore whether percent MD at ≥ 3 year follow-up in relation to serum 25-OH-D is modified by exemestane therapy.
2. To explore whether average changes over time (i.e. baseline mammogram – follow-up mammogram / years of follow-up) in percent MD in relation to serum 25-OH-D is modified by exemestane therapy.
3. To explore effect modification by calcium on the relationship between serum 25-OH-D and percent MD.
4. To investigate the interactions of two single nucleotide polymorphisms (SNPs) relevant to the vitamin D pathway on the relationship between baseline serum 25-OH-D and ≥ 3 follow-up percent MD.

### 1.3.4 Thesis Organization

This thesis is organized in a traditional thesis format. Chapter 2 provides a detailed literature review and rationale for the study that includes (i) review of the known risk factors for BC and breast density; (ii) review of the evidence supporting the use of breast density as an intermediate marker of BC risk; (iii) an overview of the hypothesized biological mechanism for vitamin D in BC etiology; and (iv) the current epidemiological evidence on the relationship between vitamin D

and BC and vitamin D and breast density. Chapter 3 provides an outline of the study methods used to achieve the study objectives and chapter 4 contains the body of the results as they relate to the stated objectives. A discussion of study results, methodological issues and implications of the findings constitutes Chapter 5.

### 1.3.5 PhD Student Contributions

I was primarily responsible for conducting the literature searches which led to the development of the specific research objectives of this project and for networking to secure participation of key collaborators. In collaboration with my thesis supervisors, I also contributed intellectually to this project by co-writing grant applications for which I was a listed co-Investigator. This project was subsequently fully funded by the Canadian Breast Cancer Foundation in November 2010. Although this observational study was nested within a large RCT, this research project included the design of a new study which involved primary data collection. As such I was responsible for developing and managing a database for all information pertinent to this study including: questionnaire information, vitamin D levels, vitamin D pathway polymorphism data, and mammogram data including percent density measurements. I also acted as the main liaison between the different disciplines represented in this project (i.e. transportation and coordination of vitamin D analyses on collected blood samples, retrieval and return of mammograms from/to participating centres, coordination of central radiology review, etc.). At study initiation I was also responsible for conducting a pilot project with a few of the member centres of the NCIC Clinical Trials Group to evaluate the feasibility of mammogram collection from radiology centres and coordination with our affiliates at Hotel Dieu Hospital (HDH). Information gathered from the pilot phase of this project resulted in some important changes to the study methods utilized for residual mammogram collection and processing. Lastly, I was primarily responsible for the data analysis, interpretation and preparation of this thesis document.

## 1.4 References

1. World Cancer Research Fund and American Institute for Cancer Research. Food, nutrition and the prevention of cancer: A global perspective. *American Institute for Cancer Research*. 1997.

2. Bonassi S, Au William W. Biomarkers in molecular epidemiology studies for health risk prediction. *Mutation Research*. 2002;511(1):73.

3. Goss PE, Ingle JN, Alés-Martínez JE, et al. Exemestane for breast-cancer prevention in postmenopausal women. *New England Journal of Medicine*. 2011(364):2381.

# Chapter 2

# Literature Review

To frame the background for the stated objectives of this research project, this chapter reviews the established risk factors for breast cancer (BC), both modifiable and non-modifiable, and describes the estimated burden of this disease within the Canadian population. An overview of what breast density is and its' known relationship with BC and plausibility as an intermediate marker is provided. This literature review also summarizes what is known about vitamin D, its importance to health and the evidence on the role of vitamin D in BC etiology. Further, the current evidence on the vitamin D and breast density relationship is reviewed. Lastly, this chapter reviews what is currently known about BC chemopreventive agents, such as exemestane, and the potential role that genetic polymorphisms in the vitamin D pathway may play on the underlying relationship with breast density/BC.

## 2.1 Epidemiology of Breast Cancer

According to Canadian Cancer Statistics, BC is the most common cancer diagnosed in Canadian women and was estimated to account for more than 22,000 new cases and 5,200 deaths in 2012 [1]. After lung cancer, BC is the leading cause of cancer deaths accounting for almost 14% of all cancer deaths in Canadian women. The age-standardized incidence rate for BC in Canada is 95.9/100,000 and the age-standardized mortality rate is 19.5/100,000 [1]. Approximately one in nine women will develop BC at some point during her lifetime, with a one in 28 lifetime probability of dying from the disease [1,2].

The evidence for BC prevention comes from observational epidemiological studies showing that reproductive, lifestyle and environmental factors appear to account for more BC cases than

having a genetic predisposition to the disease [1,3-5]. According to a recent report of the American Institute for Cancer Research and the World Cancer Research Fund approximately one third of BCs are thought to be preventable through diet, regular physical activity and healthy body weight [6,7]. Ecologic studies on international variation in BC incidence rates and migrant studies showing that women who move from areas of low BC incidence to areas of higher incidence assume the rates in the host country within one or two generations provide additional support that BC is modifiable and potentially preventable [4,8]. There is also recent evidence showing a decline in the incidence of BC among postmenopausal women with reduction in the use of combined estrogen/progesterone hormone replacement therapy (HRT) which is an established risk factor for the disease [9,10].

BC is not a homogeneous disease and can be divided into a number of distinct subtypes based on patient and tumour characteristics [11]. While BC is often studied as a single disease one of the most important distinctions in its' epidemiology is whether it is diagnosed in premenopausal or postmenopausal women. Premenopausal BCs are associated with more aggressive tumours that are more likely to be estrogen receptor (ER) negative, progesterone receptor (PR) negative and overexpress HER2-neu (HER2) leading to a poorer 5-year relative survival rate compared with postmenopausal BCs [11]. While premenopausal and postmenopausal BCs share many of the same risk factors, there are differences in the magnitude of the effect and, for a few notable risk factors, in the direction of the effect [11]. Such risk factors will be described in further detail below with differences between premenopausal and postmenopausal BC noted where applicable.

## 2.2 Risk Factors for Breast Cancer

The following section briefly reviews the current evidence on both non-modifiable and modifiable BC risk factors. A literature search was conducted to identify review papers and

meta-analyses where available. In addition, summary reports from reputable stakeholder groups such as the World Cancer Research Fund and Health Canada were reviewed.

### 2.2.1 Non-Modifiable Breast Cancer Risk Factors

#### *2.2.1.1 Age*

Age is the strongest risk factor for the development of BC [2,11-16]. BC incidence increases with increasing age [2,12,13]. Women older than 65 have a relative risk (RR) of 5.8 of developing BC compared with women less than 65 [15]. In Canada, incidence of BC cases occurs predominantly in women between the ages of 50 and 69 while 30% occur in women older than age 69 and 19% occur in women younger than age 50 [1]. Age, as a risk factor for BC, is modified by race and ethnicity [14]. African American women under the age of 50 have higher age-specific incidence rates for BC compared with Caucasian women and, conversely, African American women greater than 50 years of age are at reduced risk of developing BC compared with Caucasian women [14].

#### *2.2.1.2 Family History of Breast Cancer*

Family history and, in particular, a genetic predisposition to BC is the second strongest risk factor for this disease after age [13]. Most studies have demonstrated about a two to four-fold increased risk depending on the number of affected first-degree relatives [13-15,17]. The Collaborative Group on Hormonal Factors in Breast Cancer conducted a meta-analysis including data from 52 epidemiological studies to more precisely estimate the risk of BC among a large cohort of women with and without a family history of the disease [18]. The authors found that BC risk increased with increasing numbers of first-degree relatives affected. Compared with women without a family history of BC, women with one affected first degree relative had a 1.8 times higher risk of developing BC (99% CI: 1.69-1.91); women with two affected first degree relatives had a 2.93 times higher risk of developing BC (99% CI: 2.36-3.64); and women with three or more affected

first degree relatives had a 3.90 times higher risk of developing BC (CI: 2.03-7.40) [18] . When stratified by age, the risk estimates were higher for women younger than 50 years compared with women 50 years of age and older [18]. In addition, women with a family history of premenopausal bilateral BC have been reported to have a greater than 4 fold increase in risk compared with women without such a family history [17].

Genetic predisposition through the inheritance of a germ line mutation accounts for up to 10% of BC in Western countries [12]. At least five such germ line mutations have been identified to date with BRCA1 and BRCA2 accounting for a large proportion of very high risk families (≥ 4 affected family members) [12,15]. This accounts for approximately 20-25% of the overall risk for familial BC, however, these high risk alleles only account for about 5% of all BC cases [13,14]. Premenopausal BC, specifically, is strongly associated with BRCA1 mutations [11]. Most women who have inherited a high-risk mutation will develop BC before the age of 65 [12].

*2.2.1.3 Benign Breast Conditions*

Women with benign breast conditions such as severe atypical epithelial hyperplasia have a 4 to 5 fold increase in BC risk compared with women who do not have such proliferative breast changes [2,12-14,17]. A higher risk of BC of about 1.5 to 2.0 is also observed among women with benign cysts, fibroadenomas, duct papillomas, sclerosis adenosis and epithelial hyperplasia [2,12]. Approximately 40% of women with both a family history of BC and atypical hyperplasia go on to develop BC [14].

*2.2.1.4 Endogenous Hormonal Factors*

There are a myriad of established reproductive risk factors for BC which are particularly related to estrogen. These include early menarche and late menopause which increases a woman's exposure to endogenous estrogen over her lifetime [2,12,13,15,19]. Specifically, for each year younger

at menarche the risk of BC is observed to increase by a factor of 1.05 (95% CI: 1.04-1.06) and for each year older at menopause the risk of BC is observed to increase by a factor of 1.03 (95% CI: 1.03-1.03) [19]. Women who experience menopause after the age of 55 are twice as likely to develop BC as women aged 45 or younger [2,12,13,15].

Nulliparity and later age at first full-term pregnancy also confer increased risk [2,12,13,15,20]. Women who have at least one full-term birth have an approximately 25% reduced risk of BC compared with nulliparous women [13]. Further, women who have their first child after the age of 30 are at twice the risk of developing BC compared with women who have their first child before the age of 20 [12,15]. The risk of BC continues to decrease with additional full-term pregnancies with women with five or more children having about half the risk of BC compared with nulliparous women [2,12].

Breastfeeding appears to confer a reduced risk of BC [13,17,21]. The Collaborative Group on Hormonal Factors in Breast Cancer conducted a meta-analysis combining data from 47 epidemiological studies to evaluate the RR for BC associated with breastfeeding in parous women [21]. The authors reported a decrease in the RR of BC by 4.3% (95% CI: 2.9-5.8) for every 12 months of breastfeeding in addition to a 7.0% (95% CI: 5.0-9.0) decrease for each birth. This relationship did not change when stratified by menopausal status [21]. A more recent systematic review on the association between BC and breast feeding did not find a consistent protective effect observed among the studies included (30 case-control studies; 1 cohort study), however, inconsistency in how time of breastfeeding was reported made comparisons difficult as did inconsistent control of potential confounders on the relationship between breast feeding and BC risk such as OC use and BMI [22].

### 2.2.2 Modifiable Breast Cancer Risk Factors

*2.2.2.1 Exogenous Hormonal Factors*

Exogenous hormonal risk factors include oral contraceptive (OC) use and HRT [2,12,13]. There is a small increase in the risk of BC among OC users and within the 10 years following cessation of use [12,23-25]. In a pooled analysis conducted by the Collaborative Group on Hormonal Factors in Breast Cancer a modest increased risk of BC was observed in current OC users [RR= 1.24 (95% CI: 1.15-1.33)]; past OC users 1-4 years after stopping [RR=1.16 (95% CI: 1.08-1.23)]; and past OC users 5-9 years after stopping [RR=1.07 (95% CI: 1.02-1.13)] compared with never users [25]. Cumulative evidence from observational studies shows that the association between OC use and the increased risk of BC is primarily apparent in premenopausal/younger women and not in postmenopausal/older women [23]. Given that younger women, whose risk of BC incidence is rare, are often those using OCs this very modest increased risk does not result in a large number of additional cases [12,13,24,25].

Postmenopausal women who use HRT are at a higher risk of developing BC compared with postmenopausal women who have never used HRT [13,15,23,24]. The Collaborative Group on Hormonal Factors in Breast Cancer conducted the largest meta-analysis to date including 51 epidemiological studies to investigate the relationship between the risk of BC and use of HRT [24]. The authors found that among women using HRT BC risk was increased for each year of use and, conversely, that this risk largely disappeared within 5 years of cessation of use [24]. Specifically, women who had taken HRT for 5 years or longer were at a 35% higher risk of developing BC compared with never users [RR=1.35 (95% CI: 1.21-1.49)]. No differences in RR by the type of hormone therapy used (i.e. estrogen vs. estrogen and progesterone) were observed [15,24]. In the Women's Health Initiative randomized controlled trial (RCT) of combination estrogen and progestin for prevention of cardiovascular disease a statistically significant increase in BC in the

treatment arm was observed compared with placebo [RR=1.24 (95% CI: 1.00-1.59][23,26]. This magnitude of effect is consistent with the epidemiologic literature on this association.

*2.2.2.2 Lifestyle Factors*

Largely, the epidemiological evidence on the relationship between diet and dietary constituents including meat, fibre, fruit, vegetables, vitamins A and E, beta-carotene, folate intake and phyto-estrogens and BC incidence are not consistent and do not support strong associations [2,12-14,17,27-30]. Observational studies evaluating dietary exposures and BC incidence are difficult given the challenges associated with recall bias and measurement error [2]. The strongest evidence for an association between diet and BC exists for ecologic studies which have consistently shown a strong correlation between the dietary fat intake in a population and the BC incidence rates [2,12,13]. However, case-control and cohort studies do not show strong or consistent results for this association particularly after controlling for total energy intake [2,12]. Some evidence from recent meta-analyses support a protective association for BC (both pre- and postmenopausal) with blood concentrations of carotenoids [RR=0.78 (95% CI: 0.61-0.99 per 5000 µg/d)] [17,28] and with fruits and vegetables combined [RR=0.89 (95% CI: 0.80-0.99 for highest vs. lowest intake] [17,30]. Vegetables alone were also observed to have a statistically significant protective effect on BC in pre- but not postmenopausal women [30]. While one recent meta-analysis of prospective studies showed a small protective effect of dietary fibre intake on BC risk [RR=0.93 (95% CI: 0.89-0.98) for the highest vs. lowest intake] this protective association was not statistically significant when stratified by menopausal status [29].

Many observational studies and meta-analyses have observed a positive association between alcohol consumption and BC risk [2,5,12-14,31,32]. In its' 2010 report, the American Institute for Cancer Research and the World Cancer Research Fund concluded that there was convincing evidence in support of the association between alcohol consumption and increased BC risk in

both pre- and postmenopausal women[33]. Women consuming 3- 4 alcoholic drinks / day are at an increased risk of BC [RR=1.32, 95% CI: 1.19-1.45)] compared with women not consuming alcohol [5,31]. BC risk is increased by approximately 7% per alcoholic drink per day and is postulated to be the result of increased estrogen levels in the body [5,13,14,31]. The risk of BC associated with alcohol consumption was not altered in this meta-analysis when stratified by menopausal status [31].

The epidemiological literature overall does not support an association between smoking and BC risk [2,12,13,17,31]. However, a recent review [34], meta-analysis [35] and report by the Canadian Expert Panel on Tobacco Smoke and Breast Cancer [36] supports an increased risk of BC in association with smoking of long duration [RR=1.26 (95% CI: 1.00-1.58) for smoking between 1 and 40 years compared with never smokers], smoking that is started before a first birth [HR=1.45 (95% CI: 1.21-1.74) for smoking started after menarche but 11 or more years before first birth compared with never smokers] and smoking at an early age [HR: 1.23 (95% CI: 1.04-1.46) for smoking started at 15 or younger compared with never smokers] [35]. The magnitude of these effect estimates were higher in premenopausal compared with postmenopausal women. The interval in the variable 'smoking of long duration' is very wide and does not adequately evaluate the relationship between smoking of varying lengths of time and BC risk. It is also possible that there is residual confounding from other healthy lifestyle habits which are associated with both never smoking and smoking of very short duration.

Several epidemiologic studies have observed a lower risk of BC in women who participate in moderate to vigorous levels of physical activity [2,5,13,14,17,33,37]. BC risk is estimated to be reduced by approximately 30% in women who undertake a few hours per week of vigorous physical activity in comparison with women who are sedentary [13]. The association between physical activity and reduction in the risk of BC appears to be stronger for postmenopausal than

premenopausal women [14,33,37]. Similarly, there is a growing body of evidence that there is a higher risk of postmenopausal BC in women who are overweight and have weight gain in adulthood [12,14,15]. Specifically, postmenopausal women who are obese (>30 kg/m$^2$) are at two times the risk of developing BC compared with lean (BMI = 20 kg/m$^2$) postmenopausal women [12,13,27]. Paradoxically, premenopausal women who are obese appear to have a reduced incidence of the disease [12,13,33]. Lastly, there is evidence of a weak positive association between adult height and BC risk [13]. Specifically, it has been shown that a 10 cm greater height is associated with an approximately 10% increase in BC risk [13]. While height itself is not a modifiable risk factor it is positively correlated with energy intake during growth and thus might be a marker for early life exposures including nutrition that influence cancer risk[13]. The exact underlying biological mechanism for the relationship between height and BC risk is not currently known.

*2.2.2.3 Environmental Factors*

Ionizing radiation is a known risk factor for the development of BC [2,5,12,13]. RRs of exposure to ionizing radiation depend on the dose, number of exposures and age at exposure but carry an approximately six fold increase in overall BC incidence [5]. Only weak epidemiological evidence exists for a relationship between exposure to electromagnetic fields and the incidence of BC and thus is not viewed as a strong or consistent risk factor for the disease [2,13].

In summary, established risk factors for BC include older age, early menarche, nulliparity, later age at first full-term pregnancy, late menopause, OC and HRT use, diet, sedentary lifestyle, obesity, high alcohol intake, history of benign breast disease and family history of BC [2,12-14]. As noted, the risk factors are largely the same for both pre- and postmenopausal BC with stronger observed associations for premenopausal women and a few noted differences including the effects of BMI and physical activity. Lastly, one of the most important predictors for the

development of BC which will subsequently be discussed is breast density as seen on mammographic screening [2,13,14,38-45, 46, 47-53].

### 2.2.3 Breast Density

Dense breast tissue is inversely associated with the fat content in the breast and is primarily composed of fibrous connective tissue (the stroma) and the functional (or glandular) epithelial cells that line the ducts of the breast (the parenchyma) [14,38-40]. Mammographic density (MD) is defined as radiologically dense breast tissue which is a reflection of the variations in this tissue composition [14,47]. While fat appears dark on a mammogram (due to low X-ray attenuation), epithelium and stroma cells appear opaque and this is referred to as MD [38-40]. Women with greater than 60-75% density in their breasts have consistently been shown to have a 4 to 6-fold increase in BC risk than women with little or no density [44-46,54-57]. When adjusted for age and ethnicity breast density is equally accurate in predicting the risk of BC to that of the Gail Model which is a validated clinical risk-assessment tool used to calculate a woman's risk of developing invasive BC in her lifetime [14]. The Gail model is comprised largely of the non-modifiable BC risk factors described above, namely, current age, age at menarche, age at first live birth, number of live births, first degree family history of BC, history of breast biopsies and race [14].

A causal relationship between breast density and the development of BC is supported by the large increase in BC risk with greater breast density and is postulated to be due to the higher number of epithelial or stromal cells at risk of carcinogenesis [42,53]. Whether it is the interaction between the epithelial and stromal tissue, or abnormal differentiation of cells and decreased apoptosis in the mammary gland that is more important is still not determined [42,53,58] It is also hypothesized that the combined effects of cell proliferation (mitogenesis) and genetic damage to proliferating cells

by mutagens (mutagenesis) may be responsible for the increased risk of BC associated with increased breast density [44].

#### 2.2.3.1 Natural History of Breast Density

Breast density decreases with a woman's increasing age with postmenopausal women consistently observed to have lower percent MD than premenopausal women [44]. Specifically, breast density decreases, on average, 1% per year as a woman ages [59]. Checka and colleagues [54] evaluated the mammograms of 7,007 women who underwent digital screening mammography. Breast density was categorized using a common qualitative classification system called Breast Imaging Reporting and Data Systems (BIRADS)[38,39]. Percentage MD was observed to decrease as the age group of the women increased with women under the age of 50 observed to have higher breast density compared with women over the age of 50 across BIRADS categories [54]. A longitudinal study evaluating the effects of menopause on MD observed an 8 % decrease in MD during the transition to menopause [59]. A similar magnitude of decrease in MD in women pre- and post-menopause was also observed in a study evaluating breast density trends over time [60].

Very few studies were identified that provide data on the distribution of breast density in women via computer-assisted methods and no studies were identified that presented this data by menopausal status. Boyd et al. [55] conducted a series of nested case-control studies using data from three mammography screened populations to examine the association of percent MD with BC risk. Participants included in the first nested case-control study were recruited from the National Breast Screening Study. From the data presented by Boyd et al. [55] it is observed that the controls had a mean percent density of 28.4% ± 21.2 (61.5% of women were postmenopausal). Of the participants recruited from the Ontario Breast Screening Program, the controls in the second nested study had a mean percent density of 24.3% ± 17.5 (89.4% of women were

postmenopausal) and of the participants invited from the Screening Mammography Program of British Columbia it was observed that the controls had a mean percent density of 28.1% ± 18.5 (75.1% of the women were postmenopausal). From examination of the percent mammographic densities in the control populations of these nested case-control studies it is estimated that about 43% of the women had a percent density greater than 25%, about 17% of the women had breast densities greater than 50% and approximately 5% of the population had breast densities greater than 75%. It is important to note, however, that the data available were for both pre- and postmenopausal women. Additional estimates on the distribution of percent MD come from Kerlikowske and colleagues [41] who reported that about 30% of postmenopausal women had breast densities greater than 50% and from McCormack and colleagues who report this percentage to be approximately 13% for densities >50% in postmenopausal women [45].

*2.2.3.2 Risk Factors for Breast Density*

Only 30% of the variance in MD is explained by known risk factors for breast density to date[44,47,52,53,61]. Variations in MD are largely associated with the same risk factors as for BC including age, body size, parity, age at first birth, number of births, menopausal status, diet, alcohol, HRT, and history of benign breast disease [47,48,62-64]. Specifically, breast density is inversely associated with age, is lower in parous women, lower in women with multiple live births and lower in women who are postmenopausal [62]. With regards to parity, data has shown an approximately 2% decrease in breast density per full term birth [65,66]. Body weight and BMI are positively correlated with the total area of the mammogram and the area of non-dense tissue and negatively correlated with the area of dense tissue [47,48,62]. Specifically, breast density has been observed to decrease 1 % per Kg of body weight [66].

The apparent paradox between decreasing breast density with increasing age and increasing BC incidence with increasing age has been related to the Pike model of BC incidence [44-47,53].

Specifically, this model conceptualizes that rather than chronological age being the relevant measure for the age-specific incidence of BC it is the rate of breast tissue ageing or exposure that is important [44]. It is thought that the rate of breast tissue ageing is more rapid at the time of menarche, slows with pregnancy and slows further post menopause [44]. Thus, while the cumulative exposure to breast tissue ageing and the incidence of age-specific BC increases with age, the rate of increase slows after menopause [44]. It is hypothesized that differences in the rate of change in MD earlier in life may be associated to later BC risk rather than the rate of change of density with increasing age [44,46,47]. Further, cumulative exposure to breast density may reflect cumulative exposure to hormones and growth factors which affect breast tissue composition and may be important in the age-specific incidence of BC. Greater MD for a given age is associated with an increased risk of BC and fits into Pike's model breast tissue ageing [46,47].

Positive associations are observed between combined postmenopausal estrogen and progestin replacement therapy and increased MD and inverse associations are observed between selective estrogen-receptor modulators (SERMs) such as tamoxifen and breast density [53,62,64]. HRT use has been shown to increase breast density by 3 to 5% while tamoxifen therapy has been shown to reduce breast density upwards of 10% after 12-18 months [66, 67-69]. Further, the association between combined HRT and breast density is higher with increased age [64]. However, paradoxical associations between high MD and higher risks of both ER and PR positive and negative BCs makes it difficult to determine the attribution of hormonal influence on breast density [53].

Lastly, evidence supports the association between a family history of BC and more extensive MD[61]. A recent study examined the association of family history of BC risk with percent MD and found a 3.1% greater percent MD, on average, in women with one affected first degree family member and a 7.0% greater percent MD, on average, in women with two or more affected family members compared with women without a family history of the disease[61].

*2.2.3.3 Measurement of MD*

Both qualitative and quantitative approaches are used to classify MD. The most widely used qualitative classification for breast density is the BIRADS which has four categories: 'extremely fatty' (<25% dense tissue), 'scattered density' (25%-50% dense tissue), 'heterogeneous density' (51%-75% dense tissue), and 'extremely dense' (>75% dense tissue) [38,39]. Studies have demonstrated the 'extremely dense' category to be highly predictive of eventual BC incidence [38,40,41]. A recent meta-analysis demonstrated a strong association between breast density with BC risk with a reported 4-fold increase in BC risk for breast density in BIRADS category IV vs. category I (RR = 4.03, 95% CI:3.10 - 5.26) [39].

Quantitative approaches use computer-assisted technology where the area of the breast and total area of breast parenchyma are outlined in digitized mammograms. The total parenchymal area of the breast is then divided by the total breast area to determine the percentage of density [39]. This provides a continuous measure and is more objective than qualitative methods. High correlations are observed between intra- and inter-rater comparisons using measured breast density techniques [42]. Further, quantitative methods provide both an absolute measure of the area of dense tissue (absolute density) as well as the total area of the breast seen in the mammogram [38,42]. Studies using quantitative approaches to measure MD have all observed an increased risk of BC, with RRs ranging from 1.8 to 6, associated with more extensive density [38,48,52]. In the meta-analysis conducted by McCormack and colleagues, percent MD measurements showed a stronger relationship with BC compared with qualitative approaches such as BIRADS [45]. The authors reported a RR of incident BC of 1.79 (95% CI: 1.48 - 2.16), 2.11 (95CI: 1.70-2.63), 2.92 (95% CI: 2.49-3.42) and 4.64 (95% CI: 3.64-5.91) for categories 5% to 24%, 25% to 49%, 50% to 74%, and >75% compared with < 5% MD [45].

## 2.3 Vitamin D

### 2.3.1 Importance of Vitamin D in Health

Vitamin D is a fat soluble vitamin that is essential for the normal development and mineralization of a healthy skeleton [70,71]. The vitamin D receptor (VDR) gene has been discovered in most tissues and cells in the body and elicits a wide variety of biologic responses including the promotion of intestinal calcium absorption, insulin secretion and phosphate homeostasis [70,72,73]. Vitamin D is important for muscle and bone strength and deficiency is associated with cortical bone loss, increased bone turnover and increased parathyroid hormone levels, predisposing one to osteoporosis [70]. Vitamin D deficiency has also been notably determined to be the cause of rickets in children and osteomalacia in adults and is a suspected risk factor for cardiovascular disease, hypertension, hip fractures, insulin resistance, autoimmune diseases (including type 1 diabetes, rheumatoid arthritis and multiple sclerosis), schizophrenia and some cancers (breast, colon and prostate) [70-72].

### 2.3.2 Sources of Vitamin D

Vitamin D is produced naturally in the body through exposure to the sun's ultraviolet (UV) rays [70-72]. Approximately 90% of vitamin D is obtained through sunlight exposure and required circulating vitamin D levels in the body are maintained with adequate sunlight exposure [70]. There are several factors influencing UV radiation levels including season, latitude, month of year, cloud cover and ozone levels [70,71]. Blood levels of vitamin D have seasonal variation with peaks and troughs at the end of the summer and winter respectively [70]. UV levels decrease as one moves away from the equator towards the poles and, thus, UV(B) wavelengths are insufficient at producing vitamin D in winter months at latitudes above 37 degrees [71].

Foods and vitamin supplements are also sources of vitamin D, however, adequate levels are unlikely to be achieved through foods alone due to the limited sources and a lack of requirements for fortification [70,72]. The main food sources include fatty fish, eggs and fortified foods such as dairy products, including milk and margarine, and juices [70].

### 2.3.3 Vitamin D Metabolism

Upon exposure to UV(B) light, the skin converts UV(B) to pre-vitamin D3 from 7-dehydrocholesterol which is then changed in the skin to Vitamin D3 [70,72]. Vitamin D from sun exposure (D3 or cholecalciferol) and/or the diet (D2 or ergocalciferol) is metabolized in the liver to serum 25-OH-D and is the main metabolite used by clinicians to determine a person's vitamin D status [70,72-74]. Serum levels of 25-OH-D are directly related to cutaneous synthesis from exposure to sunlight and vitamin D intake from food and supplements [73]. Serum 25-OH-D is of clinical use primarily due to its long half-life (approximately 2-3 weeks) and thus provides some indication of the body's reserve of vitamin D from UV radiation and dietary intake [73]. This form of vitamin D, however, is biologically inactive and must be further metabolized by the enzyme 25-hydroxyvitamin D-1-α-hydroxylase (encoded by the CYP27B1 gene) in the kidneys to its active form, 1,25-dihydroxyvitamin D ($1,25[OH]_2D$) [72-74]. The production of $1,25(OH)_2D$ is tightly regulated by the parathyroid hormone produced from the kidneys and has a relatively short half-life (~4-6 hours). It therefore does not represent a measure of long term vitamin D levels [73].

### 2.3.4 Vitamin D Deficiency

There is no widespread agreement on the optimal levels of serum 25-OH-D in the body, however, vitamin D deficiency is defined by most as a serum level of less than 20 ng per milliliter (ng/ml), vitamin D insufficiency is recognized to be between 21-29 ng/ml and the preferred level for

serum 25-OH-D is recommended to be greater than 30 ng/ml [72,74]. Vitamin D intoxication, while rare, is generally observed when serum levels of 25-OH-D are greater than 150 ng/ml and is associated with hypercalcemia, hypercalciuria and hyperphosphatemia [70,72,74]. Based on the 2011 Institute of Medicine (IOM) report on calcium and vitamin D it is recommended that people between the ages of 1-70 have a recommended dietary allowance (RDA) of vitamin D of 600 IU/day and people above the age of 70 have an allowance of 800 IU/day in order to achieve a serum 25-OH-D level of at least 20 ng/ml [75].

While the IOM committee believes that the majority of North American populations are meeting its needs of maintaining serum 25-OH-D levels at 20 ng/ml [75] there are other reports that estimate between 30-50% of the population in both Europe and North America are vitamin D insufficient or deficient [74]. Whiting et al. [76] sought to determine the prevalence of vitamin D sufficiency in Canadians between the ages of 6-79 years. Overall, the authors found that one quarter of Canadians were vitamin D deficient based on circulating serum 25-OH-D levels and more than one-third of Canadians not taking supplements did not have sufficient vitamin D levels in winter [76]. These findings suggest that certain North American populations, particularly Canadians, are susceptible to vitamin D deficiency especially in the winter months.

Individual level factors that affect one's vitamin D status include age, estrogen level, skin pigmentation and BMI [77,78]. Postmenopausal women are at higher risk of vitamin D deficiency than younger women as aging reduces vitamin D production in the skin and estrogen deficiency decreases the metabolic activation of vitamin D and expression of VDR gene [58,77,78]. Skin pigmentation influences the amount of UV radiation that reaches our skin and can affect serum 25-OH-D concentrations [77]. Studies measuring skin pigmentation by colorimetry have found that people with darker skin types typically achieve lower serum 25-OH-D concentrations for a specified UV exposure than fairer skin types [77]. Ethnicity is often used as a proxy measure to

control for the effect of one's skin colour on vitamin D levels [77]. Lastly, BMI has been associated with the bioavailability of serum 25-OH-D [78]. One study examined whether obesity altered the cutaneous production of vitamin D3 (cholecalciferol) or the intestinal absorption of vitamin D2 (ergocalciferol) within the body [78]. The authors found that obese subjects had significantly lower serum 25-OH-D concentrations than age-matched controls and concluded that decreased bioavailability of vitamin D3, specifically, is likely due to its deposition in body fat which is less metabolically available [78].

## 2.4 Vitamin D and Breast Cancer

The ultimate interest of this research is with respect to a contribution to understanding the relationship between vitamin D and BC. The following provides a brief review of the evidence regarding the role of vitamin D in BC etiology.

### 2.4.1 Experimental Evidence

There is increasing experimental evidence to support the hypothesis that vitamin D and the VDR gene are involved in multiple pathways that may be important in the etiology of BC [72,79,27,80-87, 88]. Vitamin D affects cell proliferation, differentiation and apoptosis of both normal and transformed cells indicating that the vitamin D pathway has the potential to negatively impact cell growth regulation and proliferative activity [72,81-83]. Experimental studies have shown that $1,25(OH)_2D$, the metabolically active form of vitamin D, exerts its main actions via the VDR gene [79]. Both normal and malignant breast tissue have been shown to have a VDR gene that responds to $1,25(OH)_2D$ and these breast cells express the enzyme 25-hydroxyvitamin D 1-α-hydroxylase [72,79, 81,82,86]. $1,25(OH)_2D$ appears to have the ability to prevent angiogenesis if a cell becomes malignant, reducing the potential for the malignant cell to survive [72, 79,86]. Laboratory data have

shown that the VDR gene, as expressed in the normal mammary gland, opposes estrogen-driven proliferation and maintains differentiation, lending further evidence that it participates in negative-growth regulation of mammary epithelial cells [72]. Lastly, preclinical studies have shown that BC development in animals can be reduced by administering vitamin D compounds and conversely, VDR gene knockouts in animal models have shown an increased number of chemically induced mammary tumours [72,84].

### 2.4.2 Epidemiologic Evidence

Numerous epidemiologic studies have investigated the relationship between vitamin D and BC risk. Recent review papers [79-82,86,89,90], meta-analyses [87,88,91] and pooled analyses [92,93] reflect the number of investigations and state of evidence. Epidemiologic evidence comes from ecologic studies based on geographic variation in sun exposure and UVB radiation, observational studies based on sun exposure and UVB radiation, observational studies of diet and supplemental vitamin intake, and observational studies of vitamin D metabolites measured in blood.

#### *2.4.2.1 Vitamin D from Sunlight Exposure*

There have been several ecologic studies examining the association between sun exposure or UVB radiation and BC incidence or mortality [86,89]. These investigations are relevant because sun exposure is the major source of vitamin D and ecologic studies can facilitate the examination of large exposure contrasts across populations. Ecological studies on sun exposure and BC support an inverse association between UVB exposure and BC risk [86,89]. In one study, the inverse association between sun exposure and BC mortality was observed only for women over the age of 50 [89]. However, these ecological studies suffer from concerns relating to attributing exposure-disease relationships seen at the aggregate level to that of individuals. For example, it is possible

that populations with greater sun exposure may also benefit from healthier diets with more fruits and vegetables which may explain the ecologic association.

A recent review of the epidemiological evidence on sun exposure and the prevention of cancer supports the association between chronic sun exposure and a reduced risk of BC [94]. Several observational studies have examined the relationship between self-reported sunlight exposure and BC risk and all but one reported a protective effect of sunlight exposure on risk [95-100]. For example, a cohort study based on the National Health and Nutrition Examination Survey observed risk reductions in women living in high sunlight regions compared to low sunlight regions [95]. In a population based case-control study in Ontario, sun exposure between the ages of 10 and 19 was associated with reduced BC risk and the results did not differ between pre- and postmenopausal women [96]. Further, Anderson et al. observed protective associations between time spent outdoors and risk of BC during 4 independent life periods (>21 vs. ≤6 hours/week. Teenage years: OR = 0.71, 95% CI = 0.60-0.85; 20-30 years: OR = 0.64, 95% CI = 0.53 - 0.76; 40-50 years: OR = 0.74, 95% CI = 0.61 - 0.88; and 60-70 years: OR = 0.50, 95% CI = 0.37 - 0.66) [97]. The authors also observed a significant inverse association between a comprehensive solar vitamin D score and BC risk. Associations in this study were not modified by menopausal status [97].

*2.4.2.2 Dietary and Supplemental Vitamin D Intake*

Recent reviews and meta-analyses have considered the evidence from observational studies of diet and supplemental vitamin intake on BC risk [79,82,86-88]. For example, Perez-Lopez et al. reviewed the evidence from 9 studies (3 case-control, 5 cohort, and 1 RCT) on diet, vitamin D supplements and BC risk which together supported a protective effect of high vitamin D ingestion on BC risk [86]. However, results are not consistent across studies and the summary effect is modest, as illustrated by the summary RR estimate of 0.91 for high versus low intake from a

recent meta-analysis [88]. Further, a stronger overall inverse association was observed in the group of studies conducted among premenopausal women only compared with studies of postmenopausal women [88]. Overall, the evidence on dietary intake of vitamin D and BC is not convincing of a relationship. This is potentially due to difficulty in estimating vitamin D exposures accurately (measurement error) and reliably using self-reported questionnaire data (information bias) [89]. More importantly, since diet is unlikely to account for a large proportion of vitamin D levels circulating in the body dietary assessment through questionnaires is not a comprehensive measurement tool for total vitamin D exposure.

*2.4.2.3 Circulating Vitamin D Levels*

Circulating vitamin D metabolites and BC have been investigated in several case-control and nested-case-control studies and two pooled analyses and two meta-analyses have been conducted to date to summarize the current evidence on the relationship between serum 25-OH-D and BC risk [79,82,86,88,91,93,101,102]. Studies using metabolite markers, which are less susceptible to measurement error, have observed the largest protective effects of those examining the vitamin D→BC relationship. For example, two meta-analyses [88,91,93] and two pooled analyses [92,93] support an approximately 50% reduction for the highest versus lowest categories of serum 25-OH-D exposure. However, there is considerable heterogeneity of effects among the individual studies reviewed. For example, inverse associations between serum 25-OH-D and risk of BC have been observed in both pre- and postmenopausal women in some studies while in another study the association was observed only among postmenopausal women [91]. Overall support of an inverse association between serum 25-OH-D and BC appears strongest in case-control studies but remains unconfirmed in nested case control studies where serum vitamin D levels are measured pre-cancer diagnosis [88,91]. In addition to the difficulty in assessing temporality between serum 25-OH-D ad BC in retrospective studies, studies done to date also largely do not differentiate

between pre and postmenopausal women in whom associations may differ and suffer from a lack of control of potentially important confounders such as physical activity[91]

*2.4.2.4 Vitamin D and Calcium*

Vitamin D and calcium are metabolically interrelated [82]. Circulating $1,25(OH)_2D$ is important in calcium homeostasis, increasing cellular uptake of calcium from circulating blood [82,85]. Only a few studies have investigated the interaction between dietary intakes of calcium and vitamin D on BC risk[103-107], two of which reported inverse associations with intakes of these two vitamins and premenopausal BC [103,105,106]. To our knowledge, no studies have been conducted to date on the interaction between blood levels of vitamin D and calcium on the relationship with BC. Further studies are also warranted to examine the joint effects of vitamin D and calcium on BC risk in postmenopausal women.

The evidence regarding the relationship between vitamin D and BC supports a modest protective effect. At present, the most compelling results continue to come from ecologic studies that are prone to the ecologic fallacy. Results across different studies are not altogether consistent and this may be due to an underlying susceptibility of the populations under investigation and difficulty in measuring relevant vitamin D exposure in observational studies.

## 2.5 Breast Density as an Intermediate Endpoint on the Vitamin D→ BC Pathway

Prospective studies of vitamin D exposure and BC risk are costly and require large sample sizes and long follow-up to obtain sufficient cases to evaluate meaningful relationships and retrospective studies rely on assessment of exposures which have occurred many years previously and are subject to non-differential misclassification and information bias. As a result, traditional epidemiologic studies are limited with respect to providing further understanding of a vitamin D-

BC relationship. Analogous to the measurement of blood pressure or cholesterol as a biomarker of heart disease and subsequent therapeutic intervention, the substitution of breast density as an intermediate endpoint for BC provides several advantages: including an outcome (MD) which is more common than a cancer event and is measured on a continuous scale, and a shorter time period between exposure and intermediate event than between exposure and malignancy. In addition, if a causal exposure-cancer relationship exists that is mediated through an intermediate endpoint then a stronger relationship will be observed in a study using the intermediate endpoint [108].

In general, intermediate endpoints are defined as biological events on the causal pathway between an exposure and a health outcome [108-110]. In order to evaluate a potential intermediate endpoint there needs to be some evidence that the exposure is associated with the potential intermediate and, independently, that the potential intermediate is associated with the outcome of interest[109]. The goal of this research project is to contribute to understanding the relationship between vitamin D and BC. In this context, MD is a strong predictor of BC risk and several authors are in support of using percent MD as an intermediate marker for BC research [42,45,47,108,111-115]. Breast density might not be a useful intermediate in the pathway between all exposures and BC risk and in that sense is not a necessary step in the development of all BC [109]. For health outcomes such as cancer, in particular, there are likely multiple pathways to a cancer outcome [109]. In this research the proposed pathway is vitamin D → breast density → BC and this section presents the evidence for the role of breast density in the biologic pathway between vitamin D and BC. Use of this intermediate endpoint, rather than a BC diagnosis, will allow for a strong investigation of a segment of the postulated underlying biologic pathway.

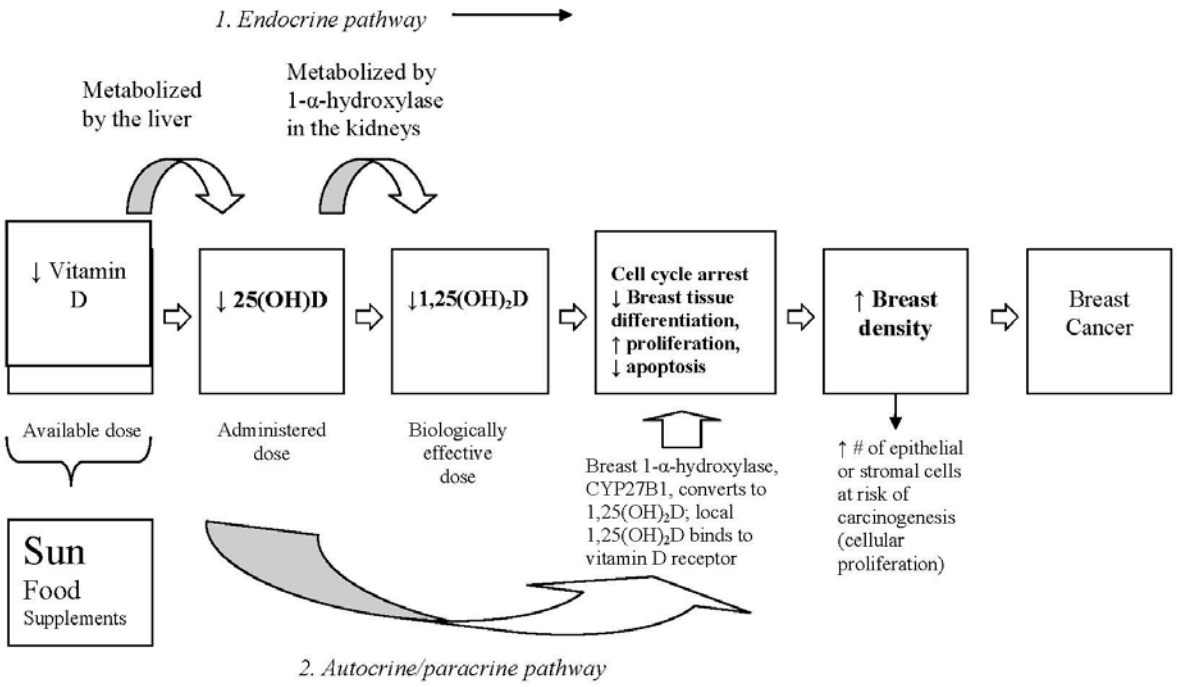
### 2.5.1 Vitamin D → Breast Density

The first component of this pathway (e.g. vitamin D → breast density) is the focus of this research. The following section will discuss both the hypothesized biological pathways in which vitamin D is thought to exert its' effects in the etiology of BC and the current epidemiological evidence on the relationship between vitamin D and breast density.

#### *2.5.1.1 Hypothesized Biological Mechanism in the Vitamin D → Breast Density Pathway*

Breast density reflects the extent of epithelial and stromal cells in the breast and thus may influence risk for carcinogenesis. There are two hypothesized pathways for vitamin D to reach and affect breast tissue (Figure 2.1). The first involves the endocrine pathway where circulating $1,25(OH)_2D$ reaches the breast tissue directly [82]. The second involves the autocrine/paracrine pathway in which serum 25-OH-D is metabolized in the breast tissue to $1,25(OH)_2D$ by 1-α-hydroxylase (CYP27B1) [13]. In the breast tissue $1,25(OH)_2D$ binds to the VDR gene and thereby influences regulation of cell proliferation, differentiation and apoptosis [79,82], [116-118]. This mechanism may explain the antiproliferative and proapoptotic properties of vitamin D which are hypothesized to reduce breast density.

**Figure 2.1 Hypothesized Role for Vitamin D in Breast Cancer Etiology**




*2.5.1.2 Current Epidemiological Evidence on the Vitamin D → Breast Density Relationship*

A literature search identified nine observational studies that examined dietary intake of vitamin D in relation to MD [119-127], and five observational studies of circulating vitamin D in relation to MD [128-132]. The results of these studies and their methodological limitations are briefly summarized below. Please refer to Table 2.1 for additional study details.

*2.5.1.2.1 Dietary and Supplemental Vitamin D Intake and Breast Density*

One of the earliest studies by Vachon and colleagues examined the association of diet, including vitamin D, and MD in 1508 women in the Minnesota Breast Cancer Family Cohort study [125]. There was no association observed between vitamin D (quartiles) and breast density among women included in this study (p for trend = 0.68), nor when stratified by menopausal status (p for trend = 0.55 in premenopausal women; p for trend = 0.96 in postmenopausal women) [125]. The authors, however, did not account for sunlight exposure which is known to be the major source of

vitamin D exposure. In a subsequent study by Holmes et al. a trend in decreasing breast density with increasing dietary intakes of vitamin D and calcium among premenopausal women was observed (p for trend = 0.02) [126]. However, vitamin D supplements or sunlight exposure was also not accounted for in this study. Berube and colleagues subsequently reported a statistically significant inverse association between dietary intake of vitamin D and MD (OR=0.24, 95% CI: 0.11-0.53) in their study population of 1092 women and observed a significant trend in decreasing breast density with increasing vitamin D (and calcium) intake (<50 IU/d, 50-99 IU/d, 100-199 IU/d and ≥ 200 IU/d) ($p<0.01$) in an analysis restricted to women classified as having low breast density (≤30%) and women with extensive breast density (≥70%) [122]. Further, these trends were also observed for both premenopausal ($p<0.01$) and postmenopausal women ($p=0.05$). However, in a subsequent study by Berube et al. in Canada that evaluated the association of vitamin D and calcium from food and/or supplements with breast density in 777 premenopausal and 783 postmenopausal women, the authors found an inverse association between total intakes of vitamin D and breast density only among premenopausal women [119]. The authors found an 8.5% lower mean breast density among premenopausal women with each increment in daily total intakes of 400 IU of vitamin D [119]. While these authors accounted for vitamin supplement use, sun exposure was not measured. The authors also speculated that there may be residual confounding by multivitamin use in this study given the potential association between other vitamins and minerals found in multivitamins and breast density. The results of a study by Diorio and colleagues also support an inverse association between dietary vitamin D with breast density in 771 premenopausal women (an association with postmenopausal women was not conducted) [123]. Although vitamin supplement use was taken into account in the analysis, measurement error is of concern as the specific timing and dose of supplement use was not collected. Another study looking at the influences of diet on MD in Hispanic and non-Hispanic populations found breast density to be associated with vitamin D intake only among pre-menopausal Hispanic women [120]. However, the sample size for this study was quite small

(premenopausal women: n=137; postmenopausal women: n=101), particularly for stratified analyses by ethnicity, and the analyses did not control for possible confounding by family history on the relationship between vitamin D and breast density. Tseng and colleagues were the first to evaluate dietary intake, including vitamin D, and breast density among women at high risk for BC [121]. The authors observed an inverse association between breast density and the highest and lowest tertile of vitamin D intake (OR=0.5, 95% CI: 0.2-1.0), [121] however, only a small sample size of 157 high-risk women were included. The authors also observed a similar effect estimate, albeit with limited statistical power, when analyses were stratified by menopausal status [121]. Similar to other investigations above, these authors did not account for sunlight exposure in their exposure assessment. The only prospective observational study identified in the literature looked at the role of dietary vitamin D intake (in childhood and adulthood) in relation to breast density and found no association [124]. Again, the authors did not take into account any measure of sun exposure and the range in vitamin D levels and breast density measurements in the study population were speculated to be too low to identify meaningful associations. The last study identified assessed dietary intake of both vitamin D and calcium on MD in postmenopausal women, adjusting for sun exposure, and did not observe a relationship [127]. The authors reported the following mean mammographic percent densities across increasing categories of vitamin D intake: 5.8%, 10.4%, 6.2%, 3.8% and 5.1% respectively (p for trend = 0.67) [127]. The authors commented that the range of breast density in the study population was narrower and with a lower overall mean breast density compared with previous studies which may have attributed to the null associations observed. Further, study participants had relatively low levels of dietary vitamin D and calcium intake (>77% had less than 200 IU/d of vitamin D from food).

In summary, of the nine studies examining dietary and supplemental intake of vitamin D and breast density six reported an inverse association [119-123,126] and three reported no association [124,125,127]. Six of these nine studies evaluated the relationship in both pre- and postmenopausal

women [119-122,124,125], four of which showed a protective association with the magnitude of the association observed to be stronger in pre-versus postmenopausal women [119-122]. In addition to the inability to evaluate the association in both pre- and postmenopausal women in all the above studies, these studies suffer from two major sources of potential bias which question the internal validity of the results obtained. First, all nine studies have the potential for exposure misclassification as the measurement of vitamin D was derived from self-reported food frequency questionnaires [119-127]. Only a few accounted for vitamin D supplement use [119,123,127] and sunlight exposure [127] which are both important determinants of total vitamin D status. Second, with the exception of the study by Mishra and colleagues [124], all studies were cross sectional in nature which does not allow for determination of temporal associations between vitamin D and breast density [119-132].

*2.5.1.2.2 Circulating Vitamin D Levels and Breast Density*

Review of the five studies that have evaluated serum or plasma 25-OH-D in association with percent MD to date was of particular focus for the current investigation. One of the first studies to look at the association between circulating 25-OH-D and percent MD in 487 women found no association, nor was an association apparent when stratified by season of blood draw or menopausal status [128]. Surprisingly, these authors observed a non-significant trend in increasing density (percent density and dense area) with increasing serum 25-OH-D [128]. However, the authors had fairly low statistical power to detect associations particularly with stratified analyses by menopausal status. In contrast, Brisson and colleagues reported that changes in serum 25-OH-D were inversely related to changes in breast density in 741 premenopausal women after consideration of seasonal variation in both serum 25-OH-D and percent MD [129]. Green and colleagues [130] examined the association between MD and plasma 25-OH-D and $1,25(OH)_2D$ in 493 eligible postmenopausal women and found no cross-sectional association between plasma 25-OH-D with MD ($p=0.69$) or between plasma $1,25(OH)_2D$ and MD ($p=0.78$) [130]. The authors

noted concern for measurement error in both their measurement of vitamin D and MD. Further, the study was underpowered to detect clinically important differences in percent MD. Chai and colleagues investigated the relationship between serum 25-OH-D and MD in 182 premenopausal Caucasian and Asian women [131]. After adjustment for confounders, the authors did not observe any association (p=0.71) [131]. Notably, the authors did not control for season of blood draw and had low power to detect statistically significant differences. The most recent study, by Sprague and colleagues [132], set out to examine a group of molecules in the vitamin D pathway, including serum 25-OH-D, in relation to MD in 238 postmenopausal women. After adjustment for age, season, BMI and other important covariates these authors did not observe an association between serum 25-OH-D and percent MD (mean percent density for 1$^{st}$ quartile = 13.6% vs. 4$^{th}$ quartile = 13.3%; p for trend=0.49) [132]. This study, however, had limited statistical power to detect small differences in breast density due to the small sample size.

In summary, five observational studies have examined serum 25-OH-D in relation to breast density to date [128, 129, 130-132]. Four found no association between serum 25-OH-D and MD after controlling for important confounding variables [128,130-132] and one reported an inverse association [129]. In two of these five studies the association was evaluated in postmenopausal women only and no association was observed [130,132]. In addition, no association was observed in the study by Knight et al. that included both pre- and postmenopausal women nor was a relationship observed when the analyses were stratified by menopausal status [128]. The major source of bias of concern is that all of these studies were cross sectional in nature, with the ascertainment of blood samples for vitamin D measurement occurring in some cases long after the date of the mammogram, which does not allow for determination of temporal associations between vitamin D and breast density [119-132]. Lastly, all studies of serum 25-OH-D and MD relied on a single measurement of vitamin D which may not have been the best representation of a person's usual vitamin D status given the considerable variation in levels by season [128-132].

*2.5.1.2.3 Summary of Evidence on the Vitamin D → Breast Density Relationship*

The totality of evidence to date on the relationship between either dietary vitamin D or circulating 25-OH-D and breast density is not conclusive. The evidence appears to support a stronger relationship between vitamin D and breast density in premenopausal as compared with postmenopausal women. There are three possible explanations hypothesized for these findings: First, it is possible that a protective effect of vitamin D on MD does only exist in premenopausal women given their higher MD compared with postmenopausal women. There may also be a complex interplay between vitamin D, estrogen and insulin-like growth factor-I (IGF-1) with the ability for vitamin D to exert its effects on MD primarily in premenopausal women in the presence of higher levels of estrogen and IGF-1[119,123]. Second, a protective effect of vitamin D on breast density in postmenopausal women may exist but it has been difficult to observe in studies conducted to date. No studies have evaluated the association between circulating vitamin D and MD among postmenopausal women at increased BC risk who may sustain higher MD than their counterparts not at elevated risk. Lastly, a protective association between vitamin D and MD may exist in postmenopausal women but methodological limitations in the studies conducted to date, as described above, have obscured findings making it difficult to draw any firm conclusions. This may include the difficulty in adequately controlling for all estrogen-related risk factors that are strongly associated with BC risk.

The current prospective study, which collected serum samples at the time of randomization and prior to MD assessment, provides the opportunity to conduct a strong observational study largely eliminating selection bias and information bias observed in previous studies. Further, this study provides the opportunity to examine whether serum 25-OH-D is associated with changes in MD over time which is of interest given the natural history of breast density over time (i.e. inverse

association with age with the sharpest decline at the transition to menopause) and which has not previously been done.

### 2.5.2 Breast Density → Breast Cancer

The latter component of this pathway, breast density → BC, is strongly supported in the literature [47-53]. Review of the literature provides three different lines of evidence in support of this pathway, namely that (1) breast density and BC share a common set of risk factors, (2) women with higher breast density are consistently associated with increased BC risk and (3) changes in breast density are associated with decreased BC risk[47-53]. The following section provides a synopsis of the relevant epidemiological evidence as it relates to the above.

#### *2.5.2.1 Current Epidemiological Evidence on the Breast Density → Breast Cancer Pathway*

As reviewed earlier, the risk factors for breast density, including age, BMI, parity, age at first birth, number of births, menopausal status, diet, alcohol, HRT, and history of benign breast disease are largely the same as the risk factors for BC.[47,48,62-64] This strengthens the hypothesis that breast density is an intermediate in the pathway to BC.

A recent meta-analysis was conducted on the association between percent MD and BC which included more than 40 epidemiological studies [45]. The authors reported an increased risk of BC with increasing breast density with a magnitude of association of 4.64 (3.64-5.91) for the most dense (>75%) compared with the least dense category (<5%) controlling for other known risk factors [45,53]. A sub-analysis was also conducted to evaluate the association between breast density and BC risk among both premenopausal and postmenopausal women. The results showed that breast density is a marker for risk in both groups with similar strength of associations [45]. Another study demonstrated that postmenopausal women with 5-25% breast density at initial evaluation

had a 5.7 (95% CI: 2.2-15.2) times greater risk of developing BC if their density did not change over an 8-year period. A trend of decreasing risk was observed with decreasing percentage density [43,48]. Specifically, postmenopausal women whose breast density decreased to less than 5% during the same interval had a 1.9 (95% CI: 0.6-6.1) times greater risk of developing BC [43,48]. However, in women with >25% density at initial evaluation, there was no clear association with a lowered BC risk with decreased breast density over time [43]. More recently, Vachon and colleagues (2010) showed that a reduction in breast density (decrease in one BIRADS category) over a period of 6 years was associated with a reduction in BC incidence (HR=0.72, 95% CI: 0.50-0.99) in a case-cohort of 19,924 women over the age of 35[133]. Due to insufficient study power the authors were unable to stratify the analyses by menopausal status.

Further supporting the breast density → BC pathway, recent evidence from BC intervention studies has demonstrated that BC risk can be predicted through changes in MD [69,111,112]. Results from the IBIS-1 study showed a reduction in BC risk by ~40% with tamoxifen therapy. Cuzick and colleagues [69] have subsequently shown that women in the tamoxifen arm with a >10% reduction in breast density had a 63% reduction in BC risk compared with the control group (OR = 0.37, 95% CI = 0.20 - 0.69, *P* = .002). Interestingly, women in the tamoxifen group whose breast density was not reduced by 10% or more did not experience a significant reduction in BC risk, relative to the control group [69]. In subgroup analyses the authors showed that both premenopausal and postmenopausal women who experienced a reduction in breast density of at least 10% were also observed to have a reduced risk of BC associated with tamoxifen although the result was not statistically significant in postmenopausal women [premenopausal women: OR=0.27 (95% CI: 0.11-0.66); postmenopausal women: OR= 0.53 (0.22-1.28)].

In addition, investigators from the Women's Health Initiative recently presented results from a nested case-control study showing that both baseline and change in MD were significantly

associated with BC risk for postmenopausal women in the estrogen progestin therapy (EPT) arm [111]. Specifically, women in the EPT arm with the greatest increase in breast density had a 3.6 fold increase in BC risk compared with those women with the lowest increase or decrease (95% CI: 1.52-8.56).

All of these studies combined strengthen the hypothesis that reducing breast density, in both pre- and postmenopausal women, can be associated with reduced risk for BC, making breast density an attractive modifiable marker for BC.

## 2.6 Breast Cancer Chemoprevention

Chemoprevention of cancer has been defined as 'the use of natural, synthetic, or biochemical agents to reverse, suppress or prevent the carcinogenic process to neoplastic disease [134]. As reviewed earlier, there is a significant positive association between estrogen (both endogenous and exogenous) and the risk of BC and thus initial efforts at chemoprevention have focused on agents to target hormonally responsive BCs (i.e. ER + and PR + BCs). This section will briefly review the two classes of drugs, SERMs and aromatase inhibitors (AIs), which have demonstrated efficacy in the reduction of BC incidence. The rationale for studying the vitamin D and breast density relationship in postmenopausal women participating in an NCIC Clinical Trials Group chemoprevention trial of the AI exemestane will be provided.

### 2.6.1 Selective Estrogen Receptor Modulators

SERMs such as tamoxifen act by selectively blocking or modulating parts of intracellular signal transduction of ERs thereby inhibiting ER binding and reducing the effects of estrogen [53,135]. Tamoxifen retains some of its estrogenic effects on certain tissues and cells which allows

preservation of bone density in postmenopausal women, however, the risk of endometrial cancer is increased [53]. SERMS are effective in preventing contralateral tumours in both pre and postmenopausal women with BC and in reducing BC incidence in women at increased risk [53,67,68,134-138]. Specifically, the tamoxifen prevention trials combined have shown a 38% overall reduction in BC incidence. As presented previously, use of tamoxifen has also been shown to reduce MD, lending further evidence that it is modifiable, and this has been shown to translate into a reduced risk for BC development among those women with the greatest reductions in MD [69]. However, given the rare but serious adverse effects of tamoxifen, including endometrial cancer, venous thromboembolism and cataracts, many women are not choosing to take this drug for prevention[38,135].

### 2.6.2 Aromatase Inhibitors

AIs act via the inhibition of the cytochrome P450 enzyme aromatase that catalyzes the conversion of androgens to estrogens, the last step in estrogen synthesis [137, 135]. AIs target aromatase which is the enzyme responsible for this conversion, lowering estrogen levels by 97% to 99% [135, 139]. AIs have been shown to be effective for the adjuvant treatment of ER+ BC in postmenopausal women and are being studied in BC prevention [53,136,140,141,142]. The NCIC Clinical Trials Group MAP.3 trial recently reported that the AI, exemestane, significantly reduced the development of invasive BC in postmenopausal women at moderately increased risk for the disease by 65% (HR = 0.35; 95% CI: 0.18 to 0.70; P = 0.002), with no serious side effects after a median duration of 3 years [142].

Exemestane binds irreversibly to aromatase causing permanent inactivation of the complex and, based on randomized trial data, appears to have a superior therapeutic index, thus offering greater efficacy and a better end-organ profile, than SERMs [136,137,141]. Although circulating estrogen

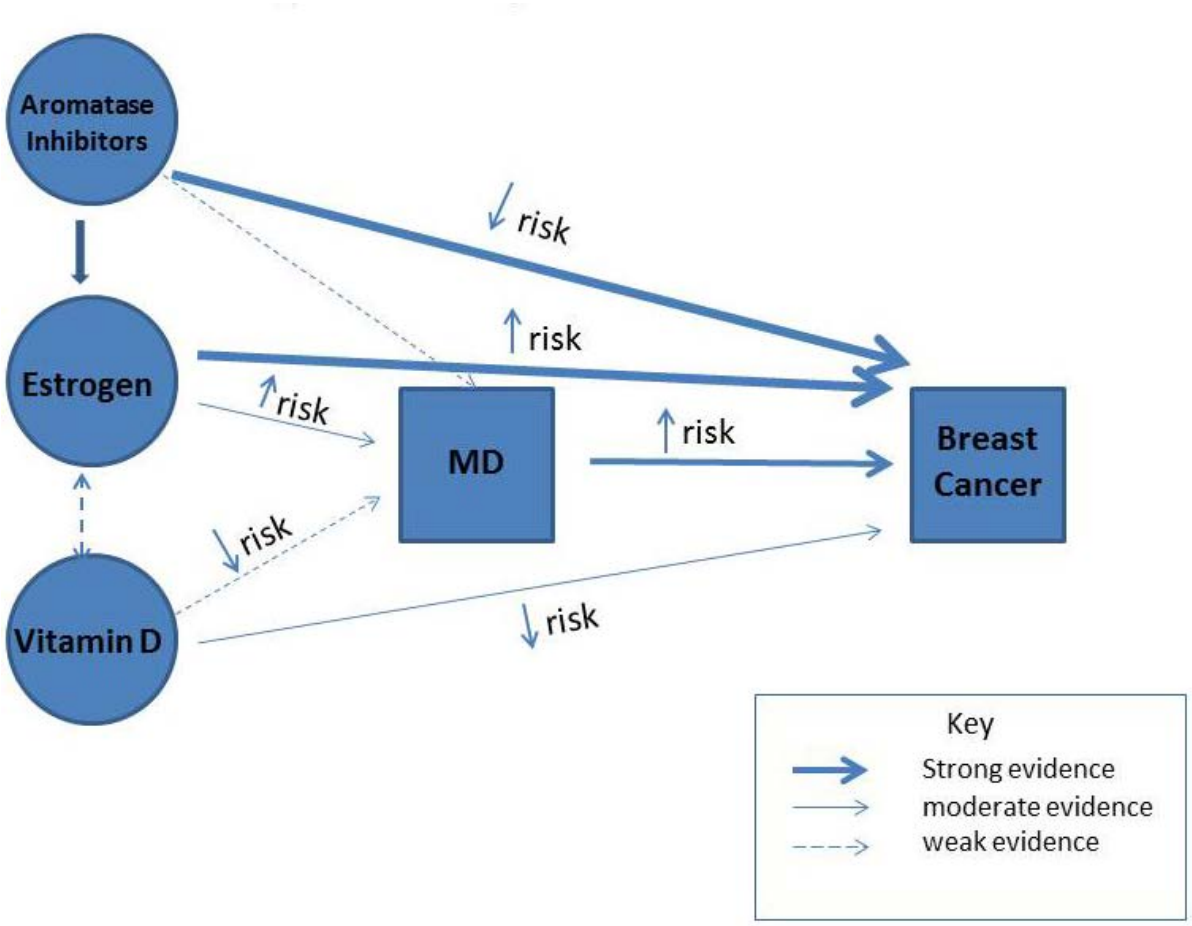
levels come mainly from ovarian production of estrogens in premenopausal women, peripheral aromatization of androgens to estrogens from adipose tissue is the main source of plasma estrogen in postmenopausal women[115,141]. Thus, while peripheral plasma levels of estrogen are lower in the postmenopausal women the breasts of both pre- and postmenopausal women have comparable estrogen concentration [141]. Thus, AIs have the ability to inhibit aromatase and reduce both peripheral and intra-breast estrogen levels [141]. As reviewed by Vachon and colleagues, the few studies to date that have evaluated the association between AIs and MD have reported inconsistent results [143]. Investigators of the NCIC Clinical Trials Group are currently investigating the relationship between exemestane and MD in their study population independent of the current thesis project.

### 2.6.3 Rationale for Investigating the Vitamin D → Breast Density Relationship in MAP.3

The primary objective of this research is to examine whether there is an association between circulating levels of vitamin D and breast density in women at increased BC risk. Evidence has been presented that demonstrates vitamin D's ability to inhibit cell proliferation and induce differentiation and vitamin D affects the cell cycle, apoptosis, hormone receptors and angiogenesis – all of which affect BC growth [144]. The study population in the current study participated in MAP.3 which demonstrated a protective effect of exemestane on the risk of invasive BC [145]. The interaction between vitamin D and exemestane on the risk of BC is an area of research which has not yet been investigated. There is some evidence that the efficacy of anti-cancer agents including paclitaxel, doxorubicin, platinum compounds and tamoxifen has been enhanced by the addition of vitamin D in human BC cell lines [144]. Conversely, there is biologic evidence to suggest that vitamin D stimulates the aromatase enzyme, CYP19, and whether this may result in a decrease in the therapeutic benefits of the AIs is of significant clinical importance [144].

A summary of the epidemiologic evidence on vitamin D and AIs on the breast density/BC relationship has been presented and is depicted below in a conceptual framework (Figure 2.2). The epidemiologic evidence on the relationship between vitamin D and MD is inconsistent across studies. This inconsistency may be, in part, the result of residual uncontrolled confounding by estrogen related risk factors which are known to be strongly associated with BC development. Exemestane, the treatment arm of the underlying RCT, binds irreversibly to aromatase causing permanent inactivation of the complex [136,137,141]. To the extent that the relevant exposure window to serum 25-OH-D is post randomization, nesting an observational study within this RCT allows us to look at the association between circulating vitamin D and breast density at ≥ 3 year follow-up as well as changes over time in MD in an estrogen suppressed group (i.e. the treatment arm of the trial). If the relationship between vitamin D and either MD at ≥ 3 year follow-up or changes over time in MD differs by trial arm results will be reported independently. If there is no modifying effect of AIs on the vitamin D → MD relationship utilizing data from this well-controlled RCT allows us to remove the possibility of confounding by a woman's estrogen levels after trial randomization since the vitamin D groups should be balanced on those hormonal factors. The ability to control for this important confounder provides an advantage over other epidemiologic investigations on this relationship to date. That said, the extent to which the relevant exposure window to serum 25-OH-D on MD is prior to randomization, the vitamin D groups may not be balanced on these hormonal factors. However, we are able to control for a myriad of variables that are related to estrogen exposure that may confound the underlying relationship between vitamin D and breast density.

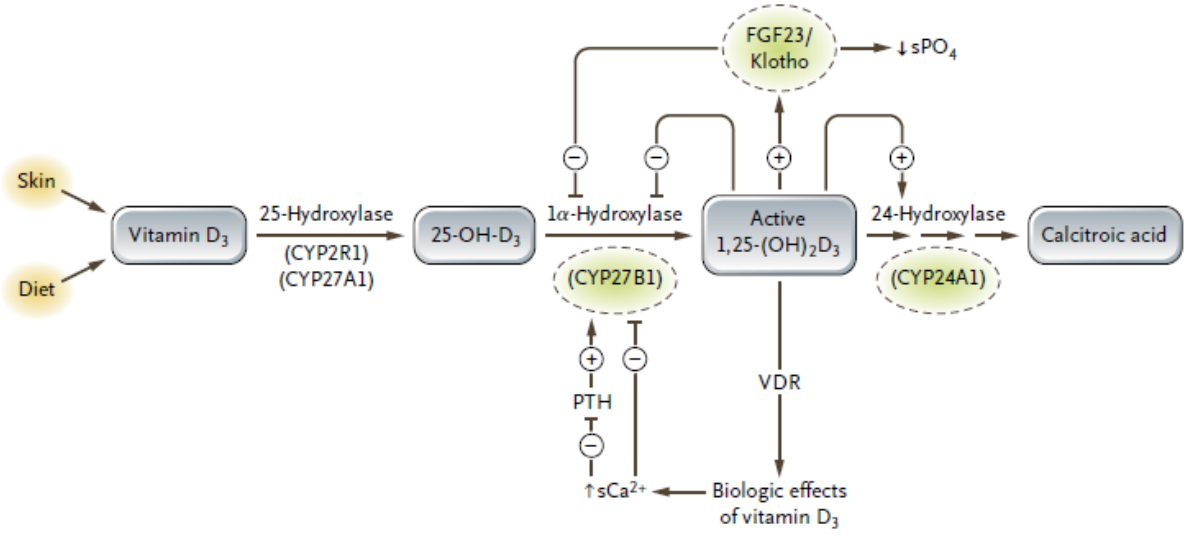
**Figure 2.2 Conceptual Framework**




## 2.7 Vitamin D Pathway Genes

There are several genes in the vitamin D pathway that are involved in the synthesis, transportation and degradation of vitamin D (Figure 2.3) [82,146-151]. The most frequently studied vitamin D pathway genes to date include the VDR, the vitamin D-binding protein gene (Gc) and genes that code for enzymes in the metabolic pathway including CYP27B1 and CYP24A1[82,146-150]. Some studies have evaluated the independent gene effects on the risk of different cancers, including breast, but few have investigated polymorphisms in these genes as potential modifiers on the vitamin D and BC relationship [146,148,152].

**Figure 2.3: Main Vitamin D Pathway Genes**




Schlingmann et al., 2011

The following sections briefly review the current evidence, predominantly from systematic reviews and meta-analyses, on the most widely studied polymorphisms in these vitamin D pathway genes to date, their relationship with BC and/or breast density and their potential effect modification on the vitamin D and BC relationship.

### 2.7.1 Polymorphisms in Vitamin D Pathway Genes

Vitamin D exerts its cellular growth and differentiation via the VDR gene, known as a nuclear transcription regulatory factor, which is located on chromosome 12q12-q14 [79,149]. The VDR gene has been the most widely studied gene in the vitamin D pathway with over 470 polymorphisms identified to date [148]. Of the many polymorphisms identified, Fok1, Bsm1, Apa1, Taq1, and Poly(A) have been the most widely studied [79,82, 148,150]. These polymorphisms are called Restriction Fragment Length Polymorphisms (RFLPs) and most, with the exception of Fok1, have an unknown functional effect [148,150,153,154].

The protein encoded by the vitamin D binding protein gene, known as the group specific component (Gc) gene, plays a key role in vitamin D metabolism. It carries vitamin D metabolites to various sites along the vitamin D pathway facilitating vitamin D actions [148,152]. Two single nucleotide polymorphisms (SNPs) (rs4588 and rs7041) in the Gc gene have repeatedly been associated with serum 25-OH-D levels [152,155]. Researchers that have used dietary and sun exposure questionnaires to measure vitamin D status have been interested in whether variants in the Gc gene plays a role in individual differences in serum 25-OH-D levels and whether there are interactions with these variants on vitamin D-disease associations. However, the evaluation of variants in the Gc gene that are involved in converting vitamin D to serum 25-OH-D is not relevant in the current study since these genetic influences precede our primary exposure measure of serum 25-OH-D.

Cytochrome P450 type 27B1 (CYP27B1) is an enzyme that plays an important role in calcium metabolism, tissue differentiation and bone growth by catalyzing the conversion of 25-OH-D to the physiologically active form of vitamin D, namely,1,25$(OH)_2$D [148,156]. Only a few common (minor allele frequency >5%) genetic variants have been identified to date with limited evidence on how these polymorphisms may affect gene function [148].

Cytochrome P450 type 24A1 (CYP24A1) is a member of the cytochrome P450 superfamily of enzymes and is involved in the degradation of 1,25(OH)2D [148,156,157]. This enzyme plays a key role in calcium homeostasis through regulation of 1,25(OH)2D. Laboratory findings have provided evidence for a biological role for CYP24A1 in humans [151,157]. However, few studies have evaluated variants in CYP24A1 in relation to vitamin D biomarkers [152].

### 2.7.2 Polymorphisms in Vitamin D Pathway Genes and Breast Cancer

As reviewed above, the metabolism of vitamin D involves many different genes and there is interest in investigating whether polymorphisms in these genes may modify BC risk [152,158]. Below is a review of the current evidence for the relevant major polymorphisms of interest in vitamin D related genes and their known relationship with BC.

#### *2.7.2.1 Polymorphisms in the VDR Gene and Breast Cancer*

It has been hypothesized that genetic polymorphisms might exert effects on VDR gene expression and protein function, thereby influencing risk of BC [79, 82,83]. Several epidemiological studies have investigated VDR gene polymorphisms and BC incidence, however, the totality of evidence has been inconsistent [79,81,82,159,160, 161,158,162,163,164]. A review of 13 case-control studies of various VDR gene polymorphisms in relation to BC risk was conducted by Cui & Rohan [82]. The authors concluded that certain polymorphisms of the VDR might modify BC susceptibility [82].

Cumulative evidence for an association with BC risk is strongest for the Fok1 restriction enzyme (rs2228570 aka 10735810) which has a polymorphic site in exon 2 at the 5'end of the VDR gene which results in truncation of the first 3-amino acids at the N-terminus and a shorter protein [79,82,159,160,162,163,164]. The shorter protein interacts with a key transcription factor more efficiently giving an increased vitamin D dependent gene transcription [79,82,159, 160,162,163,164]. Epidemiological data support an increased risk of BC in association with the Fok1 homozygous variant genotype (i.e. *ff* vs. *Ff* and *FF*) [164]. For example, a 34 percent higher BC risk was observed for women with the *ff* genotype in the Nurses' Health Study [159]. The prevalence of this less active *ff* genotype in the population is estimated to be approximately 15%. [152,158,164]. There is also some, although weaker, evidence for an association with BC for the Bsm1 restriction enzyme (rs1544410) which identifies a polymorphic site at an intron at the 3'-end which is in linkage disequilibrium with

other polymorphisms including Apa1 and Taq1 [159,160]. While functional data have been inconclusive for Bsm1 to date, a recessive model of allele influence (i.e. *bb* vs. *Bb* and *BB*) is also suggested for Bsm1 on BC risk, with the prevalence of the *bb* genotype in the population estimated to be approximately 34-38% [152,158,164]. A recent review and meta-analysis of these two VDR polymorphisms and cancer risk has been conducted [149]. The authors identified 13 independent studies on Fok1 polymorphisms and BC risk and found a significant increase in BC when comparing the Fok1 *ff* genotype with *FF* carriers (OR=1.14, 95% CI=1.03-1.27) [149]. No statistically significant associations were observed for the meta-analysis including 15 independent studies on the association between Bsm1 polymorphisms and BC [149]. The studies conducted on Apa1, Taq1 and Poly(A) to date have largely shown inconsistent associations with BC [148]. The minor allele frequencies for these three polymorphisms are estimated to be 26-28% for Apa1 *AA* [152,158], 35-36% for Taq1 *TT* [152,158] and 35% for Poly(A) *LL* [158]. Only one study was identified in the literature that looked at the association between polymorphisms in the VDR gene including Fok1 and Bsm1 and MD [165]. The study population included only premenopausal women and the authors observed no statistically significant associations for any of the VDR polymorphisms with breast density.

Few studies have examined serum 25-OH-D and VDR polymorphisms in relation to BC risk with inconsistent results; [159, 161, 158, 166, 152] and, to our knowledge, no studies have examined interactions between serum 25-OH-D and VDR polymorphisms in relation to breast density.

*2.7.2.2 Polymorphisms in Main Vitamin D Metabolism Genes*

The literature for the prevalence of CYP27B1 polymorphisms in the general population is fairly sparse to date. In one study, the frequency for the least prevalent genotype of the CYP27B1 rs4646536 polymorphism (*CC)* was reported as approximately 16% [167]. To date, only one study was identified that set out to evaluate the association between three polymorphisms in the

CYP27B1 gene and BC risk, however, these polymorphisms were excluded from subsequent analyses as they were in significant departure from Hardy-Weinberg equilibrium [152]. Lastly, three studies were identified that have evaluated polymorphisms in the CYP24A1 gene in relation to BC risk [152,158,168]. McCullough et al. [158] evaluated the association between CYP24A1 rs2296241, among other vitamin D pathway genes, and BC risk and found no overall association among postmenopausal women. Another study evaluated the association between BC risk and over 500 SNPs in 12 vitamin D related genes including SNPs in the CYP24A1 gene and found no association with BC risk after adjusting for multiple comparisons [168]. Only one study has investigated both the independent gene effects and the interaction between four variants in CYP24A1 with vitamin D from diet and sunlight and BC risk in both pre and postmenopausal women[152]. The authors did not observe any significant associations with CYP24A1 polymorphisms and risk for BC nor did they observe any significant interactions between vitamin D and genetic variants of CYP24A1 with BC risk [152]. However, when stratified by menopausal status, the authors did observe an increased BC risk for postmenopausal women with the CYP24A1 rs2181874 GA genotype (OR=1.21; 95% CI: 1.01-1.45) [152]. The minor genotype frequencies for these variants observed in their control population were as follows: 6% for CYP24A1 rs2181874 *AA* genotype; 21% for CYP24A1 rs2296241 *GG* genotype; 3% for CYP24A1 rs4809958 *GG* genotype, and 3% for CYP24A1 rs6013905 *CC* genotype [152].

In summary, the few studies that have examined genetic variants in the vitamin D pathway in relation to BC risk or breast density have had largely inconsistent results [159, 161, 158, 152,166] and, to our knowledge, no studies have examined interactions between serum 25-OH-D and SNPs in the vitamin D pathway in relation to breast density particularly among postmenopausal women.

### 2.7.3 Selection of Vitamin D Pathway Gene Polymorphisms for the Current Study

One of the stated secondary objectives of this thesis was to explore the interactions of two vitamin D pathway gene polymorphisms on the relationship between baseline serum 25-OH-D and follow-up MD. Several polymorphisms in various vitamin D pathway genes are emerging in the literature as contenders for having an association with BC risk. Limited financial resources for this secondary objective permitted us the evaluation of only two polymorphisms in this study. Based on the known functionality of the various polymorphisms in the four main vitamin D pathway genes, the estimated prevalence of genotype/allele frequency in the general population, the current epidemiological literature on the association between these polymorphisms in relation to BC and breast density and the limited evidence on known interactions between polymorphisms and serum 25-OH-D in relation to BC/breast density we selected polymorphisms related to VDR (Fok1 rs2228570) and metabolism (CYP24A1 rs2181874) genes.

## 2.8 Summary

Experimental evidence supports an inverse association between vitamin D and BC risk. However, epidemiological studies investigating the association between vitamin D from diet and sun exposure and BC have reported inconsistent results, particularly among postmenopausal women. Measures of circulating vitamin D metabolites reflect both cutaneous synthesis of vitamin D and dietary intake. Studies of vitamin D metabolites in blood and BC risk have reported the largest effect estimates, yet there is considerable heterogeneity in study results. Measurement of serum 25-OH-D reflects vitamin D exposure in the preceding few weeks and is subject to dramatic seasonal variation; as a result multiple measures collected in a prospective setting are required.

Retrospective studies of this relationship are limited by exposure misclassification and the potential for information and selection bias. The prospective nature of the NCIC CTG MAP.3

trial, which collected blood samples and BC risk factor information at the time of randomization, provided an opportunity to conduct a strong observational study eliminating the biases inherent in retrospective evaluations of such an association. This study design also allows the examination of an interaction between vitamin D and exemestane on breast density, which not only will add to the current understanding of the mechanisms that may lead to reduced breast density and/or BC risk but will also have clinical importance.

The use of intermediate endpoints in place of a cancer endpoint provides several advantages with respect to study design. Experimental and epidemiologic evidence support the use of breast density as an intermediate in the vitamin D→BC relationship. The ultimate interest of this research is still with respect to a contribution to understanding the relationship between vitamin D and BC. If a relationship with breast density is observed in this study it would provide strong support for a causal relationship between vitamin D and BC operating via this pathway. If a relationship with breast density is not observed, this does not mean that vitamin D does not impact BC risk but that it may do so via an alternative pathway that may inform subsequent investigations. This design, utilizing a biomarker of exposure and using an intermediate event as the outcome is a strong approach to quantifying a relationship, if one does truly exist. In addition, the nature of the exposure and outcome measures will facilitate evaluation of dose response patterns and the identification of specific levels of serum 25-OH-D which may provide preventive effects.

## 2.9 References

1. Canadian Cancer Society's Steering Committee on Cancer Statistics. *Canadian Cancer Statistics 2012*. Toronto, ON: Canadian Cancer Society; 2012.

2. Kelsey JL, Gammon MD. The epidemiology of breast cancer. CA Cancer J Clin. 1991;41(3):146-165.

3. Canadian Cancer Society. www.cancer.ca. Published 2008.

4. World Cancer Research Fund / American Institute for Cancer Research. Food, Nutrition and the Prevention of Cancer: a Global Perspective. Washington, DC: AICR, 1997.

5. National Cancer Institute: PDQ® Breast Cancer Prevention. Bethesda, MD: National Cancer Institute. Date last modified 2013. Available at: http://cancer.gov/cancertopics/pdq/prevention/breast/HealthProfessional.

6. World Cancer Research Fund International. http://www.wcrf.org. Published 2013.

7. Canadian Breast Cancer Foundation. http://www.newswire.ca/en/story/1105269/one-third-of-breast-cancers-are-preventable. Published 2013.

8. Colditz GA. Epidemiology and prevention of breast cancer. Cancer Epidemiology Biomarkers & Prevention. 2005;14(4):768-772. doi: 10.1158/1055-9965.EPI-04-0157.

9. Clarke CA, Glaser SL, Uratsu CS, Selby JV, Kushi LH, Herrinton LJ. Recent declines in hormone therapy utilization and breast cancer incidence: Clinical and population-based

evidence. Journal of Clinical Oncology. 2006;24(33):e49-e50. doi: 10.1200/JCO.2006.08.6504.

10. Ereman R, Prebil L, Mockus M, et al. Recent trends in hormone therapy utilization and breast cancer incidence rates in the high incidence population of marin county, california. BMC Public Health. 2010;10(1):228. http://www.biomedcentral.com/1471-2458/10/228.

11. Phipps A, Li C. Breast cancer biology and clinical characteristics. In: Li C, ed. Springer New York; 2010:21-46. http://dx.doi.org/10.1007/978-1-4419-0685-4_2. 10.1007/978-1-4419-0685-4_2.

12. McPherson K, Steel CM, Dixon JM. ABC of breast diseases: Breast cancer---epidemiology, risk factors, and genetics. BMJ. 2000;321(7261):624-628.

13. Key TJ, Verkasalo PK, Banks E. Epidemiology of breast cancer. The Lancet Oncology. 2001;2(3):133-140.

14. Vogel VG. Epidemiology, genetics, and risk evaluation of postmenopausal women at risk of breast cancer. Menopause. 2008;15(4 Suppl):782-789.

15. Singletary SE. Rating the risk factors for breast cancer. Annals of Surgery. 2003;237(4):474.

16. Phipps A, Mirick D, Li C, Davis S. Environmental and occupational exposures. In: Li C, ed. Springer New York; 2010:183-221. http://dx.doi.org/10.1007/978-1-4419-0685-4_9. 10.1007/978-1-4419-0685-4_9.

17. Kristan J. Aronson P, Sally Campbell M, Janet Faith M, et al. Summary report: Review of lifestyle and environmental risk factors for breast cancer. Report of the Working Group on Primary Prevention of Breast Cancer. *Canadian Breast Cancer Initiative*. Minister of Public Works and Government Services Canada, 2001.

18. Familial breast cancer: Collaborative reanalysis of individual data from 52 epidemiological studies including 58?209 women with breast cancer and 101?986 women without the disease. Lancet. 2001;358(9291):1389-1399. http://linkinghub.elsevier.com/retrieve/pii/S0140673601065242.

19. Menarche, menopause, and breast cancer risk: Individual participant meta-analysis, including 118?964 women with breast cancer from 117 epidemiological studies. Lancet Oncol. 2012;13(11):1141-1151. http://linkinghub.elsevier.com/retrieve/pii/S1470204512704254.

20. Phipps A, Li C. Endogenous hormones. In: Li C, ed. Springer New York; 2010:73-87. http://dx.doi.org/10.1007/978-1-4419-0685-4_4. 10.1007/978-1-4419-0685-4_4.

21. Breast cancer and breastfeeding: Collaborative reanalysis of individual data from 47 epidemiological studies in 30 countries, including 50?302 women with breast cancer and 96?973 women without the disease. Lancet. 2002;360(9328):187-195. http://linkinghub.elsevier.com/retrieve/pii/S0140673602094540.

22. Li Yang and Kathryn H. Jacobsen. A systematic review of the association between breastfeeding and breast cancer. Journal of Women's Health. 2008;17(10):1635.

23. Li C, Beaber E. Exogenous hormones. In: Li C, ed. Springer New York; 2010:89-117. http://dx.doi.org/10.1007/978-1-4419-0685-4_5. 10.1007/978-1-4419-0685-4_5.

24. Breast cancer and hormone replacement therapy: Collaborative reanalysis of data from 51 epidemiological studies of 52?705 women with breast cancer and 108?411 women without breast cancer. Lancet. 1997;350(9084):1047-1059. http://linkinghub.elsevier.com/retrieve/pii/S0140673697082330.

25. Breast cancer and hormonal contraceptives: Collaborative reanalysis of individual data on 53 297 women with breast cancer and 100 239 women without breast cancer from 54 epidemiological studies. Lancet. 1996;347(9017):1713-1727. http://linkinghub.elsevier.com/retrieve/pii/S0140673696908065.

26. Writing Group for the Women's Health Initiative Investigators. Risks and benefits of estrogen plus progestin in healthy postmenopausal women: Principal results from the women's health initiative randomized controlled trial. JAMA. 2002;288(3):321-333. doi: 10.1001/jama.288.3.321.

27. Fabian CJ. If vitamin D prevents breast cancer, how does it do it, and how much does it take? ASCO EDUCATIONAL BOOK. 2009;2009(1):71-74.

28. Aune D, Chan DS, Vieira AR, et al. Dietary compared with blood concentrations of carotenoids and breast cancer risk: A systematic review and meta-analysis of prospective studies. The American Journal of Clinical Nutrition. 2012(a);96(2):356-373. doi: 10.3945/ajcn.112.034165.

29. Aune D, Chan DSM, Greenwood DC, et al. Dietary fiber and breast cancer risk: A systematic review and meta-analysis of prospective studies. Annals of Oncology. 2012(b);23(6):1394-1402. doi: 10.1093/annonc/mdr589.

30. Aune D, Chan DSM, Vieira AR, et al. Fruits, vegetables and breast cancer risk: A systematic review and meta-analysis of prospective studies. Breast Cancer Res Treat. 2012(c);134(2):479-493. http://dx.doi.org/10.1007/s10549-012-2118-1. doi: 10.1007/s10549-012-2118-1.

31. Alcohol, tobacco and breast cancer - collaborative reanalysis of individual data from 53 epidemiological studies, including 58 515 women with breast cancer and 95 067 women without the disease. Br J Cancer. 0000;87(11):1234-1245. http://dx.doi.org/10.1038/sj.bjc.6600596.

32. Seitz HK, Pelucchi C, Bagnardi V, Vecchia CL. Epidemiology and pathophysiology of alcohol and breast cancer: Update 2012. Alcohol and Alcoholism. 2012. doi: 10.1093/alcalc/ags011.

33. World Cancer Research Fund / American Institute for Cancer Research. Continuous Update Project Report. Food, Nutrition, Physical Activity, and the Prevention of Breast Cancer. AICR, 2010.

34. Terry PD, Rohan TE. Cigarette smoking and the risk of breast cancer in women: A review of the literature. Cancer Epidemiology Biomarkers & Prevention. 2002;11(10):953-971.

35. Gaudet MM, Gapstur SM, Sun J, Diver WR, Hannan LM, Thun MJ. Active smoking and breast cancer risk: Original cohort data and meta-analysis. Journal of the National Cancer Institute. 2013;105(8):515-525. doi: 10.1093/jnci/djt023.

36. Johnson KC, Miller AB, Collishaw NE, et al. Active smoking and secondhand smoke increase breast cancer risk: The report of the canadian expert panel on tobacco smoke and breast cancer risk (2009). Tobacco Control. 2011;20(1):e2-e2. doi: 10.1136/tc.2010.035931.

37. Friedenreich C. Physical activity and breast cancer: Review of the epidemiologic evidence and biologic mechanisms. In: Senn H, Otto F, eds. Vol 188. Springer Berlin Heidelberg; 2011:125-139. http://dx.doi.org/10.1007/978-3-642-10858-7_11. 10.1007/978-3-642-10858-7_11.

38. Santen RJ, Boyd NF, Chlebowski RT, et al. Critical assessment of new risk factors for breast cancer: Considerations for development of an improved risk prediction model. Endocr Relat Cancer. 2007;14(2):169-187.

39. Cummings SR, Tice JA, Bauer S, et al. Prevention of breast cancer in postmenopausal women: Approaches to estimating and reducing risk. J Natl Cancer Inst. 2009;101(6):384-398.

40. Yaffe MJ. Mammographic density. measurement of mammographic density. Breast Cancer Research. 2008;10(3):209.

41. Kerlikowske K, Ichikawa L, Miglioretti DL, et al. Longitudinal measurement of clinical mammographic breast density to improve estimation of breast cancer risk. J Natl Cancer Inst. 2007;99(5):386-395.

42. Byrne C, Spernak S. What is breast density? Breast Cancer Online. 2005;8(10):1-7.

43. van Gils CH, Hendriks JH, Holland R, et al. Changes in mammographic breast density and concomitant changes in breast cancer risk. European Journal of Cancer Prevention. 1999;8(6):509-515.

44. Martin L, Boyd N. Mammographic density. potential mechanisms of breast cancer risk associated with mammographic density: Hypotheses based on epidemiological evidence. Breast Cancer Research. 2008;10(1):201.

45. McCormack VA, dos Santos Silva I. Breast density and parenchymal patterns as markers of breast cancer risk: A meta-analysis. Cancer Epidemiology, Biomarkers & Prevention. 2006;15(6):1159-1169.

46. Ginsburg OM, Martin LJ, Boyd NF. Mammographic density, lobular involution, and risk of breast cancer. British Journal of Cancer. 2008;99:1369-1374.

47. Boyd NF, Rommens JM, Vogt K, et al. Mammographic breast density as an intermediate phenotype for breast cancer.[erratum appears in lancet oncol. 2005 nov;6(11):826]. Lancet Oncology. 2005;6(10):798-808.

48. Harvey JA, Bovbjerg VE. Quantitative assessment of mammographic breast density: Relationship with breast cancer risk. Radiology. 2004;230(1):29-41.

49. Vachon CM, Sellers TA, Carlson EE, et al. Strong evidence of a genetic determinant for mammographic density, a major risk factor for breast cancer. Cancer Res. 2007;67(17):8412-8418.

50. Mitchell G, Antoniou AC, Warren R, et al. Mammographic density and breast cancer risk in BRCA1 and BRCA2 mutation carriers. Cancer Res. 2006;66(3):1866-1872.

51. Boyd NF, Lockwood GA, Martin LJ, et al. Mammographic densities and risk of breast cancer among subjects with a family history of this disease. J Natl Cancer Inst. 1999;91(16):1404-1408.

52. Boyd NF, Dite GS, Stone J, et al. Heritability of mammographic density, a risk factor for breast cancer. N Engl J Med. 2002;347(12):886-894.

53. Becker S, Kaaks R. Exogenous and endogenous hormones, mammographic density and breast cancer risk: Can mammographic density be considered an intermediate marker of risk? Recent Results in Cancer Research. 2009;181:135-157.

54. The relationship of mammographic density and age: Implications for breast cancer screening. Am J Roentgenol. 2012;198(3):W292-W295. http://dx.doi.org/10.2214/AJR.10.6049. doi: 10.2214/AJR.10.6049.

55. Boyd NF, Guo H, Martin LJ, et al. Mammographic density and the risk and detection of breast cancer. N Engl J Med. 2007;356(3):227-236. http://dx.doi.org/10.1056/NEJMoa062790. doi: 10.1056/NEJMoa062790.

56. Boyd N, Martin L, Minkin S. Mammographic density as a potential surrogate marker for breast cancer. In: Li C, ed. Springer New York; 2010:321-341. http://dx.doi.org/10.1007/978-1-4419-0685-4_15. 10.1007/978-1-4419-0685-4_15.

57. Boyd NF, Martin LJ, Bronskill M, Yaffe MJ, Duric N, Minkin S. Breast tissue composition and susceptibility to breast cancer. J Natl Cancer Inst. 2010;102(16):1224-1237.

58. Welsh J, Wietzke JA, Zinser GM, Byrne B, Smith K, Narvaez CJ. Vitamin D-3 receptor as a target for breast cancer prevention. J Nutr. 2003;133(7):2425S-2433.

59. Boyd N, Martin L, Stone J, Little L, Minkin S, Yaffe M. A longitudinal study of the effects of menopause on mammographic features. Cancer Epidemiology Biomarkers & Prevention. 2002;11(10):1048-1053.

60. Kelemen LE, Pankratz VS, Sellers TA, et al. Age-specific trends in mammographic density: The minnesota breast cancer family study. Am J Epidemiol. 2008;167(9):1027-1036.

61. Martin LJ, Melnichouk O, Guo H, et al. Family history, mammographic density, and risk of breast cancer. Cancer Epidemiology Biomarkers & Prevention. 2010;19(2):456-463.

62. Douglas JA, Roy-Gagnon MH, Zhou C, et al. Mammographic breast density--evidence for genetic correlations with established breast cancer risk factors. Cancer Epidemiology, Biomarkers & Prevention. 2008;17(12):3509-3516.

63. Titus-Ernstoff L, Tosteson AN, Kasales C, et al. Breast cancer risk factors in relation to breast density (united states). Cancer Causes & Control. 2006;17(10):1281-1290.

64. El-Bastawissi AY, White E, Mandelson MT, Taplin SH. Reproductive and hormonal factors associated with mammographic breast density by age (united states). Cancer Causes & Control. 2000;11(10):955-963.

65. Boyd NF, Martin LJ, Li Q, et al. Mammographic density as a surrogate marker for the effects of hormone therapy on risk of breast cancer. Cancer Epidemiology Biomarkers & Prevention. 2006;15(5):961-966.

66. Boyd N. What accounts for variations in percent density. Written communication, Nov 2010.

67. Cuzick J. Chemoprevention of breast cancer. Breast Cancer. 2008;15(1):10-16. http://dx.doi.org/10.1007/s12282-007-0006-z. doi: 10.1007/s12282-007-0006-z.

68. Cuzick J, Powles T, Veronesi U, et al. Overview of the main outcomes in breast-cancer prevention trials. The Lancet. 2003;361(9354):296-300.

69. Cuzick J, Warwick J, Pinney E, et al. Tamoxifen-induced reduction in mammographic density and breast cancer risk reduction: A nested Case–Control study. Journal of the National Cancer Institute. 2011. doi: 10.1093/jnci/djr079.

70. Stroud ML, Stilgoe S, Stott VE, Alhabian O, Salman K. Vitamin D - a review. Aust Fam Physician. 2008;37(12):1002-1005.

71. Kimlin MG. Geographic location and vitamin D synthesis. Mol Aspects Med. 2008;29(6):453-461.

72. Holick MF. Vitamin D deficiency. N Engl J Med. 2007;357(3):266-281.

73. Zerwekh JE. The measurement of vitamin D: Analytical aspects. Ann Clin Biochem. 2004;41(Pt 4):272-281.

74. Holick MF. Vitamin D status: Measurement, interpretation, and clinical application. Ann Epidemiol. 2009;19(2):73-78.

75. Ross AC, Manson JE, Abrams SA, et al. The 2011 report on dietary reference intakes for calcium and vitamin D from the institute of medicine: What clinicians need to know. Journal of Clinical Endocrinology & Metabolism. 2011;96(1):53-58. doi: 10.1210/jc.2010-2704.

76. Whiting SJ, Langlois KA, Vatanparast H, Greene-Finestone LS. The vitamin D status of canadians relative to the 2011 dietary reference intakes: An examination in children and adults with and without supplement use. The American Journal of Clinical Nutrition. 2011;94(1):128-135. doi: 10.3945/ajcn.111.013268.

77. Rockell J, Skeaff M, Green T. Association between quantitative measures of skin color & plasma 25-hydroxyvitamin D (25OHD). FASEB J. 2008;22(1_MeetingAbstracts):157.5.

78. Wortsman J, Matsuoka LY, Chen TC, Lu Z, Holick MF. Decreased bioavailability of vitamin D in obesity. Am J Clin Nutr. 2000;72(3):690-693.

79. Rohan T. Epidemiological studies of vitamin D and breast cancer. Nutrition Reviews. 2007;65(1):80-83.

80. Schwartz GG, Skinner HG. Vitamin D status and cancer: New insights. Current Opinion in Clinical Nutrition & Metabolic Care. 2007;10(1):6-11.

81. Colston KW. Vitamin D and breast cancer risk. Best Practice & Research Clinical Endocrinology & Metabolism. 2008;22(4):587-599.

82. Cui Y, Rohan TE. Vitamin D, calcium, and breast cancer risk: A review. Cancer Epidemiology, Biomarkers & Prevention. 2006;15(8):1427-1437.

83. Fleet JC. Molecular actions of vitamin D contributing to cancer prevention. Mol Aspects Med. 2008;29(6):388-396.

84. Freedman OC, Goodwin PJ. The role of vitamin D in breast cancer recurrence. ASCO EDUCATIONAL BOOK. 2009;2009(1):79-83.

85. Chlebowski RT, Madduri D, Dungo M. Calcium plus vitamin D supplementation, 25-hydroxyvitamin D, and breast cancer risk. ASCO EDUCATIONAL BOOK. 2009;2009(1):75-78.

86. Perez-Lopez FR, Chedraui P, Haya J. Review article: Vitamin D acquisition and breast cancer risk. Reproductive Sciences. 2009;16(1):7-19.

87. Gissel T, Rejnmark L, Mosekilde L, Vestergaard P. Intake of vitamin D and risk of breast cancer--a meta-analysis. Journal of Steroid Biochemistry & Molecular Biology. 2008;111(3-5):195-199.

88. Chen P, Hu P, Xie D, Qin Y, Wang F, Wang H. Meta-analysis of vitamin D, calcium and the prevention of breast cancer. Breast Cancer Res Treat. 2010 Jun;121(2):469-77. doi: 10.1007/s10549-009-0593-9.

89. IARC. Vitamin D and Cancer. IARC Working Group Reports Vol.5, International Agency for Research on Cancer, Lyon, 25 November 2008.

90. Chlebowski RT. Vitamin D and breast cancer: Interpreting current evidence. 2011;13(217). doi: 10.1186/bcr2846.

91. Yin L, Grandi N, Raum E, Haug U, Arndt V, Brenner H. Meta-analysis: Serum vitamin D and breast cancer risk. Eur J Cancer. 2010;46(12):2196-2205. doi: 10.1016/j.ejca.2010.03.037.

92. Garland CF, Gorham ED, Mohr SB, et al. Vitamin D and prevention of breast cancer: Pooled analysis. J Steroid Biochem Mol Biol. 2007;103(3-5):708-711.

93. MOHR SB, GORHAM ED, ALCARAZ JE, et al. Serum 25-hydroxyvitamin D and prevention of breast cancer: Pooled analysis. Anticancer Research. September 2011;31(9):2939-2948.

94. van dR, Coebergh JW, de Vries E. Is prevention of cancer by sun exposure more than just the effect of vitamin D? A systematic review of epidemiological studies. Eur J Cancer. 2013;49(6):1422-1436. http://linkinghub.elsevier.com/retrieve/pii/S0959804912008854?showall=true.

95. John EM, Schwartz GG, Dreon DM, Koo J. Vitamin D and breast cancer risk: The NHANES I epidemiologic follow-up study, 1971–1975 to 1992. Cancer Epidemiology Biomarkers & Prevention. 1999;8(5):399-406.

96. Knight JA, Lesosky M, Barnett H, Raboud JM, Vieth R. Vitamin D and reduced risk of breast cancer: A population-based case-control study. Cancer Epidemiology Biomarkers & Prevention. 2007;16(3):422-429.

97. Anderson LN, Cotterchio M, Kirsh VA, Knight JA. Ultraviolet sunlight exposure during adolescence and adulthood and breast cancer risk: A population-based case-control study among ontario women. American Journal of Epidemiology. 2011;174(3):293-304. doi: 10.1093/aje/kwr091.

98. Kuper H, Yang L, Sandin S, Lof M, Adami H, Weiderpass E. Prospective study of solar exposure, dietary vitamin D intake, and risk of breast cancer among middle-aged women. Cancer Epidemiology Biomarkers & Prevention. 2009;18(9):2558-2561. doi: 10.1158/1055-9965.EPI-09-0449.

99. Millen AE, Pettinger M, Freudenheim JL, et al. Incident invasive breast cancer, geographic location of residence, and reported average time spent outside. Cancer Epidemiology Biomarkers & Prevention. 2009;18(2):495-507. doi: 10.1158/1055-9965.EPI-08-0652.

100. John EM, Schwartz GG, Koo J, Wang W, Ingles SA. Sun exposure, vitamin D receptor gene polymorphisms, and breast cancer risk in a multiethnic population. American Journal of Epidemiology. 2007;166(12):1409-1419. doi: 10.1093/aje/kwm259.

101. Bertone-Johnson ER, Chen WY, Holick MF, et al. Plasma 25-hydroxyvitamin D and 1,25-dihydroxyvitamin D and risk of breast cancer. Cancer Epidemiol Biomarkers Prev. 2005;14(8):1991-1997.

102. Chlebowski RT, Johnson KC, Kooperberg C, et al. Calcium plus vitamin D supplementation and the risk of breast cancer. J Natl Cancer Inst. 2008;100(22):1581-1591.

103. Levi,F., Paschez,C., Lucchini,F., and La Vecchia,C. Dietary intake of selected micronutrients and breast-cancer risk. International Journal of Cancer. 2001;91:260.

104. Abbas SN,A., Linseisen J, Chang-Claude J. Dietary vitamin D and calcium intake and premenopausal breast cancer risk in a german case-control study. Nutrition and Cancer. 2007;59(1):54.

105. Shin M, Holmes MD, Hankinson SE, Wu K, Colditz GA, Willett WC. Intake of dairy products, calcium, and vitamin D and risk of breast cancer. Journal of the National Cancer Institute. 2002;94(17):1301-1310. doi: 10.1093/jnci/94.17.1301.

106. Lin J, Manson JE, Lee I, Cook NR, Buring JE,Zhang SM. INtakes of calcium and vitamin d and breast cancer risk in women. Archives of Internal Medicine. 2007;167(10):1050-1059. doi: 10.1001/archinte.167.10.1050.

107. Anderson LN, Cotterchio M, Vieth R, Knight JA. Vitamin D and calcium intakes and breast cancer risk in pre- and postmenopausal women. The American Journal of Clinical Nutrition. 2010;91(6):1699-1707. doi: 10.3945/ajcn.2009.28869.

108. Merlo DF, Sormani MP, Bruzzi P. Molecular epidemiology: New rules for new tools? Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis. 2006;600(1-2):3-11.

109. Schatzkin A. Intermediate markers as surrogate endpoints in cancer research. Hematol Oncol Clin North Am. 2000;14(4):887-905. doi: http://dx.doi.org/10.1016/S0889-8588(05)70317-8.

110. Schatzkin A, Freedman LS, Schiffman MH, Dawsey SM. Validation of intermediate end points in cancer research. J Natl Cancer Inst. 1990;82(22):1746-1752.

111. Celia Byrne, Giske Ursin, Christopher F. Martin, Jennifer D. Peck, Elodia B. Cole, Gerardo Heiss, Anne McTiernan, Donglin Zeng, Rowan T. Chlebowski, Dorothy S. Lane, JoAnn E. Manson, Jean Wactawski-Wende, Shagufta Yasmeen, Norman F. Boyd, Martin J. Yaffe, Etta D. Pisano. Change in mammographic density with estrogen and progestin therapy: A measure of breast cancer risk in the women's health initiative. American Association for Cancer Research. http://www.aacr.org/home/public--media/aacr-press-releases.aspx?d=1964. Published 2010.

112. J Cuzick, J Warwick, L Pinney, R Warren, S Cawthorn, A Howell and S Duffy. Change in breast density as a biomarker of breast cancer risk reduction; results from IBIS-1. Cancer Research. 2009: 69(2)suppl(1) doi: 10.1158/0008-5472.SABCS-61

113. Pike MC. The role of mammographic density in evaluating changes in breast cancer risk. Gynecol Endocrinol. 2005;211(Suppl):1.

114. Brisson J, Berube S, Diorio C. [Breast density: A biomarker to better understand and prevent breast cancer]. Bull Cancer. 2006;93(9):847-855.

115. Fabian CJ, Kimler BF, Zalles CM, et al. Reduction in proliferation with six months of letrozole in women on hormone replacement therapy. Breast Cancer Research & Treatment. 2007;106(1):75-84.

116. Kemmis CM, Salvador SM, Smith KM, etc. Human mammary epithelial cells express CYP27B1 and are growth inhibited by 25-hydroxyvitamin D-3, the major circulating form of vitamin D-3. J.Nutr. 2006;136(4):887.

117. Welsh J. Targets of vitamin D receptor signaling in the mammary gland. J Bone Miner Res. 2007;22(2):86.

118. Colston K, Hansen C. Mechanisms implicated in the growth regulatory effects of vitamin D in breast cancer. Endocr Relat Cancer. 2002;9(1):45-59.

119. Berube S, Diorio C, Masse B, et al. Vitamin D and calcium intakes from food or supplements and mammographic breast density. Cancer Epidemiol Biomarkers Prev. 2005;14(7):1653-1659.

120. Thomson CA, Arendell LA, Bruhn RL, etc. Pilot study of dietary influences on mammographic density in pre- and postmenopausal hispanic and non-hispanic white women. Menopause. 2007;14:1-8.

121. Tseng M, Byrne C, Evers K, Daly M. Dietary intake and breast density in high-risk women: A cross-sectional study. Breast Cancer Research. 2007;9(5):R72.

122. Berube S, Diorio C, Verhoek-Oftedahl W, Brisson J. Vitamin D, calcium, and mammographic breast densities. Cancer Epidemiol Biomarkers Prev. 2004;13(9):1466-1472.

123. Diorio C, Berube S, Byrne C, et al. Influence of insulin-like growth factors on the strength of the relation of vitamin D and calcium intakes to mammographic breast density. Cancer Res. 2006;66(1):588-597.

124. Mishra G, McCormack V, Kuh D, etc. Dietary calcium and vitamin D intakes in childhood and throughout adulthood and mammographic density in a British birth cohort. British Journal of Cancer. 2008;99:1539-1543.

125. Vachon CM, Kushi LH, Cerhan JR, Kuni CC, Sellers TA. Association of diet and mammographic breast density in the minnesota breast cancer family cohort. Cancer Epidemiol Biomarkers Prev. 2000;9(2):151-160.

126. Holmes MD, Hankinson SE, Bryne C. Mammographic density and diet. American Journal of Epidemiology. 2001;153:S109.

127. Bertone-Johnson, E.R., Chlebowski, R.T., Manson, J.E., Wactawski-Wende, J., Aragaki, A.,Tamimi, R.M.,Rexrode, K.M., Thomson, C.A., Rohan, T.E., Peck, J.D., Pisano, E.D., Martin, C.F., Sarto, G., McTiernan, A,. Dietary vitamin D and calcium intake and mammographic density in postmenopausal women. Menopause. 2010;17(6):1152-1160.

128. Knight JA, Vachon CM, Vierkant RA, Vieth R, Cerhan JR, Sellers TA. No association between 25-hydroxyvitamin D and mammographic density. Cancer Epidemiol.Biomarkers Prev. 2006;15:1988-1992.

129. Brisson J, Berube S, Diorio C, Sinotte M, Pollak M, Masse B. Synchronized seasonal variations of mammographic breast density and plasma 25-hydroxyvitamin D. Cancer Epidemiol Biomarkers Prev. 2007;16(5):929-933.

130. Green AK, Hankinson SE, Bertone-Johnson ER, Tamimi RM. Mammographic density, plasma vitamin D levels and risk of breast cancer in postmenopausal women. International Journal of Cancer. 2010;127(3):667-674. doi: 10.1002/ijc.25075

131. Chai, W., Maskarinec, G., Cooney, R.V. Serum 25-hydroxyvitamin D levels and mammographic density among premenopausal women in a multiethnic population. Eur J Clin Nutr. 2010;64(6):652-654.

132. Sprague, B.L., Trentham-Dietz, A., Gangnon, R.E., Buist, D.S.M., Burnside, E.S., Bowles, E.J.A., Stanczyk, F.Z., Sisney, G.S., Skinner , H.G. The vitamin D pathway and mammographic breast density among postmenopausal women. Breast Cancer Res Treat. 2012;131(1):255-265.

133. Celine M. Vachon, Thomas A. Sellers, Christopher G. Scott, Karthik Ghosh, Kathleen R. Brandt, Janet E. Olson, Matthew R. Jensen, Sandhya Pruthi, Marilyn J. Morton, Daniel J. Serie, V. S. Pankratz. Longitudinal breast density and risk of breast cancer. American Association for Cancer Research. http://www.abstractsonline.com/Plan/ViewAbstract.aspx?sKey=a20329af-72bf-4866-9104-17aff952ddb9&cKey=af56444a-db35-4e9e-bbbc-dc069dfd318d . Published 2010.

134. Cazzaniga M, Bonanni B. Breast cancer chemoprevention: Old and new approaches. Journal of Biomedicine and Biotechnology. 2012.

135. Goss PI, Richardson H, Chlebowski R, etc. National cancer institute of canada clinical trials group MAP.3: Evaluation of exemestane to prevent breast cancer in postmenopausal women. Clin Breast Cancer. 2007;7(11):895-900.

136. Goss PE, Strasser-Weippl K. Prevention strategies with aromatase inhibitors. Clinical Cancer Research. 2004;10(1 Pt 2):372S-9S.

137. Cuzick J. Aromatase inhibitors for breast cancer prevention. J Clin Oncol. 2005;23(8):1636-1643.

138. Visvanathan K, Chlebowski RT, Hurley P, et al. American society of clinical oncology clinical practice guideline update on the use of pharmacologic interventions including tamoxifen, raloxifene, and aromatase inhibition for breast cancer risk reduction. J Clin Oncol. 2009.

139. Mousa NA, Crystal P, Wolfman WL, Bedaiwy MA, Casper RF. Aromatase inhibitors and mammographic breast density in postmenopausal women receiving hormone therapy. Menopause. 2008;15(5):875-884.

140. Fabian CJ, Kimler BF, Zalles CM, etc. Reduction in proliferation with six months of letrozole in women on hormone replacement therapy. Breast Cancer Research & Treatment. 2007;106(1):75-84.

141. Goss PE. Breast cancer prevention--clinical trials strategies involving aromatase inhibitors. Journal of Steroid Biochemistry & Molecular Biology. 2003;86(3-5):487-493.

142. Goss PE, Ingle JN, Alés-Martínez JE, et al. Exemestane for breast-cancer prevention in postmenopausal women. New England Journal of Medicine. 2011(364):2381.

143. Vachon CM, Suman V, Brandt KR, et al. Mammographic breast density response to aromatase inhibition. Clinical Cancer Research. 2013. doi: 10.1158/1078-0432.CCR-12-2789.

144. Aung TT, Chandana SR, etc. The role of vitamin D in breast cancer. Oncol Rev. 2009;3:19-25.

145. Dizdar O, Bulut N, Altundag K. Vitamin D intake may be a predictor of response to aromatase inhibitors in postmenopausal women with hormone receptor positive breast cancer. Breast Cancer Research & Treatment. 2008;109(2):403.

146. Signorello LB, Shi J, Cai Q, et al. Common variation in vitamin D pathway genes predicts circulating 25-hydroxyvitamin D levels among african americans. PLoS ONE. 2011;6(12):e28623. http://dx.doi.org/10.1371%2Fjournal.pone.0028623.

147. Matthias Wjst, Janine Altmüller, Theresia Faus-Kessler, Christine Braig, Margret Bahnweg, Elisabeth André. Asthma families show transmission disequilibrium of gene variants in the vitamin D metabolism and signalling pathway. Respir Res. 2006;7(1):60.

148. McCullough ML, Bostick RM, Mayo TL. Vitamin D gene pathway polymorphisms and risk of colorectal, breast, and prostate cancer. Annu Rev Nutr. 2009;29(1):111-132. http://dx.doi.org/10.1146/annurev-nutr-080508-141248. doi: 10.1146/annurev-nutr-080508-141248.

149. Raimondi S, Johansson H, Maisonneuve P, Gandini S. Review and meta-analysis on vitamin D receptor polymorphisms and cancer risk. Carcinogenesis. 2009;30(7):1170-1180.

150. Kostner K, Denzer N, Muller CSL, Klein R, Tilgen W, Reichrath J. The relevance of vitamin D receptor (VDR) gene polymorphisms for cancer: A review of the literature. Anticancer Research. 2009;29(9):3511-3536.

151. Schlingmann KP, Kaufmann M, Weber S, et al. Mutations in CYP24A1 and idiopathic infantile hypercalcemia. N Engl J Med. 2011;365(5):410-421. http://dx.doi.org/10.1056/NEJMoa1103864. doi: 10.1056/NEJMoa1103864.

152. Anderson LN, Cotterchio M, Cole DEC, Knight JA. Vitamin D-related genetic variants, interactions with vitamin D exposure, and breast cancer risk among caucasian women in ontario. Cancer Epidemiology Biomarkers & Prevention. 2011;20(8):1708-1717. doi: 10.1158/1055-9965.EPI-11-0300.

153. Valdivielso JM, Fernandez E. Vitamin D receptor polymorphisms and diseases. Clinica Chimica Acta. 2006;371(1):1-12. http://www.sciencedirect.com/science/article/pii/S0009898106001331. doi: http://dx.doi.org/10.1016/j.cca.2006.02.016.

154. Uitterlinden AG, Fang Y, van Meurs JBJ, Pols HAP, van Leeuwen JPTM. Genetics and biology of vitamin D receptor polymorphisms. Gene. 2004;338(2):143-156. http://www.sciencedirect.com/science/article/pii/S0378111904003075. doi: http://dx.doi.org/10.1016/j.gene.2004.05.014.

155. McGrath JJ, Saha S, Burne THJ, Eyles DW. A systematic review of the association between common single nucleotide polymorphisms and 25-hydroxyvitamin D concentrations. J Steroid Biochem Mol Biol. 2010;121(1):471-477.

http://www.sciencedirect.com/science/article/pii/S096007601000172X. doi: http://dx.doi.org/10.1016/j.jsbmb.2010.03.073.

156. Genetics home reference: Your guide to understanding genetic conditions (CYP27B1). http://ghr.nlm.nih.gov/gene/CYP27B1. Published 2013.

157. Jones G, Prosser DE, Kaufmann M. 25-hydroxyvitamin D-24-hydroxylase (CYP24A1): Its important role in the degradation of vitamin D. Arch Biochem Biophys. 2012;523(1):9-18. doi: 10.1016/j.abb.2011.11.003.

158. McCullough ML, Stevens VL, Diver WR, Feigelson HS, etc. Vitamin D pathway gene polymorphisms, diet, and risk of postmenopausal breast cancer: A nested case-control study. Breast Cancer Research. 2007;9:1.

159. Chen WY, Bertone-Johnson ER, Hunter DJ, etc. Associations between polymorphisms in the vitamin D receptor and breast cancer risk. Cancer Epidemiol.Biomarkers Prev. 2005;14(10).

160. Guy M, Lowe LC, Bretherton-Watt D, et al. Vitamin D receptor gene polymorphisms and breast cancer risk. Clin Cancer Res. 2004;10(16):5472-5481.

161. Abbas SN,A., Linseisen J, etc. Vitamin D receptor gene polymorphisms and haplotypes and ostmenopausal breast cancer risk. Breast Cancer Research. 2008;10:1.

162. Sinotte M, Rousseau F, Ayotte P, Roberts R. Vitamin D receptor polymorphisms (Fok1,Bsm1) and breast cancer risk: Association replication in two case-control studies within french canadian population. Endocrine-related cancer. 2008;15:975.

163. McKay JD, McCullough ML, Ziegler RG, et al. Vitamin D receptor polymorphisms and breast cancer risk: Results from the national cancer institute breast and prostate cancer cohort consortium. Cancer Epidemiol Biomarkers Prev. 2009;18(1):297-305.

164. Tang C, Chen N, Wu M, etc. Fok1 polymorphism of vitamin D receptor gene contributes to breast cancer susceptibility: A meta-analysis. Breast Cancer Research (2009) 117:391–399. DOI 10.1007/s10549-008-0262-4

165. Diorio C, Sinotte M, Brisson J, Berube S, Pollak M. Vitamin D pathway polymorphisms in relation to mammographic breast density. Cancer Epidemiol Biomarkers Prev. 2008;17(9):2505-2508.

166. Lowe LC, Guy M, Mansi JL, et al. Plasma 25-hydroxy vitamin D concentrations, vitamin D receptor genotype and breast cancer risk in a UK caucasian population. Eur J Cancer. 2005;41(8):1164-1169.

167. Zeljic K, Supic G, Stamenkovic Radak M, Jovic N, Kozomara R, Magic Z. Vitamin D receptor, CYP27B1 and CYP24A1 genes polymorphisms association with oral cancer risk and survival. Journal of Oral Pathology & Medicine. 2012(10):779. doi: 10.1111/j.1600-0714.2012.01164.x.

168. Dorjgochoo T, Delahanty R, Lu W, et al. Common genetic variants in the vitamin D pathway including genome-wide associated variants are not associated with breast cancer risk among chinese women. Cancer Epidemiology Biomarkers & Prevention. 2011;20(10):2313-2316. doi: 10.1158/1055-9965.EPI-11-0704.

**Table 2.1 Summary of Epidemiological Evidence on Vitamin D and Breast Density**

| Authors, year, place of study | Study design | Sample size | Relationship of Interest | Measures of Effect | Covariates (adjusted for) | Menopausal Status (pre vs post) |
|---|---|---|---|---|---|---|
| *Dietary and Supplemental Vitamin D Intake and Breast Density* | | | | | | |
| Vachon CM et al. 2000, USA | cross-sectional | 1508 | breast density and dietary factors including vitamin D across quartiles of vitamin D | Mean (95% CI)<br>35 (32-37)<br>p for trend = 0.68 (overall )<br>42 (35-48)<br>p for trend = 0.55 (premenopausal women)<br>32 (30-34)<br>p for trend = 0.96 (postmenopausal women) | caloric intake, age, age2, BMI, WHR, physical activity, age at menarche age at first birth/# births, alcohol intake, smoking, family history, HRT use, oral contraceptive use | Pre & Post |
| Holmes MD et al. 2001, USA | cross-sectional | 885 | dietary vitamin D intake and breast density in premenopausal women | Mean % density across quintiles = 45,41,38,42,33<br>p for trend = 0.02 | age and BMI | Pre |
| Berube S et al. 2004, USA | cross-sectional | 543 | relation of dietary vitamin D to mammographic breast densities across quartiles of vitamin D (< 50, 50-99, 100-199 > 200 IU/day) | OR across categories of vitamin D were 1.00 (referent), 0.51 0.37, and 0.24<br>p for trend = 0.0005 (overall)<br>p for trend = 0.003 (premenopausal women)<br>p for trend = 0.05 (postmenopausal women) | age, BMI, age at menarche, number of births/age, at first birth, oral contraceptive use menopausal status, HRT use, family history, education, alcohol, total caloric intake, smoking status | Pre & Post |

**Table 2.1 *continued*** **Summary of Epidemiological Evidence on Vitamin D and Breast Density**

| Authors, year, place of study | Study design | Sample size | Relationship of Interest | Measures of Effect | Covariates (adjusted for) | Menopausal Status (pre vs post) |
|---|---|---|---|---|---|---|
| Berube S et al. 2005, Canada | cross-sectional | 1560 | vitamin D from food and/or supplements, in relation to breast density in premenopausal (n=777) and postmenopausal (n=783) women | p=0.004 (total vitamin D intake in premenopausal women) p=0.76 (total vitamin D intake in postmenopausal women) | age, BMI, age at menarche, # births, age at first birth, oral contraceptive use and duration, HRT use and duration, breast biopsies, family history, education, alcohol, total caloric intake, physical activity, smoking | Pre & Post |
| Diorio C et al. 2006, Canada | cross-sectional | 771 | association of dietary and supplemental vitamin D with breast density in premenopausal women (p values for 100 IU/d) | (p=0.004) | alcohol, energy intake BMI, age at menarche, age at first birth, number of full-term pregnancies, number of breast biopsies, OC use and duration, HRT use and duration, family history, physical activity, education, smoking status | Pre |
| Thomson CA et al. 2007, USA | cross-sectional | 238 | dietary variables including vitamin D and mammographic density in pre- and post menopausal Hispanic and non-Hispanic white [NHW] women | $p < 0.01$ (pre-men Hispanic) $p > 0.05$ (pre-men NHW $p > 0.05$ (postmen Hispanic) $p > 0.05$ (postmen NHW) | BMI and energy intake | Pre & Post |

**Table 2.1 *continued*** **Summary of Epidemiological Evidence on Vitamin D and Breast Density**

| Authors, year, place of study | Study design | Sample size | Relationship of Interest | Measures of Effect | Covariates (adjusted for) | Menopausal Status (pre vs post) |
|---|---|---|---|---|---|---|
| Tseng M et al. 2007, USA | cross-sectional | 157 | dietary vitamin D and breast density in high-risk women (tertiles), Q3 vs. Q1 | OR (95% CI) 0.5 (0.2-1.0) p = 0.05 No difference in effect by menopausal status | age, BMI, caloric intake, age at menarche, family history, menopausal status, and hormone therapy use | Pre & Post |
| Mishra G et al. 2008, United Kingdom | cohort | 1161 | dietary vitamin D intake and mammographic density | Adj regression coefficient = 0.04 95% CI: -0.03-0.11 Per 1 SD ↑ vit D intake | age at mammography, mammographic view, total energy intake, BMI reproductive and lifestyle factors, and calcium | Pre & Post |
| Bertone-Johnson, ER et al., 2010, USA | cross-sectional | 808 | dietary intake of vitamin D and calcium and mammographic density in postmenopausal women. Vitamin D categories were (<100 IU/day, 100-199, 200-399, 400-599, > 600) | Mean % MD across categories of ↑ vit D 5.8, 10.4, 6.2, 3.8, 5.1 p for trend = 0.67 | age, race/ethnicity, BMI, age at menarche, parity, OC use and duration, previous HT use/duration, HT randomization arm, family history of breast cancer, education, alcohol intake, smoking, total calorie intake, physical activity, Gail risk, use of multivitamins, total calcium, vitamin D supplements, solar irradiation and season of mammogram | Post |

**Table 2.1 *continued*** **Summary of Epidemiological Evidence on Vitamin D and Breast Density**

| Authors, year, place of study | Study design | Sample size | Association | Estimates | Covariates (adjusted for) | Menopausal Status (pre vs post) |
|---|---|---|---|---|---|---|
| *Circulating Vitamin D Levels and Breast Density* | | | | | | |
| Knight JA et al. 2006, USA | cross-sectional | 487 | circulating 25-OH-D and mammographic density (least-square means and SEs reported) | P = 0.59 (% density) P = 0.83 (dense area) | age, BMI, parity, age first birth and physical activity | Pre & Post |
| Brisson J et al. 2007, Canada | cross-sectional | 741 | correlation between mean breast density and mean plasma 25-OH-D in pre-menopausal women taking seasonal variation into account | r = -0.90, ($R^2$ = 0.81) | age at menarche, number of pregnancies, age at first birth, duration of breast-feeding, oral contraceptive use, HRT, phase of menstrual cycle, alcohol intake, mean daily caloric intake, family history, breast biopsies, smoking status, education, physical activity, dietary intake of vitamin D and calcium, IGF-1 levels | Pre |
| Green, et al. 2010 USA | cross-sectional | 493 | plasma 25-OH-D and 1, 25$(OH)_2$D and mammographic density mean % MD across serum 25-OH-D quartiles | 25-OH-D p=0.69 1,25$(OH)_2$D p=0.78 | age and month at blood draw, fasting status and time of day, HRT use, BMI, family history of BC, HRT duration, alcohol consumption, age at first birth parity and age at menarche | Post |

**Table 2.1 *continued*** **Summary of Epidemiological Evidence on Vitamin D and Breast Density**

| Authors, year, place of study | Study design | Sample size | Association | Estimates | Covariates (adjusted for) | Menopausal Status (pre vs post) |
|---|---|---|---|---|---|---|
| *Circulating Vitamin D Levels and Breast Density* | | | | | | |
| Chai et al., 2010, USA | cross-sectional | 182 | serum 25-OH-D and mammographic density in multi-ethnic premenopausal women | p=0.71 | BMI, Asian ethnicity, age at mammogram, age at birth of first child, parity and age at menarche | Pre |
| Sprague et al., 2012, USA | cross-sectional | 238 | serum 25-OH-D and mammographic density in postmenopausal women | Mean % density across vit D quartiles 13.6, 14.3, 11.2, 13.3 p for trend = 0.49 | age, BMI, parity, family history of BC, vigorous physical activity smoking, season | Post |

# Chapter 3

# Study Method

## 3.1 Study Design

This observational study was nested within a large randomized trial of exemestane and BC prevention. Canadian and American women residing at northern latitudes with appropriate blood samples and mammograms, and who were followed for a minimum of 3 years were selected from the larger trial. Baseline vitamin D exposure was represented by the metabolite serum 25-OH-D. This measure was based on an average measure from two stored blood samples taken at study entry and one year follow-up. Percent MD was evaluated in the participant's initial and final follow-up mammogram. The primary objectives were to examine the relationship between baseline serum 25-OH-D and percent MD at follow-up, and absolute change in percent MD from baseline. It was hypothesized that women with lower levels of serum 25-OH-D at baseline would have higher percent MD at follow-up compared with women with higher baseline levels of serum 25-OH-D. Further, women with lower levels of serum 25-OH-D were postulated to have no or smaller decreases in percent MD since baseline compared with women with higher serum 25-OH-D levels. It was also of clinical interest to examine whether percent MD at follow-up or changes in percent MD over time in relation to serum 25-OH-D was modified by exemestane therapy. From a methodological point of view, since study participants in this nested observational study were part of a larger trial testing a chemopreventive drug it was important to evaluate whether the relationship between serum 25-OH-D and percent MD was similar in each of the trial arms (placebo vs. exemestane) in order to pool the results. From a biological point of view, the evidence reviewed on the relationship between vitamin D and MD to date supports a stronger effect in premenopausal compared with postmenopausal women. If these results are due to a potential interaction with estrogen it is hypothesized that a stronger relationship between serum

25-OH-D and percent MD will be observed in the placebo group than in the exemestane group who are estrogen suppressed. Other secondary objectives of the current study included the exploration of interactions of calcium and, independently, of two vitamin D pathway polymorphisms on the relationship between baseline serum 25-OH-D and follow-up MD. As calcium and vitamin D are metabolically interrelated it was hypothesized that the association between lower levels of serum 25-OH-D and higher percent MD, if observed, would be strengthened in the presence of lower calcium levels. Lastly, it was hypothesized that women with lower levels of serum 25-OH-D and the recessive *ff* genotype of the Fok1 polymorphism or the *GA* genotype of the CYP24A1 rs2181874 polymorphism would have higher percent MD compared with women with lower levels of serum 25-OH-D and the more dominant genotypes based on current evidence.

This chapter will provide an overview of the trial participants included in this study, the data collection phase including the processes involved in obtaining mammograms and blood samples for subsequent measurement and the statistical methods employed to evaluate the study objectives.

### 3.1.1 Study Participants

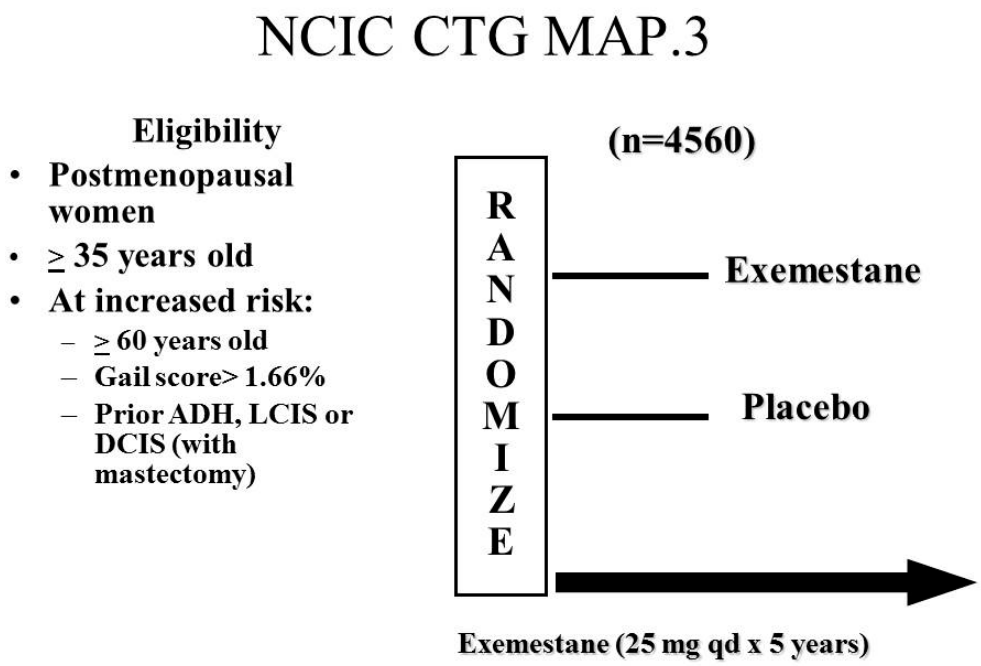
#### *3.1.1.1 NCIC Clinical Trials Group MAP.3 Participants*

The NCIC Clinical Trials Group conducted a phase III international, multi-centred, randomized controlled trial (RCT) comparing exemestane, an aromatase inhibitor (AI), with placebo in postmenopausal women at higher than average risk for BC (MAP.3); the study schema for MAP.3 is shown in Figure 3 below. Participants were postmenopausal women who were at moderately high risk for the development of BC based on age, Gail score (i.e. 5-year probability of BC) and previous benign breast conditions. For example, the average Gail score of a 59 year

old North American Caucasian woman is 1.7% [1]; the average Gail score of a 59 year old woman in MAP.3 was 2.98% (n=288). Women were excluded from the trial if they had prior malignancies, relevant comorbid conditions, or hormonal treatment within 3 months prior to randomization. All randomized participants in the parent RCT were required to have baseline mammography and were to be followed for a minimum of 5 years with scheduled follow-up visits including a yearly mammogram.

Recruitment to MAP.3 occurred between February 2004 and March 2010 for a total accrual of 4,560 women. Participants were recruited using a variety of strategies including local media, flyers/brochures, focused mass mailings and letters of invitation placed in mammography suites, family medicine waiting rooms, and radiology departments. Several of the trial investigators also held educational sessions about the trial with local physician groups to gain trial endorsement in the hope that they would speak with their patients about participation. Results of the primary objectives of the trial were published in the New England Journal of Medicine in June 2011 [2] and reported that invasive BC was significantly reduced in postmenopausal women who were on exemestane therapy compared with placebo (annual incidence of BC, 0.19% with exemestane vs. 0.55% with placebo; Hazard Ratio (HR), 0.35; 95% CI, 0.18 to 0.70).

**Figure 3.1 MAP.3 Trial Schema**




*3.1.1.2 Inclusion and Exclusion Criteria for the Current Study*

Upon study initiation, MAP.3 clinical trial participants were identified from randomizing centres located in a northern latitude, namely Canada and Buffalo, New York, and who had a follow-up period from the time of their baseline mammogram to their most recent mammogram of at least 3 years. Three years was identified as the cut off in order to ensure sufficient prospective follow-up while maximizing the number of available participants. Of this group of potential participants, women were subsequently deemed ineligible for the current study if they: (*a*) had a diagnosis of BC while on study; (*b*) did not consent to optional serum or whole blood collection as part of the larger clinical trial; (*c*) did not currently have blood samples in the NCIC Clinical Trials Group Tumour Bank; and (*d*) did not have all mammograms done at the same radiology facility. The decision to request all mammograms done for a given participant at the same radiology facility was made with respect to the feasibility of subject recruitment and ascertainment of outcome data

in order to maximize the sample size and facilitate adequate evaluation of the scientific objectives of this project.

### 3.1.2 Participant Information Forms

Information on established risk factors for BC including age, BMI, ethnicity, reproductive history (including age at menarche, age at menopause and parity), hormonal treatment history (including OC and HRT use and duration), medical history (including personal and family history of BC) and other demographic variables of interest (including education and smoking history) was available from two initial evaluation forms that were utilized for data collection for the parent trial. The first was completed by clinical trials staff at each participating centre, with information extracted from the participant's medical records, and the second was from a reproductive history and socio-demographic questionnaire that was completed via participant interview (see Appendices 1 and 2). Information on blood levels of calcium was also available on participants at baseline. Reported calcium levels were based on standard biochemistry lab assays performed at each MAP.3 centre. The only established BC risk factors that were not available for participants in this study were for physical activity and alcohol intake.

Data collected as part of the clinical trial was submitted to and reviewed by clinical trials staff at the central office in Kingston, ON. Information on established risk factors used in this nested observational study was originally reviewed by a research associate and corroborated against supporting documentation submitted by the randomizing centre (i.e. dates and results on imaging, including mammograms, hematology reports, etc.). Data was entered into a computerized trial database that included statistical checks to identify any outliers or biologically implausible values that needed clarification/correction. Lastly, all data was reviwed by the statistical

programmer/trial biostatistician prior to primary trial analysis. The distributions of this covariate data used in this nested study will also be reviewed for quality control/validity purposes.

### 3.1.3 Mammogram Collection, Digitization and De-Identification

As part of the MAP.3 trial, a baseline bilateral mammogram (2 view screening) was required within 12 months prior to participant randomization and then every 12 months from the time of the initial mammogram. Mammograms, therefore, were available for this study starting as early as 2003.

Over the course of accrual to MAP.3, many radiology clinics/departments in Canada were in the process of changing their film screen mammography machines to digital mammography equipment. While film screen mammography is a sensitive diagnostic tool for the detection of breast abnormalities and BCs, digital mammography offers several advantages including a decreased dose of radiation to the patient, the ability to manipulate the image on specialized high resolution monitors to enhance image quality, the ability to transfer images between centres electronically to improve patient care through timely review by specialists and, most importantly, improved BC detection given better resolution of the image [3]. Knowledge of this shift in technology led us to anticipate the receipt of both film screen (i.e. analog) and digital mammograms from MAP.3 trial participants. Recognition of this technology shift from film screen to digital mammography was quite important in light of the objectives of this study. It is known that percent MD is higher on film screen mammograms than on digital mammograms for the same woman [4,5]. As such, it was important to look at whether the relationship between serum 25-OH-D and MD at follow-up was modified by the format of the mammogram. Further, in order to calculate a change in MD over time both the baseline and follow-up mammograms were

required to be in the same format. If this was not taken into account a large amount of misclassification in the outcome measure of change in percent MD over time would occur.

*3.1.3.1 Chart Review*

The review of MAP.3 mammogram reports from participant case report forms started in the spring of 2011. At the initiation of chart review our intention was to retrieve mammograms for a given participant (baseline and last mammogram taken while either on protocol treatment or once treatment was discontinued) that were in the same format (i.e. all analog or all digital). This would allow us to evaluate both our primary (relationship between serum 25-OH-D and MD at follow-up) and secondary objectives (relationship between serum 25-OH-D and a change over time in percent MD), the latter of which required the baseline and follow-up mammogram to be in the same format so an appropriate change in MD over time could be calculated. After data review of all the charts of potentially eligible women, however, it became evident that the format of the mammograms done was unknown from the radiology reports, with the exception of one site. We contacted the Canadian Association of Radiologists[6] who oversees the Mammography Accreditation Program in Canada, to see whether there was any mechanism in place that would allow us to identify which mammograms were film versus digital based on the name of the radiology clinic and the date of the mammogram. While the change in mammographic technology occurred in some provinces in Canada as early as 2001 we were informed that radiology departments were not concurrently changing their equipment and in many cases radiology departments would continue to offer and utilize both film screen and digital mammography depending on the patient population.

A decision was made to request all mammograms that were done at the same radiology facility (provided there was a baseline and at least one follow-up mammogram $\geq$ 3 years) during the course of the clinical trial for each potential participant in the hopes that there would be a baseline

and at least one follow-up mammogram in the same format. The process for selection of the two mammograms included in the current study was done at the Hotel Dieu Hospital (HDH) in Kingston, ON based on known format at the time of receipt. Subsequent to this decision, radiology clinic addresses were extracted from all mammogram reports of potentially eligible participants and were maintained in an Access database at Queen's University.

*3.1.3.2 Request for Mammograms*

Prior to the mass mailing of requests for participant mammograms to all relevant centres the study methods for mammogram collection were piloted in one Canadian and one American centre that recruited the largest number of participants to the overall parent trial. This pilot phase of the overall study provided valuable information regarding the availability of participant mammograms and the feasibility of collection from centres and the source radiology facilities. This offered insight on the expected duration of data collection and projections on overall response rates. Further, this pilot phase provided the opportunity to refine the communication with centres to ensure that written instructions were clear and translated into the accurate retrieval of required mammograms (i.e. correct breast image; correct mammographic view) and accompanying information (i.e. participant identifier; date of the mammogram). This helped to minimize errors in data collection and overall outcome measurement.

Formal requests for remaining participant mammograms were subsequently sent out to all 20 randomizing centres in Canada and Buffalo, New York in the summer of 2011 (see Appendix 3) and communication with centres and retrieval of mammograms continued until completion in January 2012 for a total of 8 months of active retrieval. An explicit process for mammogram retrieval was provided to the relevant contacts at the randomizing centres to ensure timely and well-coordinated retrieval and return to the source radiology facilities where participant mammograms were done and housed (~200 clinics in Canada and Buffalo). Specifically, each

randomizing centre was provided with an information package (see Appendix 4) including: (a) a spreadsheet of the participants from their site for whom mammograms were to be requested including the contact information for the relevant radiology departments/clinics; (b) template letters to each of the radiology departments/clinics where the mammograms were taken and resided; (c) courier instructions for the transfer of mammograms to and from HDH in Kingston; and (d) a transfer of mammogram checklist to ensure package completeness. For digital mammograms, we requested the raw image data since it is known to more closely relate to breast composition than the processed images. However, the processed image data was acceptable if the raw data from the digital image was not available.

During the process of mammogram retrieval we had many discussions with centres and REBs about concerns relating to the release of mammograms with participant identifiers, despite participant consent to such release, and had to work with centres on an individual basis to ensure the release of mammograms conformed to local policies. Based on these early concerns, a service agreement was subsequently developed early in the data collection phase of this study between Queen's University and HDH via the Office of Research Services to ensure that hospital personnel working on this study kept all personal health information received for study participants confidential and behind locked doors (see Appendix 5).

*3.1.3.3 Mammogram Collection, Digitization and De-Identification*

As mammograms were received, the HDH coordinators (M Pitcher & D Parfett) de-identified and digitized (film only) mammograms and prepared all mammograms required for central radiology review by our study radiologist (Dr. Jabs). Based on a literature review and a discussion with the study radiologist, it was decided that only the view of the left breast would be processed and subsequently measured for percent MD in all study participants. Previous studies have demonstrated high correlations between percent MD in the left and right breast.[7-16].Researchers in

this area of investigation have either randomly chosen a breast for measurement purposes, consistently used the left or right breast, or used the average percentage density from both breasts for analysis purposes. If the left view of the breast was not available for any given participant, the same view of the right breast would be used for the estimate.

As previously discussed, images arrived in two formats: film based or digital. Film based mammograms were digitized by a mammographic quality digitizer as required for subsequent use in the computer program that Dr. Jabs used for measuring percent MD. Specifically, the iCad digitizer was used to produce a dicom digital image for all film based mammograms, with the GE RA600 and PACS Cube used to remove patient demographics, annotate the NCIC Clinical Trials Group unique participant identifier and burn images to CD, as required, for all images. For digital images received, all personal identifying information was also removed and annotated with the MAP.3 NCIC Clinical Trials Group subject serial number prior to breast density measurement if not already done so by the radiology clinic or randomizing centre. All images were reviewed on a mammographic quality workstation with a resolution of 5 mega pixels. This equipment is owned and operated by HDH in Kingston, ON.

A web based mammogram tracking system was developed with the NCIC Clinical Trials Group for use by research personnel both at Queen's University and at HDH to closely monitor and log the receipt and return of participant mammograms from centres (see Appendix 6). In addition, this web based tracking system was used to log the format of each mammogram, the date of de-identification and digitization (for film only), and the decision to process and retain for breast density measurement by the study radiologist (Dr. Jabs). This information allowed us to ensure that the correct two mammograms were processed (both in the same format, where possible, as well as maximizing follow-up time for subsequent evaluation). Further, the web-based system was used to generate scheduled reminder reports to randomizing centres who had outstanding

mammograms still due at HDH. Lastly, the web based tracking system was used to trigger the funding reimbursement to centres who had submitted their required mammograms for the purpose of this project. Overall response rates for mammogram collection were calculated for each of the primary objectives and will be reported. Centres were considered compliant in their retrieval of mammograms if they: (a) provided at least one follow-up mammogram ≥ 3years from the randomizing mammogram for each requested participant, and (b) provided the randomizing mammogram as well as at least one follow-up mammogram ≥ 3years regardless of the ability to use these mammograms in the analysis which was dependent on matching formats.

*3.1.3.4 Mammogram Return to Centres*

Once all film based mammograms were digitized for breast density measurement, they were returned by HDH to the mammography radiology clinic or institution that provided them via Federal Express. The date of return to the clinic was recorded in the web based tracking system. No mammograms were misplaced or lost during the course of this study with one exception for which a digital copy was subsequently found. As the digital mammograms we received were copies of the originals held at the home radiology facility the images will be destroyed by HDH after study publication.

*3.1.3.5 Measurement of Percent Mammographic Density*

Once all mammograms were received and digitized the measurement of percent MD was carried out by a single radiologist at HDH who specializes in mammography reading and measurement (D. Jabs). Dr. Jabs used an observer-assisted, quantitative technique called interactive thresholding developed and described extensively by others [17,18]. Specifically, the thresholding program, Cumulus, was purchased and installed at HDH for study purposes. In addition to her clinical expertise, Dr. Jabs took a formal training course in the use of Cumulus run by the

developers of this software before review of study mammograms (MD Measurement: Cumulus Course; Sunnybrook Health Sciences Centre, Toronto, ON).

Cumulus was used to measure percent MD from the left cranial-caudal view which was calculated as the dense area divided by the total area of the breast (dense and non-dense tissue) multiplied by 100. Dr. Jabs was blinded to treatment assignment (placebo vs. exemestane) and study visit (baseline vs. follow-up). While mammograms for a given participant were presented as a set, the order of the view of the digitized mammograms within an individual was random [17]. In addition, a random sample of 10% of the baseline and follow-up mammograms was read twice during central radiology review in order to calculate a test-retest reliability measure for percent MD. All percent MD measurements were completed and data transferred from HDH to Queen's University in May 2012.

### 3.1.4 Blood Collection

The MAP.3 trial included collection of blood on all participants at baseline, year 1 and year 5 (or off protocol treatment) for protocol specified and future research purposes. At each visit, non-fasting blood was collected into serum separator tubes and after approximately 30 minutes was centrifuged by the enrolling centre. A total of approximately 6 mL of serum for each participant per visit was divided into 3 aliquots and frozen at – 20°C on site. In addition, whole blood was collected at baseline from consenting participants (via separate consent form) for future DNA analyses. For those subjects who consented to optional banking, blood was collected into EDTA tubes and a total of approximately 3 mL of whole blood was made into two aliquots in each of two cryovials and was frozen at – 20°C on site. The date and time of collection for each serum and whole blood sample was recorded.

*3.1.4.1 Storage of Participant Biospecimens*

The NCIC Clinical Trials Group maintains a tumour/tissue (including plasma and serum) bank under the auspices of its' Tissue/Tumour Data Bank (TTDR – see http://www.ctg.queensu.ca/TissueBank/index.html). Serum and whole blood collected from institutions participating in MAP.3 was shipped to the NCIC Clinical Trials Group Tumour Bank, located in the Department of Pathology at Kingston General Hospital, within 2 months of collection where they are kept frozen at -80°C.

*3.1.4.2 Preparation and Transfer of Serum Samples*

Retrieval of mammograms was paramount to identifying the participants to be included in this study. Once those women were identified we were able to move forward with measurement of the primary exposure, namely serum 25-OH-D, in collected samples. Staff at the NCIC Clinical Trials Group Tumour Bank carried out the retrieval, thawing and aliquoting of our required MAP.3 serum samples in preparation for transfer to the laboratory of Dr. Glenville Jones. Dr. Jones is the head of the Biochemistry Department at Queen's University and expert in the field of vitamin D metabolism.

*3.1.4.3 Measurement of Serum 25-OH-D*

The principal assay that was used in this study to determine the quantity of serum 25-OH-D in participant samples was the LC-MS/MS technique which combines liquid chromatography and mass spectrometry [19, 20]. LC-MS/MS assays provide reliable measurements of both serum 25-OH-D2 and serum 25-OH-D3 leading to a more precise and accurate measure of total serum 25-OH-D [19]. While the DiaSorin radioimmunoassay (RIA) is another popular method of measuring serum 25-OH-D it is reported to have problems in precisely and accurately estimating total serum

25-OH-D and does not perform at the level of LC-MS/MS assays [19]. Another advantage to LC-MS/MS compared with RIA is the minimal sample preparation that LC-MS/MS requires thus increasing efficiency as more samples can be processed per day.[20].

Dr. Kaufmann, a postdoctoral fellow working with Dr. Jones, spent several months calibrating the state-of-the-art LC-MS/MS equipment and carrying out required quality control procedures in preparation for the receipt of participant samples from the NCIC Clinical Trials Group Tumour Bank and subsequent measurement of serum 25-OH-D. The levels of the primary vitamin D metabolite, serum 25-OH-D, were measured using 100 µL of baseline and first year serum samples from each eligible participant. Extra samples from the NCIC Clinical Trials Group Tumour Bank for the participants included could not be ascertained given the various demands on the samples by the MAP.3 trial team and other Investigators. Thus, inter-assay coefficients of variation (CV) for study participants at different time points during the full analysis could not be measured. However, both an independent quality control sample as well as triplicates of each participant's serum sample was included in each batch to evaluate the repeatability of the samples. An overall % CV for each of the triplicates and quality control samples was calculated. Further, precision of the sample measurements was evaluated by the inclusion of a gold standard sample within batches (A DEQAS sample). DEQAS, which stands for 'Vitamin D External Quality Assessment Scheme,' helps laboratories ensure the analytical reliability of their serum 25-OH-D measurements by providing validated samples against which their assay performance can be compared [21]. Again, an overall % CV for the DEQAS samples was calculated and the serum 25-OH-D measurements obtained for the DEQAS samples in our analysis was compared with the validated DEQAS samples to check overall analysis performance. In the current study, both inter- and intra-assay %CV were within acceptable ranges at < 10% (see Table 3.1). The serum 25-OH-D measures obtained in this study population are, on average, reading slightly

lower than the mean of all reporting lab measurements (with a mean discrepancy of 1.86 over all the samples analyzed) and lower than other LC/MS assays (with a mean discrepancy of 6.38 over all the samples analyzed).

**Table 3.1: Coefficients of Variation of Serum 25-OH-D Measurements**

| **Cumulative Inter-assay %CV (n)** | **%CV (total serum 25-OH-D)** |
|---|---|
| Quality control (n=83) | 6.0 |
| DEQAS samples (n=55)* | 5.7 |
| **Intra-assay %CV (n)** | **%CV (total serum 25-OH-D)** |
| Triplicates (n=1144 )** | 1.11 |

* Mean %CV for 5 DEQAS samples run 11 times
**Mean %CV based on 3 replicates over 114 4 samples

At the time of completion of serum 25-OH-D measurement, the results were provided to us and included isolated measurements of serum 25-OH-D2 and serum 25-OH-D3 as well as total serum 25-OH-D per sample and associated standard deviations. We were also provided with the results of the quality control samples for every sample run. All values for serum 25-OH-D levels at baseline and year 1 for each participant were provided in nanogram per milliliter (ng/mL).

*3.1.4.4 Preparation and Transfer of Whole Blood Samples*

Staff at the NCIC Clinical Trials Group Tumour Bank also carried out the retrieval, thawing and aliquoting of required MAP.3 whole blood samples for transfer to Dr. Harriet Feilotter's laboratory. For each study participant, 200 µL of one 1.5 ml whole blood sample was aliquoted for the purposes of this study. DNA extractions from the whole blood and subsequent genotyping were carried out through the Queen's Laboratory for Molecular Pathology (QLMP).

*3.1.4.5 DNA Extraction and Genotyping of Two Polymorphisms in the Vitamin D Pathway*

As a reminder, we were interested in exploring the association between: (1) Fok1, a polymorphism in the gene encoding the VDR protein, and MD, and (2) a polymorphism in the vitamin D metabolism gene CYP24A1 and MD. DNA extraction was carried out by personnel (G Pare) in the QLMP who were blinded to the measurement of percent MD among participants. Genomic DNA was isolated from each of the 200µl whole blood samples using either manual extraction with the Qiagen DNeasy Blood and Tissue kit (n=214 samples) or automated extraction with the QiaSymphony DNA mini kit (n=336 samples) for increased efficiency. DNA was quantified by spectrophotomety using the Nanodrop ND 1000 (Germany). Unfortunately, at the time of DNA extraction it was discovered that serum was not available for some participants (n=18 samples) and thus were discarded from subsequent genetic analyses. At the completion of DNA extraction all remaining whole blood samples were returned to the NCIC Clinical Trials Group Tumour Bank for future research studies.

Polymorphisms for the VDR and CYP24A1 genes were genotyped using commercial TaqMan assays (assay IDs: rs2181874: C_15931654_10; rs2228570: C_12060045_20) with TaqMan Genotyping Master Mix on a ViiA™ 7 Real-Time PCR System (Life Technologies), following the manufacturer's instructions. Briefly, 20 ng purified DNA was mixed with the TaqMan Genotyping Master Mix and its specific SNP assay primers. Samples were subjected to one period of 10 minutes at 95°C, then to 40 cycles of 15 seconds at 95°C, followed by 60 seconds at 60°C. The real-time PCR was concluded with 60 seconds at 60°C.

In each real-time PCR run 10% of samples from a previous batch were included to provide a measure of reproducibility. In comparing agreement between the initial study results and the quality control samples there was 100% concordance observed. Once completed, the data

provided to us included the genotyping results for SNP rs2181874 (CYP24A1) and for SNP rs2228570 (Fok1), the allele and genotype frequencies for each polymorphism in the study population, and information on the replicates. The obtained genotype frequencies for each of these polymorphisms were very similar to that observed in a similar Canadian population[22]. These two polymorphisms were tested for deviation from Hardy-Weinberg equilibrium[23]. While the polymorphism in the CYP24A1 gene was observed to be in equilibrium, departure from Hardy-Weinberg equilibrium was detected for the Fok1 polymorphism of the VDR gene in this study ($p<0.05$). It is possible that deviation from Hardy-Weinberg equilibrium is the result of the underlying population structure in the current study of predominantly Caucasian women[24]. It is noteworthy that other investigations of this genetic polymorphism in fairly homogeneous populations have observed similar deviations[22,25]. In absence of any obvious problems with genotyping, with 100 percent concordance between initial and repeat analyses and genotype frequencies in the expected range, this polymorphism was retained for analysis.

### 3.2 Statistical Analysis

All statistical analyses were carried out using SAS version 9.2 [26]. Statistical significance was defined as a p value $< 0.05$ and all tests were two-sided. Descriptive analyses were conducted to describe the characteristics of the study population. Analysis of variance (ANOVA) was used to evaluate the relationship between each covariate and serum 25-OH-D and, independently, percent MD. Due to the stringent data collection undertaken by clinical trial staff on the study participants missing data was minimal for all potential confounders examined in the current study (n=3). Given the adequate sample size and minimal missing data in this study all multivariate analyses were conducted only with participants with complete data. A comparison with previous literature in this area was undertaken.

### 3.2.1 Serum 25-OH-D

In trying to obtain a person's typical serum 25-OH-D level a few years prior to outcome assessment (MD) recall that there were two samples, randomization and year 1, per participant measured for serum 25-OH-D. In designing this study it was felt that using an average of these two samples may provide a more stable exposure estimate than the randomization measure alone given the wide variation in levels depending on the month/season the sample was drawn. The distributions of serum 25-OH-D at the time of randomization and at year 1 were examined and were observed to follow an approximately normal distribution. Means and standard deviations (SD) are presented.

In looking at the months of sample collection between the randomization and year 1 samples for a given participant, approximately 50% were taken in the same calendar month from one year to the next. This was not surprising as, per the clinical trial protocol, participants were to have these blood draws one year apart from each other. For the other 50% of women, however, their randomization and year 1 samples were not within the same calendar month to varying degrees of difference. For those women whose year 1 sample was taken in the same month as the randomization sample the average of the two values was taken for analysis purposes. However, for those women whose year 1 sample was not taken in the same month as the randomization sample seasonality needed to be accounted for prior to taking the average. In order to do this the year 1 serum sample value was converted to a standard deviate (i.e. Z score) for the month of the sample [S = (year 1 serum 25-OH-D - month mean) / month SD]. That standard deviate was then converted to the corresponding value for the randomization sample month [adj= mean for randomization sample month + (S* SD for randomization sample month)]. The randomization and adjusted serum 25-OH-D values were then averaged [Average Serum 25-OH-D = (randomization serum 25-OH-D + adj serum-25-OH-D) / 2]. These calculated average measures

*per participant* were used as the primary exposure measures in the regression analyses and analyses controlled for the randomization sample month and other confounding variables.

### 3.2.2 Mammographic Density

The two primary outcome measures of interest in this study were 1) percent MD at ≥ 3 year follow-up and 2) the average change in percent MD over time. The average change in MD was calculated by subtracting the percent MD at ≥ 3 year follow-up from the baseline MD at the time of recruitment into the underlying clinical trial, *divided* by the number of years that the participant had been followed at the time of the follow-up mammogram. The distributions of these two measures of MD were examined. The distribution of data for percent MD at ≥ 3 year follow-up was highly right skewed and thus was log transformed to improve the normality of the data for subsequent analyses. Consistent with methods employed in previous studies of MD [27], a percent MD of 0 was converted to 0.5 for the purposes of log transformation. Geometric means, derived by exponentiating the means of the log of MD, are presented for the follow-up MD overall and by format of the mammogram (film vs. digital). The distribution of the average change in MD variable was observed to follow an approximately normal distribution and means and SDs are reported.

### 3.2.3 Regression Analyses

Least squares regression was used to quantify the relationships between continuous measures of serum 25-OH-D and log transformed percent MD at ≥ 3 year follow-up and the change in percent MD over time. In bivariate analysis, serum 25-OH-D was not observed to have a linear relationship with percent MD. Regression diagnostics, including tests for normality and Q-Q plots, were carried out to confirm these observations. Consistent with other literature in this area,

a categorical representation of the average measure of serum 25-OH-D was considered. Previous studies have typically examined the relation between serum 25-OH-D and MD either across quartiles of exposure or using known clinical cut points [13,28-31]. Similarly, we categorized the serum measurements by generally accepted clinical cut points for levels of deficiency through sufficiency [32] while ensuring adequate numbers of participants in each category for analysis purposes. The serum 25-OH-D levels were categorized as follows: (1) < 25 ng/mL; (2) 25-34.9 ng/mL); (3) 35-44.9 ng/mL; (4) ≥ 45 ng/mL. Least squares means of log transformed percent density, adjusted for all confounding variables, were calculated across these categories of serum 25-OH-D using generalized linear models. The overall *p*-value for the models was derived from the F-test.

A main analysis with a dichotomous outcome measure for percent MD ≥ 3 year follow-up based on a clinically meaningful breast density was also evaluated. As previously reviewed, there is a strong association between increasing breast density, evaluated by BIRADS classification, with increasing BC risk [33]. Thus, it was of clinical interest to evaluate whether vitamin D was associated with BIRADS categories of risk using the percent MD measurements of the study participants. Given that the distribution of percent MD in this cohort of women was highly right skewed, with few women with high MD, only the association between serum 25-OH-D and percent MD in the lowest and above BIRADS categories of risk could be evaluated with sufficient study power. Specifically, a dichotomous outcome of percent MD (non-transformed) based on BIRADS category 1 vs. categories 2-4 were used (i.e. <25% MD vs. ≥ 25% MD). It was felt that this dichotomous percent MD variable more aptly captured the clinically meaningful and biologically relevant changes of differences in breast density that likely affect BC risk. Said another way, it is unlikely that vitamin D will affect the risk of BC with differences in breast density of 5 to 10%; however, BC risk at a population level may well be affected if vitamin D is associated with differences in breast density ≥ 25% (higher risk) compared with breast density <

25% (no or low risk). Multivariate logistic regression analysis, with reported odds ratios (OR) and 95% confidence intervals (CI), was used to estimate the effect of serum 25-OH-D levels between women classified as low density (<25% MD) or 'low risk' and higher density (≥ 25% MD) or 'higher risk' at ≥ 3 year follow-up. For the second primary outcome measure, the average change in breast density over time was calculated as [(baseline MD - ≥ 3 follow-up MD) / number of years of follow-up]. This regression analysis was based on a dichotomous change in breast density outcome [decrease in percent MD over time ('low risk') vs. no change or an increase in percent MD over time ('higher risk')]. A categorical representation of serum 25-OH-D as described above was again used as the primary exposure variable. As the frequency of the outcome (percent MD ≥ 25%) is not rare in this population the OR derived from the logistic regression will not estimate the relative risk (RR) [34]. Alternative methods for estimating an adjusted RR have been proposed including the use of the log-binomial model, however, these models have drawbacks including convergence problems and narrower CIs [34]. The OR derived from the logistic regression was felt to be useful for identifying relationships between vitamin D and breast density and careful attention will be paid in the interpretation of the measures of effect observed. The likelihood ratio test was used to test the significance ($p < 0.05$) of the vitamin D-breast density relationship in the logistic regression analyses.

### 3.2.4 Secondary Study Objectives

The secondary objectives of this study investigated interactions of the serum 25-OH-D and percent MD relationship with calcium, the randomization arms of the underlying trial (i.e. exemestane vs. placebo) and with two SNPs in the vitamin D pathway (i.e. Fok1 rs2228570 and CYP24A1 rs2181874). Both serum 25-OH-D and calcium were dichotomized based on the distribution of these exposures to provide sufficient power for subsequent analyses. Specifically, the bottom two serum 25-OH-D categories were combined as were the top two categories (i.e.

<35 ng/mL vs. ≥35 ng/mL). In addition, calcium was dichotomized at the median value observed among participants (i.e. low vs. high calcium). Consistent with previous literature, the genotype frequencies of the relevant polymorphisms in the Fok1 VDR (*ff* vs. *Ff* vs. *FF*) and CYP24A1 (GG vs. GA vs. AA) genes were evaluated for their association with ≥3 year follow-up MD as well as for possible interactions with serum 25-OH-D and ≥3 year follow-up MD [22,35-37]. Based on known functionality, the Fok1 variable was also grouped into a dichotomous variable to ensure adequate power to detect an association. The recessive model (*ff* vs. *Ff* + *FF*) of allele frequency was evaluated for an association with ≥3 year follow-up MD [37]. The AA genotype of the CYP24A1 polymorphism occurs in less than 10% of the population based on current findings [22]. Again, both the genotype and allele frequencies of the polymorphism in the CYP24A1 gene of interest were evaluated with ≥3 year follow-up MD. The CYP24A1 polymorphism was also grouped into a dichotomous variable with the rare homozygous genotype combined with the heterozygote (GG vs. GA & AA) in order to ensure sufficient power to detect an association [22].

Interactions were examined by the inclusion of product terms in the multivariate linear and logistic regression models using a dichotomous vitamin D exposure measure with the outcome of percent MD ≥ 3 year follow-up. Interactions in the generalized linear models were considered statistically significant if *p*-values were <0.05 for the product terms. The likelihood ratio test was used to test the significance ($p < 0.05$) of interactions in the logistic regression analyses.

### 3.2.5 Assessment of Confounding

As reviewed in Chapter 2, there are a large number of known and suspected risk factors for BC, particularly as they relate to estrogen exposure. The underlying trial prospectively collected information on a comprehensive list of these risk factors including age, BMI, month of blood

collection, age at menarche, age at menopause, parity, age at first birth, HRT use, OC use, education, smoking status, first degree family history of BC, ethnicity, race and calcium. In addition, the randomization arm to which each participant was assigned was provided.

As previously described, if exemestane has a modifying effect on the vitamin D and MD relationship results will be reported independently by randomization arm of the trial. In the absence of effect modification, this study has the ability to evaluate some of the primary objectives in the absence of confounding by estrogen levels. For primary objective 1, looking at the association between serum 25-OH-D and $\geq$ 3 year follow-up percent MD, whether there is the potential for confounding by estrogen is dependent on the relevant time window of exposure for serum 25-OH-D to exert its effects on percent MD. If all of the effects of baseline serum 25-OH-D on breast density occur after trial randomization then there should not be any confounding by estrogen since the vitamin D groups should be balanced on these hormonal factors. However, if the vitamin D levels measured at baseline are representative of one's typical level years prior to randomization which, in turn, is reflective of the biologically relevant window of exposure for vitamin D to exert its' effects on percent MD then randomization cannot control for estrogen levels during the previous exposure window. For the second primary objective, it was of interest to evaluate whether levels of serum 25-OH-D collected at randomization and year 1 were associated with changes in percent MD over the course of trial participation. In this case, the process of randomization should balance the vitamin D groups on relevant hormonal factors eliminating potential confounding by such variables.

Overall, in using the intermediate marker of MD as the primary outcome in this study it was of paramount importance to control for all possible breast density/BC risk factors to produce unbiased effect measures on the relationship between vitamin D and MD. This may provide an

advantage over other studies that may have had insufficient control for potential confounding variables particularly as they related to estrogen [13,29,30,38,39]. In the present study, all variables potentially related to breast density/BC based on previous studies of the vitamin D and MD relationship and from review of the literature on risk factors were identified as potential confounders. This literature review also informed the methodological decisions made with respect to representation of these variables included in the confounder assessment model and subsequent regression analyses.

Covariate information was obtained from the two baseline clinical trial participant information forms. For model selection, these variables were represented based on either: (a) previously established clinical cut points (i.e. BMI); (b) the underlying distribution among study participants (i.e. age at menarche); or categorization in a manner consistent with previously reported literature in this area (i.e. combined parity/age at first birth variable) [13,40]. While we did not have information on the physical activity levels of the study participants this limitation was hopefully mitigated by having information on other factors (i.e. BMI) that are known to be correlated with physical activity. We were unable to assess race and ethnicity as potential confounding variables as there was insufficient numbers of non-Caucasian women in our study population (n=35). A sensitivity analysis was done to evaluate the robustness of the results observed excluding these women. A change in estimate approach for confounder assessment is a robust procedure for identifying true confounders on the underlying exposure-disease association[41]. However, as stated above, consideration of all available breast density/BC risk factors that could possibly introduce confounding ultimately on the exposure-outcome relationships under investigation was of importance in the current investigation. Thus, for confounder selection, backward elimination was the procedure chosen to create a parsimonious model of covariates that was associated with breast density [42,43]. It has been reported that backward elimination with a traditional cut off value

of 5% may result in biased estimates of effect due to the under selection of important confounding variables and should be avoided [42]. Thus, the model was created using backward elimination at a liberal *p*-value of 0.15 for inclusion to ensure that all potential confounders were included as recommended in the literature [43]. This strategy was carried out using both continuous log transformed MD and, independently, dichotomous non-transformed MD for each of the primary study outcomes (≥3 year follow-up MD and average change in MD over time). For each of these outcomes, the model that adjusted for the larger number of covariates was used for all subsequent analyses. The same sub-set of covariates across models was included for easier interpretation of results and comparison with previous literature. In addition, age and month of blood collection were included in all analyses given their known strong association with vitamin D and/or breast density/BC risk. A sensitivity analysis using a change in estimate approach was carried out using the continuous log transformed MD outcome as a means of comparison with the backward elimination procedure. While the change in estimate approach retained fewer variables in the model compared with the backward elimination procedure the parameter estimates and standard errors of the serum 25-OH-D variable were virtually unchanged.

### 3.2.6 Sample Size

At study inception, we foresaw being able to include approximately 500 MAP.3 participants from northern latitudes in the current observational study. Detectable effects were estimated in order to provide perspective on the adequacy of the sample size. Detectable effect estimates, all with two-tailed significance of 0.05 and 80% power, were based on analysis of both a continuous and categorical representation of exposure (serum 25-OH-D) on a continuous outcome (percent MD)[44]. The literature in this area supports a linear relationship between percent MD and BC risk[45-47]. A study by Ursin et al, for example, supports a strong gradient in BC risk with increasing density among three different ethnic cohorts[45]. Further, in one meta-analysis the

authors observed a linear increase in the RRs of BC with increasing percent MD using quantitative techniques similar to ours[47]. Specifically, compared with women with <5% density, women with 5–24% MD had an RR = 1.79, 95% CI: 1.5–2.2; women with 25–49% MD had an RR=2.11, 95% CI: 1.7–2.6; women with 50–74% MD had an RR=2.92, 95% CI: 2.5–3.4; and women with ≥ 75% MD had an RR=4.64, 95% CI: 3.6–5.9[47]. Detectable effects considering a dichotomous outcome of percent MD was also estimated.

*3.2.6.1 Detectable Effects Based on Continuous Exposure and Outcome Variables*

Detectable slope estimates were first based on analysis of a continuous representation of serum 25-OH-D on a continuous outcome of percent MD. A correlation of 0.1 between dependent and independent variables was used in the calculation and the detectable estimates varied little with correlations between 0.05 and 0.3. The independent variable is treated as a standardized variable and, therefore, the slope of the regression line is interpretable as the difference in percent MD for a one standard deviation change in serum 25-OH-D. A case-control study nested within the Canadian National Breast Screening Study was used to obtain an estimate of the distribution of percent MD, where a mean of 26.8% and standard deviation of 19.2% was observed among control subjects[48].

A sample size of 500 would facilitate detection of a slope of ± 2.4% in this analysis of the effect of serum 25-OH-D on percent MD. With respect to the clinical importance of this magnitude of difference in MD – the average difference in percent MD between BC cases and controls is approximately 5% [49]. Therefore, the study is able to detect clinically meaningful differences in percent MD.

*3.2.6.2 Detectable Effects Based on a Categorical Exposure and Continuous Outcome*

Detectable effect estimates were next based on an analysis of a categorical representation of serum 25-OH-D on a continuous outcome of percent MD. In this study, serum 25-OH-D measurements were categorized using generally accepted clinical cut points of deficiency to sufficiency. Specifically, the categories were (1) < 25 ng/mL; (2) 25-34.9 ng/mL; (3) 35-44.9 ng/mL; and (4) ≥45 ng/mL. From a recent study examining the vitamin D status of Canadians it was estimated that approximately 25% of the population is vitamin D deficient (< 20 ng/mL)[50]. Thus, it was assumed that approximately 25% of the study population would be exposed to low levels (<25 ng/mL) of serum 25-OH-D and approximately 25% would be exposed to high levels (≥45 ng/mL ). Detectable effects were estimated for the contrast between participants with expected low levels of vitamin D (exposed) compared with participants with expected high levels of vitamin D (unexposed). Assuming equal numbers across categories of exposure, 125 participants were expected to be vitamin D deficient (exposed). Using a standard deviation of 19.2 again for the anticipated distribution of percent MD, this study is able to detect a 6.8% difference in mean MD at ≥ 3 year follow-up across serum 25-OH-D categories.

*3.2.6.3 Detectable Effects Based on Categorical Exposure and Outcome Variables*

Detectable estimates were also based on an analysis of a categorical representation of serum 25-OH-D on a dichotomous outcome of percent MD (≥ 25% vs. < 25%). It was estimated that approximately 40% of postmenopausal women in this study population of 500 would have ≥ 25 percent MD based on recent estimates from similar populations (n=200)[48]. Detectable effects are based on a comparison of the lowest versus highest vitamin D categories and 100 events (i.e. percent MD ≥ 25%) in this contrast. We will be able to detect an OR of 2.1 for percent MD ≥ 25 in participants with low serum 25-OH-D compared to participants with high serum 25-OH-D.

*3.2.6.4 Detectable Effects by Potential Effect Modifiers*

Detectable effects within the strata defined by potential effect modifiers (exemestane, calcium and genetic polymorphisms) are dependent on the distributions of these variables. Half of the study sample will have been randomized to receive exemestane resulting in a sample size of 250 within strata. Detectable effect estimates based on an analysis of a dichotomous representation of serum 25-OH-D [deficient (<25 ng/mL) vs. sufficient (≥25 ng/mL)] on a dichotomous outcome of percent MD (< 25% vs. ≥ 25%) within strata of the treatment variable (exemestane vs. placebo) were calculated. Again, it was estimated that approximately 40% of postmenopausal women in this study population of 250 would have ≥ 25 percent MD based on recent estimates from similar populations (n=100)[48]. Detectable effects are based on a comparison of the lowest versus highest vitamin D categories and 100 events (i.e. percent MD ≥ 25%) in this contrast. We will be able to detect an OR of 2.16 within strata for percent MD ≥ 25 in participants with low serum 25-OH-D compared to participants with high serum 25-OH-D.

At the other extreme of the distribution of interaction terms is the Fok1 (rs2228570 aka 10735810) polymorphism, with an estimated 15% having the homozygous variant *ff* genotype[37]. Detectable effect estimates based on an analysis of a dichotomous representation of serum 25-OH-D (<25 ng/mL vs. ≥25 ng/mL) on a dichotomous outcome of percent MD (< 25% vs. ≥ 25%) within estimated strata (n=75) defined by the Fok1 variable were also calculated. It was estimated again that approximately 40% of postmenopausal women in this study population of 75 would have ≥25 percent MD[48]. Detectable effects are based on a comparison of the lowest versus highest vitamin D categories and 30 anticipated events (i.e. percent MD ≥ 25%) in this contrast. We will be able to detect an OR of 3.94 within this stratum for percent MD ≥ 25 in participants with low serum 25-OH-D compared to participants with high serum 25-OH-D.

### 3.3 Ethics

The randomizing centers that participated in the MAP.3 clinical trial received research ethics board (REB) approval from their overseeing institutions prior to starting the RCT. MAP.3 participants also provided individual informed consent for trial participation which included consent for mandatory and optional blood collection and retrieval of mammograms for future research purposes. A copy of the MAP.3 informed consent template document can be found in Appendix 7. Ethics approval for this project was initially granted from the Health Sciences REB at Queen's University in October 2010 (Appendix 8) and annual re-approval has been granted since that time.

Support for this observational study was sought and gained from the MAP.3 trial committee including the use of stored serum and whole blood samples that were collected as part of the trial. The NCIC Clinical Trials Group Breast Tissue Correlative Sciences committee, who provides oversight for the use of such subject specimens, also provided support. Blood samples used for this study were identified by a code number only with all other personal identifying information kept confidential by NCIC CTG. All identifying information was held in strict confidence in either the secure NCIC Clinical Trials Group environment or in locked filing cabinets in a locked office at HDH which was accessible only to designated study personnel. Password-protected computerized data files contained no identifying information and all results of this study are presented in aggregate form such that individual participant's results are not identifiable. Service agreements between NCIC CTG, Queen's University and our study collaborators (HDH for the purposes of mammogram collection and processing; Dr. Glenville Jones whose laboratory personnel conducted our serum 25-OH-D analysis; and Dr. Harriet Feilotter whose laboratory personnel conducted the DNA isolation and genetic analysis) were developed and signed by relevant parties under the direction of the Office for Research Services at Queen's University.

These research services agreements ensured all parties were aware of the requirement to keep strictly confidential all personal and personal health information of the study participants provided by NCIC Clinical Trials Group (Appendices 5,9 and 10).

Some of the time delays experienced in the data collection portion of this study pertained to participant consent to retrieve mammograms with personal identifying information. While the MAP.3 informed consent document provided to centres for their use included the parametres for data collection necessary for the current study (i.e. serum and whole blood samples and mammograms) some individual centres removed the requirement for participants to consent to future mammogram retrieval at the time of joining MAP.3. This resulted in the need for them to amend their informed consent document, seek REB approval and re-consent required MAP.3 participants before they were able to retrieve mammograms for the current study. In addition, a few other centres with whom we communicated about mammogram retrieval viewed the need to seek additional REB approval before releasing mammograms to HDH which contained personal identifying information. We were successful in assisting the centres in obtaining the necessary ethics approval and in re-consenting required participants in order to gain access to their mammograms for percent MD measurement. All participants who were contacted to provide consent for this additional aspect under the auspices of the parent trial provided consent.

### 3.4 References

1. Breast Cancer Risk Assessment Tool. National Institutes of Health. http://www.cancer.gov/bcrisktool/ Published 2011.

2. Goss PE, Ingle JN, Alés-Martínez JE, et al. Exemestane for breast-cancer prevention in postmenopausal women. New England Journal of Medicine. 2011(364):2381.

3. What is mammography? Radiology Regional Centre. http://radiologyregional.com/radiologyregional/index.php?option=com_content&view=article&id=47&Itemid=93. Published 2012

4. Boyd N. Percent density on film screen vs. digital mammography. Written communication, Feb 2011.

5. van Gils CH. Follow-up from AACR frontiers in cancer prevention conference. Written communication, Jan 2013.

6. Mammography Accreditation Program. Canadian Association of Radiologists. http://www.car.ca/en/accreditation/map.aspx. Published 2009.

7. Berube S, Diorio C, Masse B, et al. Vitamin D and calcium intakes from food or supplements and mammographic breast density. Cancer Epidemiol Biomarkers Prev. 2005;14(7):1653-1659.

8. Tseng M, Byrne C, Evers K, Daly M. Dietary intake and breast density in high-risk women: A cross-sectional study. Breast Cancer Research. 2007;9(5):R72.

9. Berube S, Diorio C, Verhoek-Oftedahl W, Brisson J. Vitamin D, calcium, and mammographic breast densities. Cancer Epidemiol Biomarkers Prev. 2004;13(9):1466-1472.

10. Diorio C, Berube S, Byrne C, et al. Influence of insulin-like growth factors on the strength of the relation of vitamin D and calcium intakes to mammographic breast density. Cancer Res. 2006;66(1):588-597.

11. Mishra G, McCormack V, Kuh D, etc. Dietary calcium and vitamin D intakes in childhood and throughout adulthood and mammographic density in a British birth cohort. British Journal of Cancer. 2008;99:1539-1543.

12. Vachon CM, Kushi LH, Cerhan JR, Kuni CC, Sellers TA. Association of diet and mammographic breast density in the minnesota breast cancer family cohort. Cancer Epidemiol Biomarkers Prev. 2000;9(2):151-160.

13. Knight JA, Vachon CM, Vierkant RA, Vieth R, Cerhan JR, Sellers TA. No association between 25-hydroxyvitamin D and mammographic density. Cancer Epidemiol.Biomarkers Prev. 2006;15:1988-1992.

14. Brisson J, Berube S, Diorio C, Sinotte M, Pollak M, Masse B. Synchronized seasonal variations of mammographic breast density and plasma 25-hydroxyvitamin D. Cancer Epidemiol Biomarkers Prev. 2007;16(5):929-933.

15. Green AK, Hankinson SE, Bertone-Johnson ER, Tamimi RM. Mammographic density, plasma vitamin D levels and risk of breast cancer in postmenopausal women. International Journal of Cancer. 2010;127(3):667-674. doi: 10.1002/ijc.25075

16. Byrne C, Colditz GA, Willett WC, Speizer FE, Pollak M, Hankinson SE. Plasma insulin-like growth factor (IGF) I, IGF-binding protein 3, and mammographic density. Cancer Research. 2000;60(14):3744-3748.

17. Yaffe MJ. Mammographic density. measurement of mammographic density. Breast Cancer Research. 2008;10(3):209.

18. McCormack VA, Highnam R, Perry N, dos Santos Silva I. Comparison of a new and existing method of mammographic density measurement: Intramethod reliability and associations with known risk factors. Cancer Epidemiology, Biomarkers & Prevention. 2007;16(6):1148-1154.

19. Jones G, Horst R, Carter G, etc. Contemporary diagnosis and treatment of vitamin D-related disorders. J Bone Miner Res. 2007;22(2):V11.

20. Chen H, McCoy LF, Schleicher RL, Pfeiffer CM. Measurement of 25-hydroxyvitamin D3 (25OHD3) and 25-hydroxyvitamin D2 (25OHD2) in human serum using liquid chromatography-tandem mass spectrometry and its comparison to a radioimmunoassay method. Clinica Chimica Acta. 2008;391(1-2):6-12.

21. D E Q A S VitaminD ExternalQualityAssessmentScheme. http://www.deqas.org/. No publication date.

22. Anderson LN, Cotterchio M, Cole DEC, Knight JA. Vitamin D-related genetic variants, interactions with vitamin D exposure, and breast cancer risk among caucasian women in ontario. Cancer Epidemiology Biomarkers & Prevention. 2011;20(8):1708-1717. doi: 10.1158/1055-9965.EPI-11-0300.

23 Hardy-weinberg principle.

http://en.wikipedia.org/wiki/Hardy%E2%80%93Weinberg_principle. Published 2013.

24. Wigginton JE, Cutler DJ, Abecasis GR. A note on exact tests of hardy-weinberg equilibrium. Am J Hum Genet. 2005;76(5):887-893. http://linkinghub.elsevier.com/retrieve/pii/S0002929707607356.

25. Falchetti A, Sferrazza C, Cepollaro C, et al. FokI polymorphism of the vitamin D receptor gene correlates with parameters of bone mass and turnover in a female population of the italian island of lampedusa. Calcif Tissue Int. 2007;80(1):15-20. http://dx.doi.org/10.1007/s00223-005-0295-1. doi: 10.1007/s00223-005-0295-1.

26. SAS Institute Inc. 2009. *SAS/STAT® 9.2 User's Guide, Second Edition*. Cary, NC: SAS Institute Inc.

27. Bertone-Johnson ER. Percent mammographic density readings of zero. Written communication, June 2012.

28. Neuhouser ML, Bernstein L, Hollis BW, et al. Serum vitamin D and breast density in breast cancer survivors. Cancer Epidemiology Biomarkers & Prevention. 2010;19(2):412-417.

29. Sprague, B.L., Trentham-Dietz, A., Gangnon, R.E., Buist, D.S.M., Burnside, E.S., Bowles, E.J.A., Stanczyk, F.Z., Sisney, G.S., Skinner , H.G. The vitamin D pathway and mammographic breast density among postmenopausal women. Breast Cancer Res Treat. 2012;131(1):255-265.

30. Chai, W., Maskarinec, G., Cooney, R.V. Serum 25-hydroxyvitamin D levels and mammographic density among premenopausal women in a multiethnic population. Eur J Clin Nutr. 2010;64(6):652-654.

31. Bertone-Johnson, E.R., Chlebowski, R.T., Manson, J.E., Wactawski-Wende, J., Aragaki, A.,Tamimi, R.M.,Rexrode, K.M., Thomson, C.A., Rohan, T.E., Peck, J.D., Pisano, E.D., Martin, C.F., Sarto, G., McTiernan, A,. Dietary vitamin D and calcium intake and mammographic density in postmenopausal women. Menopause. 2010;17(6):1152-1160.

32. Ross AC, Manson JE, Abrams SA, et al. The 2011 report on dietary reference intakes for calcium and vitamin D from the institute of medicine: What clinicians need to know. Journal of Clinical Endocrinology & Metabolism. 2011;96(1):53-58. doi: 10.1210/jc.2010-2704.

33. Cummings SR, Tice JA, Bauer S, et al. Prevention of breast cancer in postmenopausal women: Approaches to estimating and reducing risk. J Natl Cancer Inst. 2009;101(6):384-398.

34. McNutt L, Wu C, Xue X, Hafner JP. Estimating the relative risk in cohort studies and clinical trials of common outcomes. American Journal of Epidemiology. 2003;157(10):940-943. doi: 10.1093/aje/kwg074.

35. McCullough ML, Stevens VL, Diver WR, Feigelson HS, etc. Vitamin D pathway gene polymorphisms, diet, and risk of postmenopausal breast cancer: A nested case-control study. Breast Cancer Research. 2007;9:1.

36. McCullough ML, Bostick RM, Mayo TL. Vitamin D gene pathway polymorphisms and risk of colorectal, breast, and prostate cancer. Annu Rev Nutr. 2009;29(1):111-132. http://dx.doi.org/10.1146/annurev-nutr-080508-141248. doi: 10.1146/annurev-nutr-080508-141248.

37. Tang C, Chen N, Wu M, etc. Fok1 polymorphism of vitamin D receptor gene contributes to breast cancer susceptibility: A meta-analysis. Breast Cancer Research (2009) 117:391–399. DOI 10.1007/s10549-008-0262-4

38. Holmes MD, Hankinson SE, Bryne C. Mammographic density and diet. American Journal of Epidemiology. 2001;153:S109.

39. Thomson CA, Arendell LA, Bruhn RL, etc. Pilot study of dietary influences on mammographic density in pre- and postmenopausal hispanic and non-hispanic white women. Menopause. 2007;14:1-8.

40. Bertone-Johnson ER, McTiernan A, Thomson CA, et al. Vitamin D and calcium supplementation and one-year change in mammographic density in the women's health initiative calcium and vitamin D trial. Cancer Epidemiology Biomarkers & Prevention. 2012;21(3):462-473.

41. Kenneth J. Rothman, Sander Greenland. Modern epidemiology. Philadelphia, PA: Lippincott-Raven Publishers; 1998.

42. Budtzâ€“JÃ¸rgensen E, Keiding N, Grandjean P, Weihe P. Confounder selection in environmental epidemiology: Assessment of health effects of prenatal mercury exposure.

Ann Epidemiol. 2007;17(1):27-35. http://linkinghub.elsevier.com/retrieve/pii/S1047279706001360?showall=true.

43. Kleinbaum DG, Kupper LL, Muller KE, et al. Applied regression analysis and other multivariable methods. Pacific Grove, CA: Brooks/Cole; 1998.

44. Dupont WD, PlummerJr. WD. Power and sample size calculations for studies involving linear regression. Control Clin Trials. 1998;19(6):589-601.

45. Ursin G, Ma H, Wu AH, et al. Mammographic density and breast cancer in three ethnic groups. Cancer Epidemiology Biomarkers & Prevention. 2003;12(4):332-338.

46. Boyd NF, Byng JW, Jong RA, et al. Quantitative classification of mammographic densities and breast cancer risk: Results from the canadian national breast screening study. J Natl Cancer Inst. 1995;87(9):670-675.

47. Boyd NF, Martin LJ, Li Q, et al. Mammographic density as a surrogate marker for the effects of hormone therapy on risk of breast cancer. Cancer Epidemiology Biomarkers & Prevention. 2006;15(5):961-966.

48. Boyd NF, Guo H, Martin LJ, et al. Mammographic density and the risk and detection of breast cancer. N Engl J Med. 2007;356(3):227-236. http://dx.doi.org/10.1056/NEJMoa062790. doi: 10.1056/NEJMoa062790.

49. Boyd N. What accounts for variations in percent density. Written communication, Nov 2010.

50. Whiting SJ, Langlois KA, Vatanparast H, Greene-Finestone LS. The vitamin D status of canadians relative to the 2011 dietary reference intakes: An examination in children

and adults with and without supplement use. The American Journal of Clinical Nutrition. 2011;94(1):128-135. doi: 10.3945/ajcn.111.013268.

# Chapter 4

# Results

## 4.1 Organization of Results

The presentation of the results of this study is provided in five main subchapters. Each subchapter provides an overview of the results from relevant statistical analyses and concludes with a brief summary of the observed results. Specifically, the subchapters are as follows: (A) Description of Study Participants; (B) MD Data; (C) Serum 25-OH-D Data; (D) Covariates; and (E) Results of the Study Objectives. All relevant tables and figures are included within each subchapter for ease of reference.

# Subchapter A

## 4.2 Description of Study Participants

As previously indicated, the retrieval of mammograms from randomizing centres was paramount to defining the study population. This section outlines the requests for and the results of mammogram collection from participating centres including overall response rates and the number of eligible study participants available for percent MD measurement. Further, the length of follow-up time for study participants based on the time they were randomized to the parent trial to the time of their follow-up mammogram used for study purposes is described.

### 4.2.1 Study Participants

A total of 896 Canadian and Buffalo (New York) MAP.3 clinical trial participants who had a follow-up period from the time of their baseline mammogram to their most recent mammogram of

at least 3 years and who consented to optional serum and whole blood collection as part of the parent trial were identified as potentially eligible for this study. Among these women, 216 were subsequently deemed ineligible from chart review as they had one or more of the following: (a) had a diagnosis of BC while on study (n=13); (b) did not provide or currently have blood samples in the NCIC Clinical Trials Group Tumour Bank (n=32); and/or (c) did not have all mammograms done at the same radiology facility (n=171). Requests for mammograms from the 680 remaining potentially eligible women were sent to centres. Review of data collection progress part way through this study was done and a decision was made to request the mammograms from an additional 65 participants from three centres who had completed their initial retrieval to ensure an adequate overall sample size to meet study objectives. These 65 participants met all inclusion criteria except that mammograms for each participant were done at varying radiology facilities.

Requests for mammograms were sent to 20 randomizing centres; 19 across Canada and one in Buffalo, New York. With the exception of a few centres, response rates to mammogram requests were very high. Overall, 77% of centres provided at least one $\geq$ 3 year follow-up mammogram for evaluation of the first primary objective. Further, 71% of centres provided both the baseline and at least one $\geq$ 3 year follow-up mammogram per requested participant for inclusion in the evaluation of the second primary objective if image formats were matching (i.e. both film or both digital). In total, of the 745 participants for whom mammograms were requested, multiple mammograms for 575 participants were received from these randomizing centres (see Table 4.1).

In consultation with the study radiologist, some mammograms or MD measurements were subsequently discarded from the analyses for the following reasons: mammograms were removed for (a) participants who were discovered to have had either a breast implant or breast reconstruction surgery (n=2); (b) participants for whom percent MD could not be accurately measured due to the receipt of poor quality images (n=5); and (c) participants who had baseline

mammograms measured but whose formats did not match the follow-up retained (n=105). For the latter participants, the percent MD measurements from their follow-up mammograms were retained for evaluation of primary objective 1. Overall, follow-up mammograms for a total of 568 eligible participants were available for the first primary objective examining the relationship between serum 25-OH-D and percent MD at ≥ 3 year follow-up. Further, a baseline and follow-up mammogram for 388 eligible participants were available for the second primary objective examining the relationship between serum 25-OH-D and change in percent MD over time. Table 4.2 below describes the length of follow-up for these participants from the time of randomization to the parent trial until the date of the follow-up mammograms utilized in this study. Approximately 75% of study participants had follow-up mammograms greater than 3 years from the time of randomization and ~ 30% of study participants had follow-up mammograms greater than 4 years from the time of randomization. On average, these women were followed for 3.7 years from the time of randomization.

### 4.2.2 Summary

Overall, there was high compliance among randomizing centres in retrieving participant mammograms for percent MD measurement in the current study. Further, there were minimal errors in data collection which negated the need to engage in multiple exchanges with centres to ascertain the appropriate mammograms. Very few mammograms were discarded from subsequent analyses due to subject ineligibility or poor image quality which overall reflects the robustness of the procedure developed for mammogram collection for this study. A total of 568 trial participants were included in this study with prospective mammographic data that ranged from 2 to 6 years from the time of randomization. For 388, baseline and follow-up mammograms were available for the investigation of change in percent MD.

**Table 4.1: Mammogram Collection for Eligible Study Participants**

| Centre | Total accrual at start of data collection | # Participants with mammograms ≥ 3 years | Total # ineligible | # Potentially eligible | Additional participants * | # Participants for whom mammograms requested | # Received at least 1 ≥ 3 year follow-up mammogram (Primary Objective 1) | Response rate (Primary Objective 1) (%) | # Received baseline and at least 1 ≥ 3 year follow-up mammogram (Primary Objective 2) | Response Rate (Primary Objective 2) (%) |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 1 | 1 | 0 | 1 | | 1 | 1 | 100 | 1 | 100 |
| 2 | 22 | 21 | 5 | 16 | | 16 | 16 | 100 | 9 | 56 |
| 3 | 74 | 42 | 21 | 21 | | 21 | 0 | 0 | 0 | 0 |
| 4 | 24 | 5 | 4 | 1 | | 1 | 1 | 100 | 1 | 100 |
| 5 | 16 | 13 | 2 | 11 | | 11 | 11 | 100 | 11 | 100 |
| 6 | 24 | 12 | 4 | 8 | | 8 | 8 | 100 | 8 | 100 |
| 7 | 30 | 25 | 5 | 20 | | 20 | 20 | 100 | 17 | 85 |
| 8 | 12 | 5 | 0 | 5 | | 5 | 5 | 100 | 5 | 100 |
| 9 | 3 | 2 | 1 | 1 | | 1 | 1 | 100 | 1 | 100 |
| 10 | 20 | 6 | 1 | 5 | | 5 | 5 | 100 | 4 | 80 |
| 11 | 39 | 25 | 2 | 23 | | 23 | 22 | 96 | 20 | 87 |
| 12 | 511 | 127 | 12 | 115 | | 115 | 110 | 96 | 98 | 85 |
| 13 | 59 | 39 | 8 | 31 | | 31 | 31 | 100 | 31 | 100 |
| 14 | 142 | 73 | 27 | 46 | 23 | 69 | 56 | 81 | 54 | 78 |
| 15 | 35 | 15 | 3 | 12 | | 12 | 12 | 100 | 12 | 100 |
| 16 | 70 | 67 | 9 | 58 | | 58 | 58 | 100 | 58 | 100 |
| 17 | 103 | 55 | 26 | 29 | 22 | 51 | 37 | 73 | 22 | 43 |
| 18 | 41 | 31 | 13 | 18 | | 18 | 18 | 100 | 16 | 89 |
| 19 | 46 | 36 | 25 | 11 | 20 | 31 | 29 | 94 | 28 | 90 |
| 20 | 528 | 296 | 48 | 248 | | 248 | 134 | 54 | 134 | 54 |
| **Total** | **1800** | **896** | **216** | **680** | **65** | **745** | **575** | | **530** | |
| **Overall Response Rate%** | | | | | | | | **77.20** | | **71.10** |

*Participants who met all inclusion criteria, with the exception that mammograms were done at varying radiology clinics, were added after initial mammogram requests were disseminated to centres to ensure an adequate overall sample size for evaluation of study objectives.

**Table 4.2: Years of Follow-Up for Study Participants by Mammogram Format**

| **Participants with Follow-Up Mammograms Only** | | | |
|---|---|---|---|
| **Years of Follow-up*** | **N (%)** | **Mean** | **SD** |
| ≥ 2 and < 3 years | 137 (24.1) | 2.62 | 0.28 |
| ≥ 3 and < 4 years | 228 (40.1) | 3.50 | 0.34 |
| ≥ 4 and < 5 years | 158 (27.8) | 4.47 | 0.33 |
| ≥ 5 years | 45 (8.0) | 5.24 | 0.31 |
| Total | 568 | 3.70 | 0.87 |
| **Participants with Baseline and Follow-Up Mammograms** | | | |
| **Years of Follow-up*** | **N (%)** | **Mean** | **SD** |
| ≥ 2 and < 3 years | 98 (25.3) | 2.63 | 0.29 |
| ≥ 3 and < 4 years | 163 (42.0) | 3.50 | 0.34 |
| ≥ 4 and < 5 years | 104 (26.8) | 4.46 | 0.34 |
| ≥ 5 years | 23 (5.9) | 5.33 | 0.39 |
| Total | 388 | 3.65 | 0.85 |

*The number of years of prospective follow-up from the time of trial randomization to the time of the follow-up mammogram utilized in all analyses

# Subchapter B

## 4.3 Mammographic Density Data

The following section provides a summary of the results from descriptive analyses of the breast density data obtained for study participants and reviewed by the study radiologist. This section concludes with a comparison of the data obtained in this study to that in the literature.

### 4.3.1 Descriptive Summary of the Mammographic Density Data

A total of 956 mammograms for 568 study participants were measured by the study radiologist and retained for analysis purposes. Specifically, there were 568 participants who each had one ≥ 3 year follow-up mammogram for whom we could evaluate the first primary objective (n=568 mammograms). Of these 568 participants, 388 participants also had a baseline mammogram in the same format as the ≥ 3 year follow-up mammogram and, thus, were evaluable for the second primary objective evaluating change in percent MD over time (n=388 additional mammograms). A descriptive summary of the percent MD data for these participant mammograms is found in Table 4.3 below. The overall distribution of breast density data for all mammograms is provided as well as independently for both the baseline and follow-up mammograms. It was of interest to examine whether a reduction in percent MD was observed from the baseline to the follow-up mammograms given that it is known that breast density decreases with increasing age. This data was further divided by the format of the mammograms to evaluate whether film based mammograms had a higher percent MD measurement compared with digital images as expected. The overall distribution of percent MD in this study population was highly right skewed and, as a result, geometric means are presented. The overall geometric mean percent MD for participant mammograms (n=956) was 4.72.

A random sample of 10% (N=102) of mammograms was re-read by the study radiologist with high intra-rater reliability (correlation coefficient = 0.95). The mean absolute difference in the percent MD measurement between the reads was, on average, 2.5% (SD = 3.08).

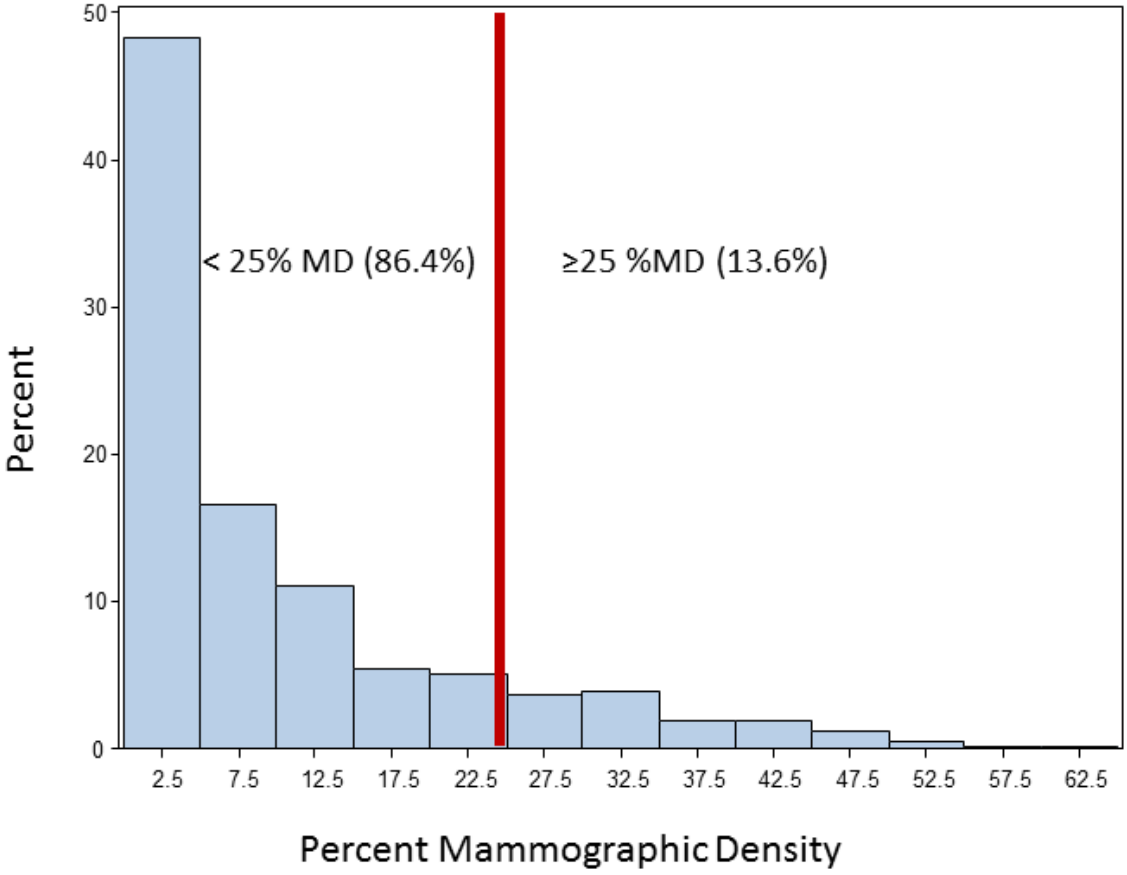
**Table 4.3: Descriptive Summary of Percent Mammographic Density Data**

| Mammograms | N | Geometric Mean | Range |
|---|---|---|---|
| **All** | 956 | 4.72 | 0-80.99 |
| **Baseline** | 388 | 5.50 | 0-80.99 |
| Film | 298 | 4.95 | 0-54.36 |
| Digital | 90 | 7.79 | 0-80.99 |
| **Follow-up** | 568 | 4.25 | 0-64.08 |
| Film | 326 | 4.17 | 0-59.50 |
| Digital | 242 | 4.36 | 0-64.08 |

*4.3.1.1 Mammographic Density Data at ≥ 3 Year Follow-Up*

There were 568 follow-up mammograms measured, 326 of which were film and 242 of which were digital, and available for the analysis of the first primary objective evaluating percent MD at ≥ 3 year follow-up. The geometric mean percent MD of the follow-up mammograms was 4.25%. As observed from Table 4.3 above, it can be noted that the geometric mean percent MD of the follow-up mammograms are similar for film-based and digital-based images. The distribution of percent MD for these follow-up mammograms was highly right skewed as observed in Figure 1 below. As previously described, an analysis with a dichotomous outcome measure for percent MD ≥ 3 year follow-up based on a clinically meaningful breast density according to BIRADs categories [category 1 (<25% MD) vs. categories 2-4 ( ≥ 25% MD)] was of interest. It is noteworthy that less than 14 percent of the mammograms included in this study had a percent MD measurement ≥ 25%.

**Figure 4.1: Distribution of Percent Mammographic Density at ≥ 3 Year Follow-Up (n=568)**

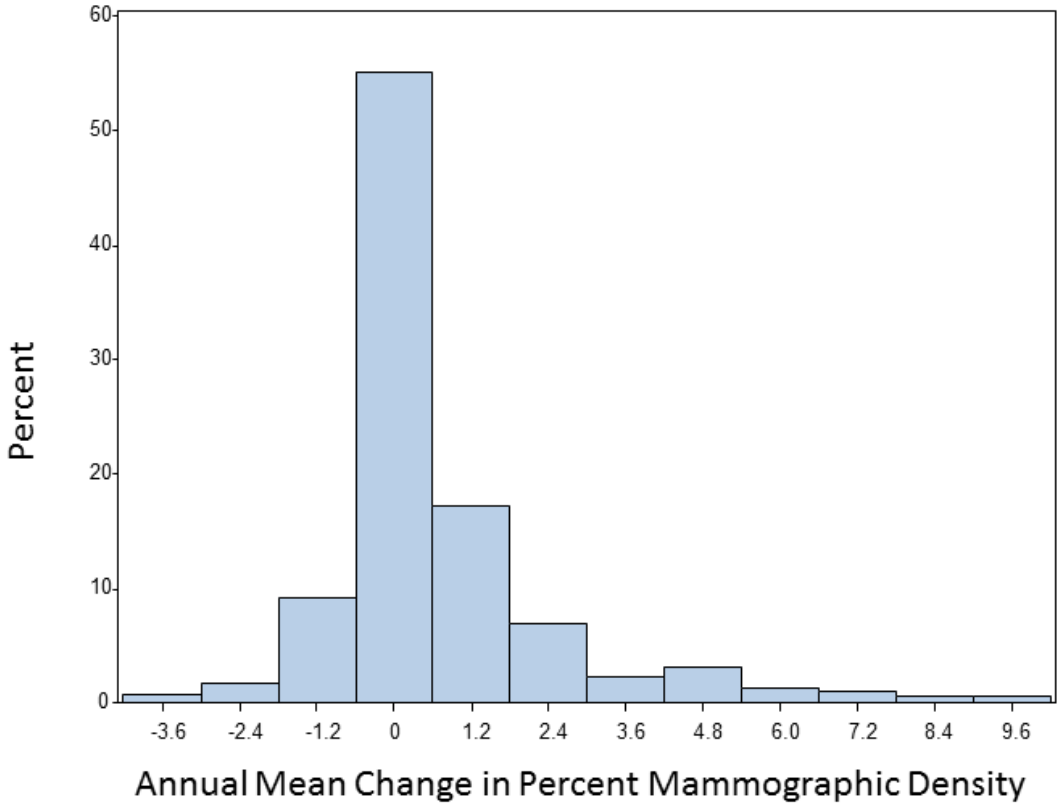



*4.3.1.2 Data on Mammographic Density Changes Over Time*

There were 388 participants who each had a baseline and a ≥ 3 year follow-up mammogram in the same format measured and available for the analysis of average change in percent MD over time. Of these participants, 298 pairs of mammograms were film-based and 90 pairs were digital-based. The geometric mean percent MD of the baseline mammograms, irrespective of format, was 5.50. In looking at these baseline mammograms, it was observed that the geometric mean percent MD was higher for the digital-based images as compared with the film-based images which was not anticipated.

As expected, a reduction in mean percent MD between baseline and follow-up mammograms was observed. In addition, when taking the format of the mammograms into account a similar pattern of reduction in mean percent MD between baseline and follow-up film and between baseline and follow-up digital images was seen. The distribution of the annual mean changes in percent MD between the baseline and follow-up images was approximately normal as observed in Figure 2 below. The annual mean change was defined as follows: (baseline percent MD - follow-up percent MD / years of follow-up). Therefore, in this figure a positive mean difference reflects an annual reduction in percent MD ('good' change) while a negative mean difference reflects an annual increase in percent MD over time ('bad' change). Among the 388 participants who had baseline and follow-up mammograms in the same format the overall mean difference was 2.7% over time (SD = 7.00) and the average difference per year was 0.70% (SD=1.81) reflecting a reduction in percent MD between baseline and follow-up images. The range in the annual mean difference in percent MD across the study participants was from a reduction of 9.8% to an increase of 3.8%.

**Figure 4.2: Distribution of the Annual Mean Difference in Percent Mammographic Density between Baseline and Follow-up Mammograms (n=388)**




### 4.3.2 Summary

The geometric mean percent MD of both the baseline and the follow-up mammograms in this study is comparable to the geometric mean percent MD in postmenopausal women reported in other literature [1-3] but without the range of density expected at a population level [4-6]. Based on prior studies, it was expected that approximately 5% of study participants would have mammographic densities ≥ 75% [4,5]. Unexpectedly, the large majority of study participants had percent MD measurements at follow-up <25% with only 13.6 percent of participants with a follow-up MD measurement ≥ 25%. Further, less than 1% of participants had a follow-up MD measurement ≥ 50% and no participants had follow-up mammograms with breast densities ≥75%.

For those participants for whom a change in MD over time can be evaluated (n=388), a decrease in the geometric mean percent MD between baseline and follow-up was observed as expected, with the magnitude of the decrease consistent with the literature [7,8]. Specifically, it is estimated that there is an approximately 1% decrease in percent MD per year as a woman ages [7,8].

Lastly, it was anticipated that percent MD on digital images would be lower than that on film images based on prior evidence [9,10]. However, it was observed that women who had digital mammography at baseline had higher percent MD compared with women who had film mammography (*p*-value = 0.02). Discussion with experts at radiology clinics during the mammogram collection phase of this study led to the hypothesis that there may be differences in risk factors among the women receiving digital vs. film-based mammography at baseline. Thus, the relationship between image format and baseline percent MD and potential confounding by variables including age, BMI, Gail score and education level was examined. The observed difference in baseline percent MDs by image format, however, was not accounted for by differences in these covariates between the study groups.

## Subchapter C

### 4.4.1 Serum 25-OH-D Data

The following section provides a summary of the results from descriptive analyses of the serum 25-OH-D data for study participants. Specifically, this section: (a) describes the seasonal pattern observed for the serum 25-OH-D measurements; (b) reviews the correspondence between the two samples measured per study participant; and (c) describes the distribution of the averaged serum 25-OH-D measurements per participant used in subsequent analyses.

### 4.4.2 Descriptive Summary of the Serum 25-OH-D Data

A total of 1144 serum samples for 575 participants were sent to Dr. Jones' laboratory and subsequently measured for total serum 25-OH-D which was the sum of serum 25-OH-D2 and serum 25-OH-D3. Two samples per participant were available for measurement with the exception of 6 participants who were discovered at the NCIC Clinical Trials Group Tumour Bank to only have one sample for use. Seven participants, and their accompanying serum samples (n=14), were subsequently discarded from the primary analysis for eligibility reasons and reasons pertaining to the quality of measurements on the outcome of interest (percent MD) as described above. Thus, 562 participants each had two serum 25-OH-D measurements and 6 participants each had one serum 25-OH-D measurement for a total of 1130 serum 25-OH-D measurements available for the analyses. As described in detail in Chapter 3, additional quality control samples from non study participants were also measured for serum 25-OH-D to check analysis performance; both inter- and intra-assay % CV were $\leq 10\%$.

Descriptive results of the serum 25-OH-D data are presented below in Table 4.4. Unadjusted for the month of blood collection, the mean serum 25-OH-D concentration in our study population was 36.5 ng/mL (SD=10.6) based on pooled baseline and year one samples (n=1130 samples). Independently, the baseline and year 1 mean values were very similar; the baseline mean serum 25-OH-D level for our study population was 36.3 ng/mL (SD=10.9) and the year 1 mean level was 36.8 ng/mL (SD=10.2). The mean levels for serum 25-OH-D2 and serum 25-OH-D3 are also presented. The ability of LC-MS/MS to reliably measure D2 and D3 leads to a more precise measurement of an individual's total serum 25-OH-D level. Availability of these individual measurements also provides insight into the specific sources of vitamin D contributing to overall serum 25-OH-D which was of interest. Recall that serum 25-OH-D2 is derived from vitamin D enriched food sources and/or supplements whereas serum 25-OH-D3 comes from sun exposure.

**Table 4.4: Descriptive Summary of Unadjusted Serum 25-OH-D Data**

| Serum Samples | N | Mean | SD | Range |
|---|---|---|---|---|
| **All Serum 25-OH-D** | 1130 | 36.53 | 10.55 | 9.40-103.74 |
| **Serum 25-OH-D2*** | 221 | 6.29 | 8.89 | 1.00-71.4 |
| **Serum 25-OH-D3** | 1130 | 35.30 | 10.33 | 8.30-74.60 |
| **Baseline Serum 25-OH-D** | 568 | 36.32 | 10.92 | 10.00-103.70 |
| **Serum 25-OH-D2*** | 106 | 7.36 | 10.74 | 1.00-71.40 |
| **Serum 25-OH-D3** | 568 | 34.95 | 10.52 | 8.30-67.30 |
| **Year 1 Serum 25-OH-D** | 562 | 36.75 | 10.16 | 9.40-74.60 |
| **Serum 25-OH-D2*** | 115 | 5.31 | 6.64 | 1.00-49.50 |
| **Serum 25-OH-D3** | 562 | 35.67 | 10.13 | 9.40-74.60 |

**A mean and SD is provided for those women who have a non-zero serum 25-OH-D2 measure*

*4.4.2.1 Seasonal Pattern of Serum 25-OH-D Measurements for all Samples*

As previously reviewed, it was anticipated that serum 25-OH-D levels would vary depending on the month/season the samples were drawn as sun exposure is an important source of vitamin D. In table 4.5 below are the mean serum 25-OH-D measurements (ng/mL) by the month the samples were drawn for the entire population of samples (N=1130). As expected, it was observed that serum 25-OH-D levels are higher in the summer months of June, July and August. Conversely, lower mean serum 25-OH-D measurements were observed in the winter months of January, February and April.

**Table 4.5: Unadjusted Mean Serum 25-OH-D (ng/mL) Measurements by Month (n=1130)**

| Month | N | Mean | SD |
|---|---|---|---|
| January | 101 | 33.4 | 9.8 |
| February | 88 | 32.7 | 9.5 |
| March | 86 | 36.8 | 12.6 |
| April | 76 | 33.8 | 10.4 |
| May | 80 | 35.9 | 10.6 |
| June | 97 | 38.1 | 12.5 |
| July | 80 | 38.9 | 8.7 |
| August | 83 | 38.2 | 11.5 |
| September | 94 | 37.4 | 9.2 |
| October | 128 | 38.3 | 10.1 |
| November | 132 | 37.6 | 8.9 |
| December | 85 | 36.0 | 10.9 |

*4.4.2.2 Correspondence between Serum 25-OH-D Measurements per Study Participant*

Recall that two serum 25-OH-D measurements per participant were obtained in an effort to best represent each individual's 'typical' serum 25-OH-D exposure level prior to MD assessment. An average measure of the two samples for a given participant was calculated if both were collected within the same month (n=296 participants). If the month of collection for each of the samples differed (n=266 participants), the serum 25-OH-D measures were adjusted to account for seasonality prior to taking their average. The single serum 25-OH-D measurement for the six participants who did not have a second sample was used as their exposure measurement.

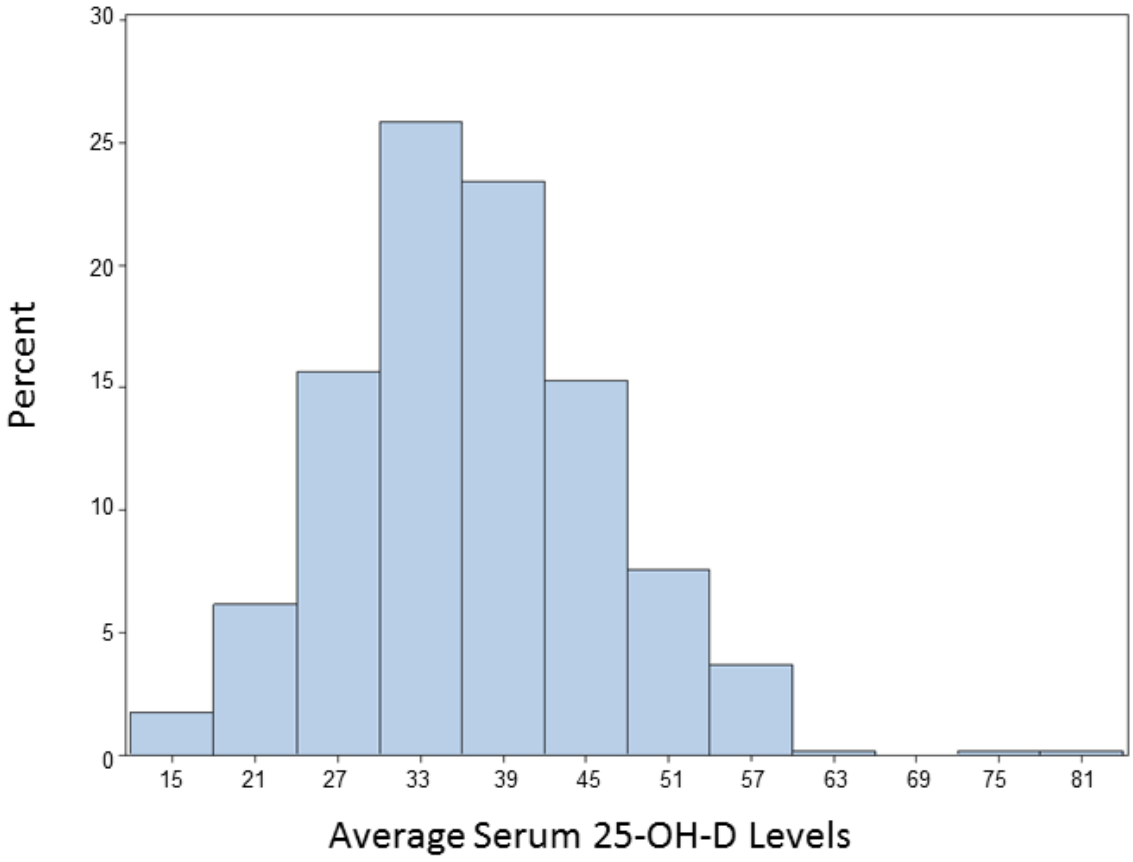
We found good correlation between the two samples (r=0.64 for baseline and seasonally adjusted samples; r=0.68 for baseline and year 1 samples within the same month). In addition, 75% of the two samples for a given participant were within 5 ng/mL of each other; ~90% were within 11 ng/ml. The average time period between the samples for the 562 participants with both a baseline and ~year 1 serum sample that was measured was 1.00 year (SD=0.17; range=0.13-2.03) which was not surprising as per the parent trial protocol participants were required to provide blood

samples at baseline (pre-randomization) and at year 1 (or at the time they came off protocol therapy).

*4.4.2.3 Distribution of Serum 25-OH-D Measurements per Study Participant (n=568)*

The calculated average measures per participant described above were the primary exposure measures used in analyses reported in subchapter E which controlled for the baseline month of serum collection and other confounding variables. In looking at these calculated average measures, the mean serum 25-OH-D concentration was 36.6 ng/mL (SD=9.5) for the 568 study participants. The distribution of these average serum measurements for study participants was approximately normal as can be seen below in Figure 3. Specifically, it was observed that the large majority of these women had serum 25-OH-D levels in the sufficient range ($n = 549 \geq$ 20ng/mL). It was noteworthy that only 3.4% of study participants were considered vitamin D deficient ($n = 19 < 20$ ng/mL).

**Figure 4.3: Distribution of Average Serum 25-OH-D Levels for Study Participants (n=568)**




### 4.4.3 Summary

The mean values for the serum 25-OH-D measurements for participants in the current study, whether they were baseline or year 1 measurements, were higher than those observed in other recent Canadian studies that have measured serum 25-OH-D in postmenopausal women [11]. The majority of women in this study have serum 25-OH-D levels in the sufficient range (> 30 ng/mL) with less than 5% of the study population having serum 25-OH-D levels that would be considered deficient (< 20 ng/mL). Thus, both the mean levels of serum 25-OH-D are higher than expected and without the range of levels expected at a population level in this study population. While the expected seasonal pattern was observed, with higher serum 25-OH-D levels in the summer months and lower levels in the winter months, this pattern was not as dramatic as that expected

based on previous literature [11]. The lowest monthly mean serum level was 32.7 ng/mL in February and is above what is considered to be adequate for health. It is also observed that a fairly high percentage (20%) of our participants had a serum 25-OH-D2 measurement which suggests either high consumption of vitamin D2 fortified foods or vitamin D2 supplement use.

## Subchapter D

### 4.5 Covariates

The following section provides a description of the characteristics of the study population including the bivariate relationship between these variables and, independently, serum 25-OH-D and percent MD. All covariates potentially related to breast density/BC were identified and evaluated as potential confounders. The relationship between each of the confounding variables identified and percent MD, adjusting for all other variables, is also provided.

#### 4.5.1 Characteristics of the Study Population

Characteristics of the study population are described in Table 4.6. Among the 568 participants, the mean age at study entry was 62 years (SD = 6.5) and the mean BMI was 28.6 (SD= 5.8), with over two-thirds of the women being overweight or obese ($\geq$ 25 Kg/m$^2$). In general, the study participants were predominantly Caucasian (97.4%), highly educated (74.4% had college education or higher) and a large percentage reported prior use of OCs (82.4%). The mean blood level of calcium among these women was 2.40 which was within normal ranges (between 2.25 - 2.5 mmol/l) with less than 5% of study participants having below normal levels at the time of randomization.

### 4.5.2 Characteristics of Study Participants by Mean Serum 25-OH-D and Percent MD

The relationship between covariates and serum 25-OH-D and, independently, percent MD was also evaluated (see Table 4.6). There was evidence of an association between serum 25-OH-D and use of bisphosphonates and also with the race of the study population, although it should be noted that less than 3% of the population were non-white. It was also observed that serum 25-OH-D was inversely related to BMI. As expected, percent MD at ≥ 3 year follow-up was inversely associated with BMI and age, although the latter did not reach statistical significance. Percent MD at ≥ 3 year follow-up was observed to be positively associated with Gail Score, parity and age at first birth, and OC use which was in the anticipated direction of effect. Conversely, percent MD at ≥ 3 year follow-up was observed to be negatively associated with HRT use; unexpectedly, those who had a prior history of HRT use had a lower percent MD at ≥ 3 year follow-up than those who had no such prior history. Similar to percent MD at ≥ 3 year follow-up, the average change in percent MD since baseline was inversely associated with age and BMI. The average change in percent MD was also associated with participant race although attention should again be paid to the fact that the population is 97% Caucasian. Lastly, the average change in percent MD over time was associated with parity and age at first birth. Specifically, women who had one full birth before the age of 24 (considered to be at lower BC risk) had a smaller change in percent MD over time, on average, compared to women who were nulliparous (considered to be at higher BC risk).

### 4.5.3 Relationship between Confounding Variables and Percent MD

As previously described in the Methods chapter, a backward elimination procedure was used to identify the set of covariates that were associated with breast density at a *p*-value of 0.15 at the time of model selection and had the potential to confound the vitamin D → MD relationships under investigation. This was done using both the continuous log transformed MD outcome

variable and, independently, the dichotomous non-transformed MD outcome variable and the final model included the covariates retained in either analysis. All analyses also included serum 25-OH-D, month of serum collection and age. For the first primary study objective with ≥ 3 year follow-up as the study outcome (N=568) the following covariates were identified as potential confounders: BMI, family history of BC, calcium, age at menarche, parity/age at first birth, smoking status, OC use and randomization arm. For the second primary study objective with average change in percent MD over time as the study outcome (N=388) the following covariates were identified as potential confounders: BMI, age at menarche, HRT use, smoking status, OC use and age at menopause.

The relationship between each of these confounding variables and percent MD at ≥ 3 year follow-up and mean change in percent MD over time (both continuous and categorical representations), adjusted for all other covariates, can be observed in Table 4.7a and 4.7b. Note that the *p*-values in these tables are reflective of the final fully adjusted model and thus may not meet the pre-specified criteria of $p < 0.15$ observed in the original backward elimination procedure. All subsequent regression analyses examining the vitamin D → breast density relationship controlled for the relevant subset of confounders.

### 4.5.4 Summary

The relationship between study population characteristics and both serum 25-OH-D and percent MD at ≥ 3 year follow-up and changes in percent MD over time were largely in the anticipated direction of effect. It was noteworthy that the population of women included in this study was predominantly Caucasian and highly educated with high BMI. It was surprising that HRT use was not associated with greater percent MD at ≥ 3 year follow-up as the literature suggests [12-14]. However, it was noted that these study participants could not have had any hormonal therapies,

including HRT, within at least 3 months prior to randomization to the parent trial. There is some evidence to suggest that the relationship between HRT use and breast density disappears after cessation of use which may explain the current findings [12]. It is also noted that women who used HRT at some time in the past were observed to have a smaller change in percent MD over time, although this was not statistically significant.

**Table 4.6: Baseline Characteristics of Study Participants According to Mean Serum 25-OH-D Levels and Percent Mammographic Density**

| Characteristic | N (%)** | Serum 25-OH-D Mean (SD) | % MD at Follow-Up Geometric Mean | N (%)*** | Mean Change % MD (SD) |
|---|---|---|---|---|---|
| Age (years) | | | | | |
| < 55 | 78 (13.7) | 35.3 (9.0) | 5.4 | 29 (7.5) | 1.3 (1.9) |
| 55-59 | 138 (24.3) | 35.7 (9.0) | 5.1 | 84 (21.6) | 1.3 (2.4) |
| 60-64 | 182 (32.0) | 37.4 (10.2) | 4.4 | 130 (33.5) | 0.5 (1.6) |
| 65-69 | 106 (18.7) | 37.6 (8.9) | 3.0 | 88 (22.7) | 0.3 (1.2) |
| >70 | 64 (11.3) | 36.0 (10.0) | 3.4 | 57 (14.7) | 0.6 (1.8) |
| *p*-value* | | 0.23 | 0.08 | | <0.01 |
| BMI ( $Kg/m^2$) | | | | | |
| <25 | 174 (30.7) | 40.1 (9.4) | 8.4 | 113 (29.2) | 1.1 (2.2) |
| ≥ 25 and < 30 | 192 (33.9) | 36.5 (9.3) | 4.5 | 135 (34.9) | 0.8 (1.9) |
| ≥ 30 and < 35 | 120(21.1) | 34.5 (9.2) | 2.8 | 89 (23.0) | 0.5 (1.3) |
| ≥ 35 | 81 (14.3 | 32.7 (8.4) | 1.6 | 50 (12.9) | 0.1 (0.9) |
| *p*-value* | | <0.01 | <0.01 | | 0.01 |
| Race | | | | | |
| White race | 553 (97.4) | 36.9 (9.4) | 4.2 | 382 (98.5) | 0.7 (1.8) |
| Other | 15 (2.6) | 26.9 (6.9) | 4.9 | 6 (1.5) | 2.6 (3.4) |
| *p*-value* | | <0.01 | 0.76 | | <0.01 |
| Education | | | | | |
| ≤ High School | 146 (25.7) | 36.2 (9.6) | 3.8 | 111 (28.6) | 0.6 (1.6) |
| College | 148 (26.1) | 35.9 (9.3) | 4.2 | 87 (22.4) | 0.6 (1.8) |
| ≥ University | 274 (48.2) | 37.2 (9.6) | 4.6 | 190 (49.0) | 0.8 (1.9) |
| *p*-value* | | 0.33 | 0.54 | | 0.56 |
| First Degree Family history of BC | | | | | |
| Yes | 316 (55.6) | 36.6 (9.4) | 4.7 | 200 (51.5) | 0.8 (2.0) |
| No | 252 (44.4) | 36.6 (9.7) | 3.7 | 188 (48.5) | 0.6 (1.6) |
| *p*-value* | | 0.95 | 0.11 | | 0.46 |

**Table 4.6 *continued*: Baseline Characteristics of Study Participants According to Mean Serum 25-OH-D Levels and Percent Mammographic Density**

| Characteristic | N (%)** | Serum 25-OH-D Mean (SD) | % MD at Follow-Up Geometric Mean | N (%)*** | Mean Change % MD (SD) |
|---|---|---|---|---|---|
| Gail Score (%) | | | | | |
| < 2.50 | 282 (49.6) | 36.5 (9.3) | 3.5 | 196 (50.5) | 0.7 (1.6) |
| ≥ 2.50 | 286 (50.4) | 36.7 (9.7) | 5.1 | 192 (49.5) | 0.7 (2.0) |
| *p*-value* | | 0.75 | 0.01 | | 0.67 |
| Calcium (mmol/L) | | | | | |
| <2.39 | 270 (47.8) | 36.0 (10.0) | 3.8 | 182 (47.0) | 0.7 (1.8) |
| ≥2.39 | 295 (52.2) | 37.2 (9.0) | 4.7 | 205 (53.0) | 0.7 (1.9) |
| *p*-value* | | 0.13 | 0.14 | | 0.91 |
| Age at Menarche (years) | | | | | |
| ≤ 11 | 126 (22.2) | 35.7 (9.4) | 3.7 | 91 (23.5) | 0.5 (1.7) |
| 12-13 | 314 (55.3) | 37.0 (9.6) | 3.9 | 212 (54.6) | 0.8 (1.8) |
| ≥14 | 128 (22.5) | 36.6 (9.5) | 5.8 | 85 (21.9) | 0.8 (1.8) |
| *p*-value* | | 0.45 | 0.07 | | 0.35 |
| Parity/Age at First Birth (years) | | | | | |
| < 24 | 206 (36.3) | 36.3 (10.1) | 2.9 | 157 (40.5) | 0.6 (1.6) |
| ≥ 24 and < 30 | 169 (29.7) | 37.1 (9.2) | 4.3 | 112 (28.9) | 0.5 (1.5) |
| ≥ 30 | 79 (13.9) | 34.9 (8.6) | 5.6 | 49 (12.6) | 1.2 (2.3) |
| Nulliparous | 114 (20.1) | 37.5 (9.5) | 7.1 | 70 (18.0) | 1.0 (2.2) |
| *p*-value* | | 0.24 | <0.01 | | 0.03 |

**Table 4.6 *continued*: Baseline Characteristics of Study Participants According to Mean Serum 25-OH-D Levels and Percent Mammographic Density**

| Characteristic | N (%)** | Serum 25-OH-D Mean (SD) | % MD at Follow-Up Geometric Mean | N (%)*** | Mean Change % MD (SD) |
|---|---|---|---|---|---|
| Age at Menopause (years) | | | | | |
| <50 | 288 (50.7) | 36.5 (9.3) | 3.9 | 201 (51.8) | 0.7 (1.7) |
| 50-54 | 219 (38.6) | 36.8 (9.8) | 5.0 | 139 (35.8) | 0.6 (2.0) |
| >55 | 61 (10.7) | 36.2 (9.4) | 3.9 | 48 (12.4) | 0.8 (1.7) |
| *p*-value* | | 0.91 | 0.24 | | 0.79 |
| HRT Use | | | | | |
| Yes | 339 (59.7) | 36.7 (9.1) | 3.7 | 248 (63.9) | 0.6 (1.6) |
| No | 229 (40.3) | 36.4 (10.0) | 5.1 | 140 (36.1) | 0.9 (2.2) |
| *p*-value* | | 0.70 | 0.03 | | 0.19 |
| OC Use | | | | | |
| Yes | 468 (82.4) | 36.7 (9.6) | 4.6 | 312 (80.4) | 0.7 (1.8) |
| No | 100 (17.6) | 36.1 (9.2) | 3.0 | 76 (19.6) | 0.8 (2.0) |
| *p*-value* | | 0.55 | 0.02 | | 0.70 |
| Smoking Status | | | | | |
| Never Smoker | 289 (50.9) | 36.7 (9.2) | 4.9 | 199 (51.3) | 0.6 (1.7) |
| Former Smoker | 246 (43.3) | 36.9 (9.9) | 3.6 | 170 (43.8) | 0.9 (2.0) |
| Current Smoker | 33 (5.8) | 33.0 (9.2) | 5.0 | 19 (4.9) | 0.7 (1.7) |
| *p*-value* | | 0.08 | 0.10 | | 0.23 |
| Prior Bisphosphonate Therapy | | | | | |
| Yes | 127 (22.4) | 39.5 (10.5) | 4.7 | 93 (24.0) | 0.7 (1.9) |
| No | 441(77.6) | 35.8 (9.0) | 4.1 | 295 (76.0) | 0.7 (1.8) |
| *p*-value* | | <0.01 | 0.43 | | 0.89 |

**Table 4.6 *continued*: Baseline Characteristics of Study Participants According to Mean Serum 25-OH-D Levels and Percent Mammographic Density**

| Characteristic | N (%)** | Serum 25-OH-D Mean (SD) | % MD at Follow-Up Geometric Mean | N (%)*** | Mean Change % MD (SD) |
|---|---|---|---|---|---|
| Prior Tamoxifen Therapy | | | | | |
| Yes | 0 (0) | --- | --- | 0 (0) | --- |
| No | 568 (100.0) | 36.6 (9.5) | 4.3 | 388 (100.0) | 0.7 (1.8) |
| *p*-value* | | n/a | n/a | | n/a |
| Randomization Arm | | | | | |
| Exemestane | 287 (50.5) | 36.6 (9.9) | 4.2 | 202 (52.1) | 0.8 (1.8) |
| Placebo | 281 (49.5) | 36.6 (9.1) | 4.3 | 186 (47.9) | 0.6 (1.8) |
| *p*-value* | | 0.95 | 0.78 | | 0.44 |

* *p*-value from Anova

** The relationship between each covariate and serum 25-OH-D and, independently, percent MD at ≥ 3 year follow- up among the 568 eligible participants

*** The relationship between each covariate and the mean change in percent MD over time among the 388 eligible participants

**Table 4.7a: Multivariate Association between Covariates and Continuous Percent Mammographic Density**

| Characteristic | N (%)** | % MD at Follow-Up Geometric Mean** | N (%)*** | Mean Change % MD*** |
|---|---|---|---|---|
| Age (years) | | | | |
| < 55 | 78 (13.7) | 4.1 | 29 (7.5) | 1.3 |
| 55-59 | 138 (24.3) | 3.9 | 84 (21.6) | 1.3 |
| 60-64 | 182 (32.0) | 4.0 | 130 (33.5) | 0.5 |
| 65-69 | 106 (18.7) | 3.3 | 88 (22.7) | 0.3 |
| >70 | 64 (11.3) | 3.5 | 57 (14.7) | 0.4 |
| *p*-value* | | 0.85 | | <0.01 |
| BMI ($Kg/m^2$) | | | | |
| <25 | 174 (30.7) | 7.9 | 113 (29.2) | 1.2 |
| ≥ 25 and < 30 | 192 (33.9) | 4.9 | 135 (34.9) | 1.0 |
| ≥ 30 and < 35 | 120(21.1) | 3.0 | 89 (23.0) | 0.6 |
| ≥ 35 | 81 (14.3) | 1.7 | 50 (12.9) | 0.2 |
| *p*-value* | | <0.01 | | <0.01 |
| First Degree Family history of BC | | | | |
| Yes | 316 (55.6) | 4.1 | | |
| No | 252 (44.4) | 3.4 | | |
| *p*-value* | | 0.21 | | |
| Calcium (mmol/L) | | | | |
| <2.39 | 270 (47.8) | 3.4 | | |
| ≥2.39 | 295 (52.2) | 4.1 | | |
| *p*-value* | | 0.23 | | |
| Age at Menarche (years) | | | | |
| ≤ 11 | 126 (22.2) | 3.5 | 91 (23.5) | 0.5 |
| 12-13 | 314 (55.3) | 3.1 | 212 (54.6) | 0.9 |
| ≥14 | 128 (22.5) | 4.7 | 85 (21.9) | 0.9 |
| *p*-value* | | 0.04 | | 0.33 |

**Table 4.7a *continued*: Multivariate Association between Covariates and Continuous Percent Mammographic Density**

| Characteristic | N (%)** | % MD at Follow-Up Geometric Mean** | N (%)*** | Mean Change % MD*** |
|---|---|---|---|---|
| Parity/Age at First Birth (years) | | | | |
| < 24 | 206 (36.3) | 2.6 | | |
| ≥ 24 ad < 30 | 169 (29.7) | 3.3 | | |
| ≥ 30 | 79 (13.9) | 3.7 | | |
| Nulliparous | 114 (20.1) | 6.1 | | |
| *p*-value* | | <0.01 | | |
| Age at Menopause (years) | | | | |
| <50 | | | 201 (51.8) | 0.7 |
| 50-54 | | | 139 (35.8) | 0.5 |
| >55 | | | 48 (12.4) | 1.1 |
| *p*-value* | | | | 0.17 |
| HRT Use | | | | |
| Yes | | | 248 (63.9) | 0.8 |
| No | | | 140 (36.1) | 0.7 |
| *p*-value* | | | | 0.94 |
| OC Use | | | | |
| Yes | 468 (82.4) | 4.8 | 312 (80.4) | 0.6 |
| No | 100 (17.6) | 2.9 | 76 (19.6) | 0.9 |
| *p*-value* | | <0.01 | | 0.14 |

**Table 4.7a *continued*: Multivariate Association between Covariates and Continuous Percent Mammographic Density**

| Characteristic | N (%)** | % MD at Follow-Up Geometric Mean** | N (%)*** | Mean Change % MD*** |
|---|---|---|---|---|
| Smoking Status | | | | |
| Never Smoker | 289 (50.9) | 3.9 | 199 (51.3) | 0.6 |
| Former Smoker | 246 (43.3) | 3.1 | 170 (43.8) | 1.1 |
| Current Smoker | 33 (5.8) | 4.2 | 19 (4.9) | 0.6 |
| *p*-value* | | 0.22 | | 0.05 |
| Randomization Arm | | | | |
| Exemestane | 287 (50.5) | 3.5 | | |
| Placebo | 281 (49.5) | 3.9 | | |
| *p*-value* | | 0.39 | | |

* F-test

** Adjusted for serum 25-OH-D, month of serum collection, age and other variables identified from backward elimination (N=568)

*** Adjusted for serum 25-OH-D, month of serum collection, age and other variables identified from backward elimination (N=388)

**Table 4.7b: Multivariate Association between Covariates and Dichotomous Percent Mammographic Density**

| | MD (N)** | | | Average Change in % MD (N)*** | | |
|---|---|---|---|---|---|---|
| Characteristic | < 25% | ≥25% | OR (95% CI) | Decrease | No change/Increase | OR (95% CI) |
| Age (years) | | | | | | |
| < 55 | 64 | 14 | 1.9 (0.5- 8.0) | 24 | 5 | 0.4 (0.1-1.2) |
| 55-59 | 116 | 22 | 1.5 (0.4-5.6) | 53 | 31 | 1.0 (0.4-2.3) |
| 60-64 | 152 | 30 | 2.1 (0.6-7.7) | 86 | 44 | 1.0 (0.4-2.2) |
| 65-69 | 99 | 7 | 1.2 (0.3-5.2) | 58 | 30 | 1.0 (0.4-2.2) |
| >70 | 60 | 4 | 1.0 | 37 | 20 | 1.0 |
| p-value* | | | 0.65 | | | 0.45 |

**Table 4.7b *continued*: Multivariate Association between Covariates and Dichotomous Percent Mammographic Density**

| Characteristic | MD (N)** | | OR (95% CI) | Average Change in % MD (N)*** | | OR (95% CI) |
|---|---|---|---|---|---|---|
| | < 25% | ≥25% | | Decrease | No change/Increase | |
| BMI (Kg/m$^2$) | | | | | | |
| <25 | 130 | 44 | 10.9 (4.4-26.8) | 74 | 39 | 1.0 (0.5-1.7) |
| ≥ 25 and < 30 | 167 | 25 | 5.0 (2.0-12.4) | 89 | 46 | 0.9 (0.5-1.6) |
| ≥ 30 | 193 | 8 | 1.0 | 95 | 44 | 1.0 |
| p-value* | | | <0.01 | | | 0.93 |
| First Degree Family history of BC | | | | | | |
| No | 224 | 28 | 0.7 (0.4-1.4) | | | |
| Yes | 267 | 49 | 1.0 | | | |
| p-value* | | | 0.32 | | | |
| Calcium (mmol/L) | | | | | | |
| <2.39 | 240 | 30 | 0.6 (0.3-1.0) | | | |
| ≥2.39 | 249 | 46 | 1.0 | | | |
| p-value* | | | 0.06 | | | |
| Age at Menarche (years) | | | | | | |
| ≤ 11 | 110 | 16 | 0.7 (0.3-1.6) | 52 | 39 | 1.6 (0.8-3.2) |
| 12-13 | 275 | 39 | 0.6 (0.3-1.1) | 148 | 64 | 0.7 (0.4-1.3) |
| ≥14 | 106 | 22 | 1.0 | 58 | 27 | 1.0 |
| p-value* | | | 0.24 | | | 0.03 |
| Parity/Age at First Birth (years) | | | | | | |
| < 24 | 186 | 20 | 0.2 (0.1-0.5) | | | |
| ≥ 24 and < 30 | 154 | 15 | 0.2 (0.1-0.4) | | | |
| ≥ 30 | 66 | 13 | 0.3 (0.1-0.7) | | | |
| Nulliparous | 85 | 29 | 1.0 | | | |
| p-value* | | | <0.01 | | | |

**Table 4.7b *continued*: Multivariate Association between Covariates and Dichotomous Percent Mammographic Density**

| Characteristic | MD (N)** | | OR (95% CI) | Average Change in % MD (N)*** | | OR (95% CI) |
|---|---|---|---|---|---|---|
| | < 25% | ≥25% | | Decrease | No change/Increase | |
| Age at Menopause (years) | | | | | | |
| <50 | | | | 141 | 60 | 1.3 (0.6-2.8) |
| 50-54 | | | | 84 | 55 | 1.9 (0.9-4.1) |
| >55 | | | | 33 | 15 | 1.0 |
| p-value* | | | | | | 0.17 |
| HRT Use | | | | | | |
| Yes | | | | 88 | 52 | 1.4 (0.9-2.3) |
| No | | | | 170 | 78 | 1.0 |
| p-value* | | | | | | 0.18 |
| OC Use | | | | | | |
| No | 94 | 6 | 0.2 (0.1-0.6) | 50 | 26 | 0.8 (0.4-1.6) |
| Yes | 397 | 71 | 1.0 | 208 | 104 | 1.0 |
| p-value* | | | <0.1 | | | 0.59 |
| Smoking Status | | | | | | |
| Never Smoker | 239 | 50 | 1.8 (0.5-6.1) | 127 | 72 | 1.5 (0.5-4.6) |
| Former Smoker | 224 | 22 | 0.8 (0.2-2.8) | 117 | 53 | 1.1 (0.3-3.4) |
| Current Smoker | 28 | 5 | 1.0 | 14 | 5 | 1.0 |
| p-value* | | | 0.04 | | | 0.39 |
| Randomization Arm | | | | | | |
| Placebo | 239 | 42 | 1.6 (0.9-2.8) | | | |
| Exemestane | 252 | 35 | 1.0 | | | |
| p-value* | | | 0.14 | | | |

* *p*-value from logistic regression

** Adjusted for serum 25-OH-D, month of serum collection, age and other variables identified from backward elimination (N=568)

*** Adjusted for serum 25-OH-D, month of serum collection, age and other variables identified from backward elimination (N=388)

# Subchapter E

## 4.6 Results of the Main Analyses

This subchapter provides the results of both the primary and secondary objectives of this thesis project and is divided into two main parts:

1. The first section presents the results of the first primary objective which was to examine the relationship between baseline serum 25-OH-D and percent MD at ≥ 3 year follow-up among the study population of postmenopausal women. Results of the secondary objectives as related to this first primary objective, namely, evaluation of the interactions with exemestane, calcium and genetic polymorphisms, are also presented.

2. The second section presents the results of the second primary objective which was to examine the relationship between baseline serum 25-OH-D and average changes in percent MD over time. The results for secondary objectives related to the exploration of interactions between serum 25-OH-D and exemestane and, independently, calcium on the relationship with changes in percent MD are presented.

Both the outcome of percent MD at ≥ 3 year follow-up and the average change in percent MD over time were analyzed as both continuous and dichotomous measures. For brevity and clarity all analyses evaluating interactions on the vitamin D → percent MD outcome were conducted using a dichotomous representation of outcomes.

### 4.6.1 Primary Objective 1: Serum 25-OH-D and Percent MD at ≥ 3 Year Follow-Up

*4.6.1.1 Analyses Using Percent Mammographic Density as a Continuous Outcome Measure*

In order to evaluate the first primary objective that examines the relationship between baseline serum 25-OH-D and percent MD at ≥ 3 year follow-up least squares regression was used. As described in the previous subchapter, all multivariable regression models used to estimate the effect of serum 25-OH-D on percent MD at follow-up were adjusted for age, month of serum sampling, BMI, family history of BC, calcium, age at menarche, parity/age at first birth, smoking status, OC use and randomization arm. Continuous measures of serum 25-OH-D were not observed to have a linear relationship with percent MD at ≥ 3 year follow-up. Thus, the average measures of serum 25-OH-D were categorized as follows: (1) < 25 ng/mL; (2) 25-34 ng/mL; (3) 35-44.9 ng/mL; and (4) ≥ 45 ng/mL. The β coefficients and geometric means from the least squares regression are presented in Table 4.8. The coefficients presented represent the difference in percent MD between those with lowest serum 25-OH-D compared with those with highest serum 25-OH-D. It was observed that the adjusted point estimates of effect for each category of serum 25-OH-D were close to the null value and none were statistically significant. Adjusted geometric mean percent MDs by increasing categories of serum 25-OH-D were 4.11%, 3.14%, 3.45% and 4.31% respectively. The overall *p*-value for the effect of serum 25-OH-D on log transformed percent MD in this analysis was 0.36.

**Table 4.8: Relationship between Serum 25-OH-D and Percent MD at ≥ 3 Year Follow-Up**

| Serum 25-OH-D (ng/mL) | N | Crude Analysis | | | Adjusted Analysis* | | |
|---|---|---|---|---|---|---|---|
| | | Coefficient** | Mean % MD *** | *p*-value | Coefficient** | Adjusted Mean % MD*** | *p*-value |
| 0-24.9 | 55 | -0.51 | 3.97 | 0.07 | -0.05 | 4.11 | 0.86 |
| 25-34.9 | 196 | -0.76 | 3.10 | **<0.01** | -0.31 | 3.14 | 0.11 |
| 35-44.9 | 216 | -0.36 | 4.66 | 0.08 | -0.22 | 3.45 | 0.25 |
| ≥ 45 | 101 | Referent | 6.62 | | Referent | 4.31 | |
| Total N | 568 | *Overall p-value = <**0.01******* | | | *Overall p-value = 0.36 ***** | | |

* Adjusted in linear regression model for age, month of serum sampling, BMI, family history of BC, calcium, age at menarche, parity/age at first birth, smoking status, OC use and randomization arm.

** The outcome was log transformed; the difference in percent MD compared to the referent can be calculated using the coefficients above as follows: $[(e^{\beta 1} - 1) * 100]$

*** Geometric means

**** F-test

*4.6.1.2 Analyses Using Percent Mammographic Density as a Dichotomous Outcome Measure*

A logistic regression model, with reported ORs, was also used to estimate the effect of serum 25-OH-D categories between women classified as low density (<25%) and higher density (≥ 25%) at ≥ 3 year follow-up. In first looking at the relationship between important covariates and this dichotomous percent density outcome it was again noted that there was a strong relationship between BMI and percent MD. In fact, women in the highest quartile of BMI (i.e. ≥ 35 Kg/m$^2$) were observed to never have percent MD ≥ 25%. Thus, BMI was categorized into the following tertiles for all logistic regression analyses in order to evaluate study objectives using a dichotomous percent density outcome: (1) 'Normal' (< 25 Kg/m$^2$); (2) 'Overweight' (≥ 25 Kg/m$^2$ and < 30 Kg/m$^2$); and (3) 'Obese' (≥ 30 Kg/m$^2$).

All multivariable regression models used to estimate the effect of serum 25-OH-D on percent MD at follow-up were adjusted for age, month of serum sampling, BMI, family history of BC,

calcium, age at menarche, parity/age at first birth, smoking status, OC use and randomization arm. While the results for the adjusted analysis were in the anticipated direction of effect (with women with lower serum 25-OH-D having higher percent MD) the ORs associated with each category of serum 25-OH-D in comparison to those with high serum 25-OH-D were not statistically significantly different from the null value. The overall *p*-value was 0.37 after adjusting for all confounding variables (see Table 4.9).

It was noted that the point estimates for the crude analysis compared with the adjusted analysis were in the opposite direction to one another. It was hypothesized that this difference in estimates was due either to missing values for covariates included in the adjusted analysis that impacted upon already limited cell size numbers or was the result of substantial confounding by one or more variables on the underlying vitamin D → breast density relationship. To evaluate this further, the crude analysis was restricted to the same number of participants included in the multivariate analysis with no missing data (N=564). The four participants excluded from this subsequent crude analysis did not impact on the cell sizes with particularly small numbers and it was observed that the ORs did not significantly change from the original crude analysis when these individuals were excluded (see Appendix 1).

To evaluate which variables had the largest confounding effects on the underlying relationship we examined whether the adjusted ORs changed by more than 10% between models with and without each variable [15]. It was observed that BMI and, to a lesser extent, month of serum sampling were strong confounders of the vitamin D → breast density relationship in this study. Specifically, women with higher BMI were observed to have lower serum 25-OH-D and, independently, women with higher BMI were observed to have lower percent MD at ≥ 3 year follow-up. This confounding had the effect of dramatically reducing the crude point estimates in compared with the fully adjusted analysis.

**Table 4.9: Relationship between Serum 25-OH-D and ≥ 25 Percent Mammographic Density at ≥ 3 Year Follow-Up**

| Serum 25-OH-D (ng/mL) | Mammographic Density | | Crude Analysis | | Adjusted Analysis* | |
|---|---|---|---|---|---|---|
| | < 25% | ≥ 25% | Odds Ratio* | 95% CI | Odds Ratio* | 95% CI |
| 0-24.9 | 48 | 7 | 0.59 | (0.23–1.50) | 1.68 | (0.54-5.25) |
| 25-34.9 | 174 | 22 | 0.51 | (0.27-0.99) | 1.00 | (0.45-2.26) |
| 35-44.9 | 188 | 28 | 0.60 | (0.32-1.13) | 0.69 | (0.33-1.45) |
| ≥ 45 | 81 | 20 | 1.00 | (referent) | 1.00 | (referent) |
| Total N | 491 | 77 | *Overall p-value = 0.23* | | *Overall p-value =0.37* | |

* ORs from logistic regression adjusted for age, month of serum sampling, BMI, family history of BC, calcium, age at menarche, parity/age at first birth, smoking status, OC use and randomization arm.

*4.6.1.3 Sensitivity Analysis to Evaluate the Effect of Mammogram Format*

Given the mixture of film and digital images received for study participants it was important to determine whether there was a relationship between mammogram format and the primary outcome measure of percent MD and, further, whether the relationship between serum 25-OH-D and percent MD differed by mammogram format. A relationship between mammogram format and log transformed percent MD at ≥ 3 year follow-up adjusted for potential confounders including age and BMI was not observed (p=0.76) (see Table 4.10). Adjusted geometric mean percent MDs by format were 3.42% for digital images and 3.56% for film images. To evaluate whether the relationship between serum 25-OH-D and percent MD differed by image format, serum 25-OH-D levels were dichotomized as < 35 ng/mL vs. ≥ 35 mg/mL based on the distribution of this exposure and to ensure a sufficient sample size for analyses purposes. There was no effect modification by mammogram format on the relationship between serum 25-OH-D and percent MD at ≥ 3 year follow-up observed (*p*-value for the interaction term = 0.52) and no

effect of serum 25-OH-D on MD in either the film or digital strata (see Table 4.11). Thus, all analyses looking at the relationship between serum 25-OH-D and percent MD at ≥ 3 year follow-up include all eligible mammograms regardless of format.

**Table 4.10: Relationship between Image Format and Percent MD at ≥ 3 Year Follow-Up**

| Format | N | Coefficient * | Adjusted Mean % MD** | *p*-value |
|---|---|---|---|---|
| Digital | 242 | -0.04 | 3.42 | 0.76*** |
| Film | 326 | Referent | 3.56 | |

* Adjusted in linear regression model for age, month of serum sampling, BMI, family history of BC, calcium, age at menarche, parity/age at first birth, smoking status, OC use and randomization arm. The outcome was log transformed; the difference in percent MD compared to the referent can be calculated using the coefficient above as follows: $[(e^{\beta 1} - 1) * 100]$

** Geometric means

*** F-test

**Table 4.11: Effect Modification by Image Format on the Relationship between Serum 25-OH-D and Percent MD at ≥ 3 Year Follow-Up**

| | FILM Mammographic density | | | DIGITAL Mammographic density | | |
|---|---|---|---|---|---|---|
| **Serum 25-OH-D** | **< 25%** | **≥ 25%** | **OR (95% CI)*** | **< 25%** | **≥ 25%** | **OR (95% CI)*** |
| < 35 ng/mL | 130 | 16 | 1.22 (0.55 – 2.70) | 92 | 13 | 1.83 (0.71–4.72) |
| ≥ 35 ng/ml | 150 | 30 | 1.0 (referent) | 119 | 18 | 1.0 (referent) |
| Total N | 280 | 46 | | 211 | 31 | |
| | | | | | | *p-value for interaction = 0.52* |

* OR from logistic regression adjusted for age, month of serum sampling, BMI, family history of BC, calcium, age at menarche, parity/age at first birth, smoking status, OC use and randomization arm.

*4.6.1.4 Secondary Objective 1: Effect Modification by Exemestane*

It was of clinical interest to examine whether the relationship between serum 25-OH-D and percent MD at ≥ 3 year follow-up differed by the randomization arm of the trial (exemestane vs. placebo). In this analysis, serum 25-OH-D levels were dichotomized as < 35 ng/mL vs. ≥ 35

mg/mL based on the distribution of this exposure and to ensure a sufficient sample size for analyses purposes. As stated in the Methods Chapter, if the relationship between serum 25-OH-D and percent MD at ≥ 3 year follow-up was observed to be different by trial arm the results of the first primary objective would be reported independently. While the interaction between serum 25-OH-D and randomization arm for percent MD was not statistically significant (*p*-value for interaction = 0.09), the data suggest a differential relationship between trial arms. In looking at the specific effect measures obtained in each stratum, the reported ORs appear to be qualitatively different (see Table 4.12). While there does not seem to be any effect of serum 25-OH-D on percent MD in the exemestane stratum the data indicates that women with lower serum 25-OH-D levels on the placebo arm of the trial were more likely to have higher percent MD (≥ 25%) compared with women with higher serum 25-OH-D levels [OR=2.28; 95% CI: 1.01-5.16)].

**Table 4.12: Effect Modification by Randomization Arm on the Relationship between Serum 25-OH-D and Percent MD at ≥ 3 Year Follow-Up**

| | **Placebo Mammographic density** | | | | **Exemestane Mammographic density** | | | |
|---|---|---|---|---|---|---|---|---|
| **Serum 25-OH-D (ng/mL)** | **< 25%** | **≥ 25%** | **Crude OR (95% CI)** | **Adjusted OR (95% CI)*** | **< 25%** | **≥ 25%** | **Crude OR (95% CI)** | **Adjusted OR (95% CI)*** |
| < 35 | 104 | 19 | 1.07 (0.56-2.07) | **2.28 (1.01-5.16)** | 118 | 10 | 0.45 (0.21-0.99) | 0.79 (0.31-2.01) |
| ≥ 35 | 135 | 23 | 1.0 (referent) | 1.0 (referent) | 134 | 25 | 1.0 (referent) | 1.0 (referent) |
| Total N | 239 | 42 | | | 252 | 35 | | |
| | | | | | | | | *p-value for interaction** = 0.09* |

* OR adjusted for age, month of sampling, BMI, family history of BC, calcium, age at menarche, parity/age at first birth, smoking status and OC use

** From fully adjusted model

If serum 25-OH-D is associated with MD in this study population that relationship may only be evident in women with higher estrogen levels. Given this hypothesis it was of interest to explore the interaction between serum 25-OH-D and other hormonally related variables including OC use, HRT use, age at menarche, parity and age at menopause. There was no evidence of an interaction

between serum 25-OH-D and any of these hormonal variables for percent MD observed with the exception of parity (*p*-value for interaction = <0.01). Contrary to what was expected, among women who were parous the effect of lower serum 25-OH-D on having higher percent MD was 2.73 (95% CI: 1.28-5.79) and among women who were nulliparous the effect of lower serum 25-OH-D on having higher percent MD was 0.31 (95% CI: 0.09-1.06).

*4.6.1.5 Secondary Objective 3: Effect Modification by Calcium*

As previously reviewed, vitamin D and calcium are metabolically interrelated. Thus, there was interest in evaluating whether there was any interaction between calcium and serum 25-OH-D on percent MD. It was hypothesized that the association between lower levels of serum 25-OH-D and higher percent MD, if observed, would be strengthened in the presence of lower calcium levels. Therefore, the relationship between serum 25-OH-D and percent MD at ≥ 3 year follow-up was examined among those with lower (below the median) and higher (above the median) levels of calcium. Both serum 25-OH-D and calcium levels were dichotomized based on their underlying distributions and to ensure a sufficient sample size for analyses purposes. There was no evidence of an interaction between serum 25-OH-D and calcium for percent MD at ≥ 3 year follow-up observed (*p*-value for the interaction term = 0.98) and no effect of serum 25-OH-D on MD in either stratum of calcium (see Table 4.13).

**Table 4.13: Effect Modification by Calcium on the Relationship between Serum 25-OH-D and Percent MD at ≥ 3 Year Follow-Up**

| Serum 25-OH-D (ng/mL) | Lower Calcium (below median) Mammographic density | | | | Higher calcium (above median) Mammographic density | | | |
|---|---|---|---|---|---|---|---|---|
| | < 25% | ≥ 25% | Crude OR (95% CI) | Adjusted OR (95% CI)* | < 25% | ≥ 25% | Crude OR (95% CI) | Adjusted OR (95% CI)* |
| < 35 | 117 | 12 | 0.70 (0.32-1.52) | 1.43 (0.57-3.58) | 105 | 16 | 0.73 (0.38-1.41) | 1.46 (0.65-3.26) |
| ≥ 35 | 123 | 18 | 1.0 (referent) | 1.0 (referent) | 144 | 30 | 1.0 (referent) | 1.0 (referent) |
| Total N | 240 | 30 | | | 249 | 46 | | |
| *p-value for interaction** = 0.98* | | | | | | | | |

* OR adjusted for age, month of sampling, BMI, family history of BC, age at menarche, parity/age at first birth, smoking status, OC use and treatment arm.

** From fully adjusted model

*4.6.1.6 Secondary Objective 4: Effect Modification by Genetic Polymorphisms*

It was of interest to examine the association between two vitamin D-related genetic polymorphisms and percent MD at ≥ 3 year follow-up. Specifically, the association between Fok1, a polymorphism in the gene encoding the VDR protein, and percent MD and between a polymorphism in the vitamin D metabolism gene CYP24A1 and percent MD were evaluated. These polymorphisms were also evaluated for possible interactions with serum 25-OH-D and percent MD. Genotyping data for a total of 550 of the 568 study participants were provided which included results for SNP rs2181874 (CYP24A1) and for SNP rs2228570 (Fok1) and the allele and genotype frequencies for each polymorphism. The genotype frequencies of the relevant polymorphisms in the Fok1 VDR (*ff* vs. *Ff* vs. *FF*) and CYP24A1 (GG vs. GA vs. AA) genes were evaluated for their association with percent MD as well as for possible interactions with serum 25-OH-D and percent MD. However, the low frequency of some of these genotypes in the study population resulted in very small cell sizes particularly in the analysis looking at the association between genotypes in women with lower (< 25%) compared with higher (≥ 25%) percent MD. Thus, results from these analyses are not provided given the inadequate power available to detect associations. Instead, results of the analyses that grouped each genetic

polymorphism into a dichotomous variable are provided below. As a reminder, for Fok1 the recessive model (ff vs. Ff + FF) of allele frequency was evaluated for an association with percent MD based on known functionality of this polymorphism. Further, for CYP24A1, the rare homozygous genotype was combined with the heterozygote (GG vs. GA & AA) and evaluated for an association with percent MD.

Least squares regression was used to evaluate the relationship between each of the vitamin D-related polymorphisms and percent MD at ≥ 3 year follow-up controlling for age, month of serum sampling, BMI, family history of BC, calcium, age at menarche, parity/age at first birth, smoking status, OC use and randomization arm. The association between each of these polymorphisms and percent MD are presented in Table 4.14. A relationship between the SNP rs2181874 (CYP24A1) and log transformed percent MD at ≥ 3 year follow-up adjusted for potential confounders was not observed (p=0.36) nor was a relationship between the SNP rs2228570 (Fok1) and log transformed percent MD at ≥ 3 year follow-up observed (p=0.68). For the CYP24A1 SNP, the adjusted geometric mean percent MDs by allele were 3.78% for study participants with the GA&AA allele and 3.33% for study participants with GG. Further, for the Fok1 SNP, adjusted geometric mean percent MDs by allele were 3.29% for ff and 3.54% for Ff+FF combined.

**Table 4.14: Relationship between Vitamin D Related Polymorphisms and Percent MD at ≥ 3 Year Follow-Up**

| Vitamin D-Related SNPs | N (=550) | Crude Analysis | | | Adjusted Analysis* | | |
|---|---|---|---|---|---|---|---|
| | | Coefficient** | Mean % MD*** | *p*-value | Coefficient** | Adjusted Mean % MD*** | *p*-value |
| **CYP24A1** | | | | | | | |
| GA+AA | 246 | 0.04 | 4.31 | 0.78**** | 0.12 | 3.78 | 0.36**** |
| GG | 304 | Referent | 4.14 | | Referent | 3.33 | |
| **Fok1** | | | | | | | |
| ff | 97 | -0.14 | 3.78 | 0.48**** | -0.07 | 3.29 | 0.68**** |
| Ff + FF | 453 | Referent | 4.31 | | Referent | 3.54 | |

* Adjusted in linear regression model for age, month of serum sampling, BMI, family history of BC, calcium, age at menarche, parity/age at first birth, smoking status, OC use and randomization arm.

** The outcome was log transformed; the difference in percent MD compared to the referent can be calculated using the coefficients above as follows: $[(e^{\beta 1} - 1) * 100]$

*** Geometric means

**** F-test

A main analysis using logistic regression, with reported ORs, was also conducted to evaluate the relationship between the vitamin D-related SNPs in women with low (<25%) vs. higher (≥ 25%) percent MD (see Table 4.15). In looking at the CYP24A1 polymorphism it was observed that women with the GA or AA genotype combined were more likely to have higher percent MD compared with women with the GG genotype although this finding was not statistically significant (p= 0.33). For the Fok1 polymorphism of interest, study participants with the ff genotype were more likely to have higher percent MD compared with women with the Ff or FF genotype but again this result did not reach statistical significance (p=0.47).

**Table 4.15: Relationship between Vitamin D-Related Polymorphisms and ≥ 25 Percent Mammographic Density at ≥ 3 Year Follow-Up**

| Vitamin D-Related SNPs | Mammographic Density | | Crude Analysis | | Adjusted Analysis* | |
|---|---|---|---|---|---|---|
| | < 25% | ≥ 25% | Odds Ratio | 95% CI | Odds Ratio | 95% CI |
| **CYP24A1** | | | | | | |
| GA+AA | 209 | 37 | 1.32 | (0.81-2.16) | 1.33 | (0.75-2.36) |
| GG | 268 | 36 | 1.00 | (referent) | 1.00 | (referent) |
| **Total N** | 477 | 73 | *Overall p-value =0.27* | | *Overall p-value =0.33* | |
| **Fok1** | | | | | | |
| ff | 84 | 13 | 1.01 | (0.53-1.93) | 1.33 | (0.62-2.84) |
| Ff + FF | 393 | 60 | 1.00 | (referent) | 1.00 | (referent) |
| **Total N** | 477 | 73 | *Overall p-value = 0.97* | | *Overall p-value =0.47* | |

* ORs from logistic regression adjusted for age, month of serum sampling, BMI, family history of BC, calcium, age at menarche, parity/age at first birth, smoking status, OC use and randomization arm.

These polymorphisms were also evaluated for possible interactions with serum 25-OH-D and percent MD. In this analysis, serum 25-OH-D levels were dichotomized as < 35 ng/mL vs. ≥ 35 mg/mL based on the distribution of this exposure and to ensure a sufficient sample size for analyses purposes. Recall also that a dichotomous representation of the outcome was utilized for analyses evaluating interactions on the vitamin D → percent MD relationship. There was no effect modification by either vitamin D-related SNPs on the relationship between serum 25-OH-D and percent MD at ≥ 3 year follow-up observed (CYP24A1: *p*-value for the interaction term = 0.98; Fok1: *p*-value for the interaction term = 0.36) (see Table 4.16). While the interaction between serum 25-OH-D and Fok1 for percent MD at ≥ 3 year follow-up was not statistically significant, the stratum specific effect measures appear to be qualitatively different.

**Table 4.16: Effect Modification by Vitamin D-Related Genetic Polymorphisms on the Relationship between Serum 25-OH-D and Percent MD at ≥ 3 Year Follow-Up**

| | CYP24A1 (GA+AA) Mammographic density | | | CYP24A1 (GG) Mammographic density | | |
|---|---|---|---|---|---|---|
| **Serum 25-OH-D** | **< 25%** | **≥ 25%** | **OR (95% CI)** | **< 25%** | **≥ 25%** | **OR (95% CI)** |
| < 35 ng/mL | 99 | 15 | 1.56 (0.65-3.77) | 114 | 13 | 1.54 (0.64-3.71) |
| ≥ 35 ng/ml | 110 | 22 | 1.0 (referent) | 154 | 23 | 1.0 (referent) |
| Total N | 209 | 37 | | 268 | 36 | |
| | | | | | | *p-value for interaction =0.98* |
| | **Fok1 (ff) Mammographic density** | | | **Fok1 (Ff + FF) Mammographic density** | | |
| **Serum 25-OH-D** | **< 25%** | **≥ 25%** | **OR (95% CI)** | **< 25%** | **≥ 25%** | **OR (95% CI)** |
| < 35 ng/mL | 39 | 4 | 0.84 (0.19-3.70) | 174 | 24 | 1.78 (0.90-3.54) |
| ≥ 35 ng/ml | 45 | 9 | 1.0 (referent) | 219 | 36 | 1.0 (referent) |
| Total N | 84 | 13 | | 393 | 60 | |
| | | | | | | *p-value for interaction =0.36* |

* OR from logistic regression adjusted for age, month of serum sampling, BMI, family history of BC, calcium, age at menarche, parity/age at first birth, smoking status, OC use and randomization arm.

*4.6.1.7 Sensitivity Analysis Restricted to Caucasian Study Participants*

Race and ethnicity have been associated with vitamin D deficiency and, independently, with BC risk [16-19]. Specifically, African American and Hispanic women, in particular, have been observed to be at greater BC risk [19]. Differences in MD have also been observed among different racial and ethnic groups [20,21]. In the current study, race and ethnicity could not be adequately assessed as potential confounding variables due to insufficient variability among study participants. Specifically, 97% (n=553) of women reported their race to be Caucasian and 96% (n=546) of women indicated their ethnicity to be non-Hispanic. To assess the robustness of the results across all study participants reported in this subchapter a sensitivity analysis was conducted that restricted the analysis to only women of Caucasian race and non- Hispanic ethnicity (n=533). The results of this sensitivity analysis were comparable with those of the larger cohort and thus are not reported in any greater detail.

*4.6.1.8 Summary of Results Evaluating Serum 25-OH-D and Percent MD at ≥ 3 Year Follow-Up*

Overall, the results reported in this section do not support a relationship between serum 25-OH-D and percent MD at ≥ 3 year follow-up in this population of women. However, the ORs observed from the logistic regression analysis were in the anticipated direction of effect. Specifically, those women with lower serum 25-OH-D levels were more likely to have higher percent MD compared with women with higher serum 25-OH-D levels although this relationship was not statistically significant. Sensitivity analyses confirmed the appropriateness of conducting these analyses using both film-based and digital-based images together which has not been extensively reported on in the literature. The evaluation of the interactions with exemestane, calcium and genetic polymorphisms on the relationship between serum 25-OH-D and percent MD were not statistically significant but sample size issues for these analyses raise concerns about the adequacy of study power which affect the interpretation of results. The results obtained from the analysis looking at the interaction between randomization arm of the trial and serum 25-OH-D on percent MD were of interest. Among women on the placebo arm of the trial, those with lower serum 25-OH-D levels were more likely to have higher percent MD than those with higher serum 25-OH-D; among women on the exemestane arm of the trial no such relationship was observed. Lastly, while the results of the analyses examining the association between vitamin D- related genetic polymorphisms and percent MD at ≥ 3 year follow-up were not statistically significant they were consistent with the direction of effect observed in previous studies evaluating BC risk [22-25].

### 4.6.2 Primary Objective 2: Serum 25-OH-D and Change in Percent MD Over Time

*4.6.2.1 Analyses Using Changes in Percent MD as a Continuous Outcome Measure*

There were 388 participants who had two mammograms in the same format: 258 participants were observed to have a decrease in percent MD over the course of the follow-up period and 130

either had no change (n=6) or an increase (n=124) in percent MD over time. The average change in breast density variable used in regression analyses was defined as [(baseline percent MD - follow-up percent MD) / years of follow-up]. A positive value was consistent with normal breast density etiology; that is, breast density decreased over time with greater decreases hypothesized to reduce BC risk. A negative value was consistent with increased breast density over time which was not what was expected based on the natural history of breast density and represents a 'bad' event. All regression models used to estimate the effect of serum 25-OH-D on the average change in percent MD over time were adjusted for the variables that were associated with changes in breast density at a *p*-value of 0.15 using a backward elimination procedure as previously described. Specifically, the regression models controlled for the effects of age, month of serum sampling, BMI, age at menarche, HRT use, smoking status, OC use and age at menopause. Continuous measures of serum 25-OH-D were not observed to have a linear relationship with average changes in percent MD over time. Thus, the average measures of serum 25-OH-D were again categorized as follows: (1) < 25 ng/mL; (2) 25-34 ng/mL; (3) 35-44.9 ng/mL; and (4) ≥ 45 ng/mL. A table of the β coefficients and means from the least squares regression is presented in Table 4.17. Contrary to what was hypothesized, the absolute mean change in percent MD over time decreased by increasing categories of serum 25- OH-D: specifically, they were 0.84%, 0.96%, 0.76% and 0.45%, respectively, for categories <25% ng/mL, 25-34 ng/mL, 35-44.9 ng/mL and ≥ 45 ng/mL adjusting for all covariates in the model. The coefficients, with a positive coefficient indicative of a better outcome, represent the difference in mean changes in percent MD over time between those with lowest serum 25-OH-D and those with highest serum 25-OH-D. The results suggest that women who have lower serum 25-OH-D levels have a larger decrease in percent MD compared with women with higher serum 25-OH-D levels which is contrary to the original hypothesis. It is observed that none of the point estimates were statistically significant for the effect of serum 25-OH-D on average change in percent MD over time in this analysis with an overall *p*-value = 0.33.

**Table 4.17: Relationship between Serum 25-OH-D and Average Change in Percent MD Over Time**

| **Average Change in Percent MD= (Baseline % MD– Follow-up % MD) /years follow-up** | | | | | | | |
|---|---|---|---|---|---|---|---|
| | | **Crude Analysis** | | | **Adjusted Analysis*** | | |
| **Serum 25-OH-D (ng/mL)** | **N** | **Coefficient**** | **Average Change in % MD** | ***p*-value ***** | **Coefficient **** | **Adjusted Average Change in % MD** | ***p*-value ***** |
| 0-24.9 | 33 | -0.04 | 0.60 | 0.91 | 0.39 | 0.84 | 0.33 |
| 25-34.9 | 138 | 0.07 | 0.71 | 0.81 | 0.51 | 0.96 | 0.06 |
| 35-44.9 | 145 | 0.10 | 0.75 | 0.69 | 0.31 | 0.76 | 0.24 |
| ≥ 45 | 72 | Referent | 0.64 | | Referent | 0.45 | |
| Total N | 388 | *Overall p-value = 0.97*** | | | *Overall p-value = 0.33*** | | |

* Adjusted in linear regression model for age, month of serum measurement, BMI, age at menarche, HRT use, smoking status, OC use and age at menopause.

** The coefficients presented represent differences in mean changes in percent MD over time compared to the referent.

*** F-test

*4.6.2.2 Analyses Using Changes in Percent MD as a Dichotomous Outcome Measure*

A main analysis using logistic regression, with reported ORs, was also conducted to estimate the effect of serum 25-OH-D levels between women who had a decrease in breast density over time ('no event') compared with those women who had no change or an increase in BD over time ('event') (see Table 4.18). Results based on this dichotomous change in percent MD outcome were not in the anticipated direction of effect. The ORs associated with each category of serum 25-OH-D, defined in the above section, suggest that women who have low levels of serum 25-OH-D are less likely to have no change or an increase in percent MD over time in comparison to those women with high levels of serum 25-OH-D. The overall *p*-value for the categorical vitamin D variable was 0.05 after adjusting for all confounding variables.

**Table 4.18: Relationship between Serum 25-OH-D and Average Change in Percent MD Over Time**

| Serum 25-OH-D (ng/mL) | Average Change in % MD | | Crude Analysis | | Adjusted Analysis* | |
|---|---|---|---|---|---|---|
| | Decrease | No change/ Increase | Odds Ratio | 95% CI | Odds Ratio | 95% CI |
| 0-24.9 | 25 | 8 | 0.48 | (0.19-1.20) | **0.30** | **(0.10 – 0.87)** |
| 25-34.9 | 98 | 40 | 0.61 | (0.33-1.10) | **0.46** | **(0.23- 0.88)** |
| 35-44.9 | 92 | 53 | 0.85 | (0.48-1.53) | 0.72 | (0.36 - 1.31) |
| ≥ 45 | 43 | 29 | 1.00 | (referent) | 1.00 | (referent) |
| Total N | 258 | 130 | *Overall p-value =0.21* | | *Overall p-value =**0.05*** | |

* OR from logistic regression adjusted for age, month of serum measurement, BMI, age at menarche, HRT use, smoking status, OC use and age at menopause

*4.6.2.3 Sensitivity Analyses to Evaluate the Effect of Mammogram Format*

Of the 388 pairs of participant mammograms used to examine the relationship between serum 25-OH-D and changes in percent MD over time, 298 pairs were film-based and 90 were digital-based mammograms. Given the mixture of film and digital images received for study participants it was of interest to examine whether there was a relationship between mammogram format and the outcome measure of change in percent MD over time and, further, whether the relationship between serum 25-OH-D and change in percent MD over time was different by mammogram format. A relationship between mammogram format and change in percent MD over time, adjusted for potential confounders, was observed ($p < 0.01$) (see Table 4.19). Adjusted mean changes in percent MDs over time by format were 1.69% for digital images and 0.61% for film images. To evaluate whether the relationship between serum 25-OH-D and average change in percent MD over time differed by image format, serum 25-OH-D levels were dichotomized as < 35 ng/mL vs. ≥ 35 mg/mL based on the distribution of this exposure and to ensure a sufficient sample size for analyses purposes. There was no effect modification by mammogram format on the relationship between serum 25-OH-D and average changes in percent MD over time observed

(*p*-value for the interaction term = 0.79) and no effect of serum 25-OH-D on MD in either the film or digital strata (see Table 4.20).

**Table 4.19: Relationship between Mammogram Format and Average Change in Percent MD over Time**

| Format | N | Coefficient * | Adjusted Average Change in % MD | *p*-value** |
|---|---|---|---|---|
| Digital | 90 | 1.07 | 1.69 | <0.01 |
| Film | 298 | Referent | 0.61 | |

* Adjusted in linear regression model for age, month of 25(OH)D measurement, BMI, age at menarche, HRT use, smoking status, OC use and age at menopause. The coefficient presented represents the difference in mean change in percent MD over time compared to the referent.

** F-test

**Table 4.20: Effect Modification by Mammogram Format on the Relationship between Serum 25- OH-D and Average Change in Percent MD Over Time**

| Serum 25-OH-D (ng/mL) | FILM Average Change in % MD | | | DIGITAL Average Change in % MD | | |
|---|---|---|---|---|---|---|
| | Decrease | No change /Increase | OR (95%CI) | Decrease | No change /Increase | OR (95% CI) |
| < 35 | 92 | 40 | **0.56 (0.32-0.97)** | 31 | 8 | 0.47 (0.16–1.40) |
| > 35 | 99 | 67 | 1.0 (referent) | 36 | 15 | 1.0 (referent) |
| | 191 | 107 | | 67 | 23 | |
| | | | | | | *p-value for interaction =0.79* |

* OR adjusted for age, month of serum collection, BMI, age at menarche, HRT use, smoking status, OC use and age at menopause

*4.6.2.4 Secondary Objective 2: Effect Modification by Exemestane*

Whether the relationship between serum 25-OH-D and the average change in percent MD over time was modified by the randomization arm of the trial (exemestane vs. placebo) was of clinical interest in this study. Serum 25-OH-D levels were again dichotomized as < 35 ng/mL vs. ≥ 35 ng/mL in order to have a sufficient sample size within stratified categories. There was no evidence of an interaction between serum 25-OH-D and randomization arm for average changes

in percent MD over time observed (*p*-value for the interaction = 037). While there was no observed effect of serum 25-OH-D on changes in percent MD over time in the exemestane stratum the data suggests that among women on the placebo arm of the trial those with lower levels of serum 25-OH-D were less likely to have no change or an increase in percent MD over time compared with women who had higher levels of serum 25-OH-D (see Table 4.21).

**Table 4.21: Effect Modification by Randomization Arm on the Relationship between Serum 25-OH-D and Average Change in Percent MD Over Time**

| Serum 25-OH-D (ng/mL) | Placebo Average Change in % MD | | | | Exemestane Average Change in % MD | | | |
|---|---|---|---|---|---|---|---|---|
| | Decrease | No change /Increase | Crude OR (95% CI) | Adjusted OR (95% CI)* | Decrease | No change /Increase | Crude OR (95% CI) | Adjusted OR (95% CI)* |
| < 35 | 59 | 23 | 0.55 (0.30-1.03) | **0.44 (0.22–0.87)** | 64 | 25 | 0.74 (0.41-1.36) | 0.68 (0.34–1.34) |
| ≥ 35 | 61 | 43 | 1.0 (referent) | 1.0 (referent) | 74 | 39 | 1.0 (referent) | 1.0 (referent) |
| Total N | 120 | 66 | | | 138 | 64 | | |
| | | | | | | | | *p-value for interaction** =0.37* |

* OR adjusted for age, month of serum collection, BMI, age at menarche, HRT use, smoking status, OC use and age at menopause

** From fully adjusted model

*4.6.2.5 Secondary Objective 3: Effect Modification by Calcium*

The relationship between serum 25-OH-D and average change in percent MD over time was examined among those with low (below the median) and high (above the median) levels of calcium. There was no evidence of an interaction between serum 25-OH-D and calcium for average changes in percent MD over time (*p*-value for the interaction term = 0.74) (see Table 4.22).

**Table 4.22: Effect Modification by Calcium on the Relationship between Serum 25-OH-D and Average Change in Percent MD Over Time**

| Serum 25-OH-D (ng/mL) | Low Calcium (below median) Average Change in Percent MD | | | | High calcium (above median)Average Change in Percent MD | | | |
|---|---|---|---|---|---|---|---|---|
| | Decrease | No change /Increase | Crude OR (95% CI) | Adjusted OR (95% CI)* | Decrease | No change /Increase | Crude OR (95% CI) | Adjusted OR (95% CI)* |
| < 35 | 60 | 22 | 0.62 (0.33-1.18) | 0.50 (0.25–1.02) | 63 | 26 | 0.68 (0.37-1.22) | 0.59 (0.30 – 1.14) |
| ≥ 35 | 63 | 37 | 1.0 (referent) | 1.0 (referent) | 72 | 44 | 1.0 (referent) | 1.0 (referent) |
| Total N | 123 | 59 | | | 135 | 70 | | |
| *p-value for interaction** = 0.74* | | | | | | | | |

* OR adjusted for age, month of serum collection, BMI, age at menarche, HRT use, smoking status, OC use and age at menopause

*4.6.2.6 Summary of Results Evaluating Serum 25-OH-D and Changes in Percent MD Over Time*

Given that breast density is known to decrease with increasing age [26] it was of interest that ~34% of study participants either had no change or an increase in percent MD between baseline and follow-up mammograms. It was hypothesized that women with lower levels of serum 25-OH-D would have no or smaller changes in percent MD over time, on average, compared with women with higher levels of serum 25-OH-D at baseline. Surprisingly, the results were in the opposite direction anticipated; namely, women with lower serum 25-OH-D levels were less likely to have no changes or an increase in percent MD over time compared with women with higher serum 25-OH-D. No effect modification by either exemestane or calcium was observed on the underlying vitamin D → change in percent MD over time relationship.

## 4.7 References

1. Bertone-Johnson, E.R., Chlebowski, R.T., Manson, J.E., Wactawski-Wende, J., Aragaki, A.,Tamimi, R.M.,Rexrode, K.M., Thomson, C.A., Rohan, T.E., Peck, J.D., Pisano, E.D., Martin, C.F., Sarto, G., McTiernan, A,. Dietary vitamin D and calcium intake and mammographic density in postmenopausal women. Menopause.

2010;17(6):1152-1160.

2. Bertone-Johnson ER, McTiernan A, Thomson CA, et al. Vitamin D and calcium supplementation and one-year change in mammographic density in the women's health initiative calcium and vitamin D trial. Cancer Epidemiology Biomarkers & Prevention.

2012;21(3):462-473.

3. Neuhouser ML, Bernstein L, Hollis BW, et al. Serum vitamin D and breast density in breast cancer survivors. Cancer Epidemiology Biomarkers & Prevention.

2010;19(2):412-417.

4. Boyd NF, Guo H, Martin LJ, et al. Mammographic density and the risk and detection of breast cancer. N Engl J Med. 2007;356(3):227-236. http://dx.doi.org/10.1056/NEJMoa062790. doi: 10.1056/NEJMoa062790.

5. Kerlikowske K, Ichikawa L, Miglioretti DL, et al. Longitudinal measurement of clinical mammographic breast density to improve estimation of breast cancer risk. J Natl Cancer Inst. 2007;99(5):386-395.

6. McCormack VA, dos Santos Silva I. Breast density and parenchymal patterns as markers of breast cancer risk: A meta-analysis. Cancer Epidemiology, Biomarkers & Prevention. 2006;15(6):1159-1169.

7. Boyd N. What accounts for variations in percent density. Written communication, Nov 2010.

8. Boyd N, Martin L, Stone J, Little L, Minkin S, Yaffe M. A longitudinal study of the effects of menopause on mammographic features. Cancer Epidemiology Biomarkers & Prevention. 2002;11(10):1048-1053.

9. Boyd N. Percent density on film screen vs. digital mammography. Written communication, Feb 2011.

10. van Gils CH. Follow-up from AACR frontiers in cancer prevention conference. Written communication, Jan 2013.

11. Whiting SJ, Langlois KA, Vatanparast H, Greene-Finestone LS. The vitamin D status of canadians relative to the 2011 dietary reference intakes: An examination in children and adults with and without supplement use. The American Journal of Clinical Nutrition. 2011;94(1):128-135. doi: 10.3945/ajcn.111.013268.

12. Rutter CM, Mandelson MT, Laya MB,Taplin S. CHanges in breast density associated with initiation, discontinuation, and continuing use of hormone replacement therapy. JAMA. 2001;285(2):171-176. doi: 10.1001/jama.285.2.171.

13. McTiernan A, Martin CF, Peck JD, et al. Estrogen-plus-progestin use and mammographic density in postmenopausal women: Women's health initiative

randomized trial. Journal of the National Cancer Institute. 2005;97(18):1366-1376. doi: 10.1093/jnci/dji279.

14. Greendale GA, Reboussin BA, Slone S, Wasilauskas C, Pike MC, Ursin G. Postmenopausal hormone therapy and change in mammographic density. Journal of the National Cancer Institute. 2003;95(1):30-37. doi: 10.1093/jnci/95.1.30.

15. Kenneth J. Rothman, Sander Greenland. Modern epidemiology. Philadelphia, PA: Lippincott-Raven Publishers; 1998.

16. Vogel VG. Epidemiology, genetics, and risk evaluation of postmenopausal women at risk of breast cancer. Menopause. 2008;15(4 Suppl):782-789.

17. Rockell J, Skeaff M, Green T. Association between quantitative measures of skin color & plasma 25-hydroxyvitamin D (25OHD). FASEB J. 2008;22(1_MeetingAbstracts):157.5.

18. Grant W. Differences in vitamin D levels may contribute to racial disparities in breast cancer. Breast Cancer Res Treat. 2013;138(3):967-968.

http://dx.doi.org/10.1007/s10549-013-2480-7. doi: 10.1007/s10549-013-2480-7.

19. Neuhouser ML, Sorensen B, Hollis BW, et al. Vitamin D insufficiency in a multiethnic cohort of breast cancer survivors. The American Journal of Clinical Nutrition. 2008;88(1):133-139.

20. Chen Z, Wu AH, Gauderman WJ, et al. Does mammographic density reflect ethnic differences in breast cancer incidence rates? American Journal of Epidemiology.

2004;159(2):140-147. doi: 10.1093/aje/kwh028.

21. Ursin G, Ma H, Wu AH, et al. Mammographic density and breast cancer in three ethnic groups. Cancer Epidemiology Biomarkers & Prevention. 2003;12(4):332-338.

22. Anderson LN, Cotterchio M, Cole DEC, Knight JA. Vitamin D-related genetic variants, interactions with vitamin D exposure, and breast cancer risk among caucasian women in ontario. Cancer Epidemiology Biomarkers & Prevention. 2011;20(8):1708-1717. doi: 10.1158/1055-9965.EPI-11-0300.

23. Tang C, Chen N, Wu M, etc. Fok1 polymorphism of vitamin D receptor gene contributes to breast cancer susceptibility: A meta-analysis. Breast Cancer Research (2009) 117:391–399. DOI 10.1007/s10549-008-0262-4

24. Chen WY, Bertone-Johnson ER, Hunter DJ, etc. Associations between polymorphisms in the vitamin D receptor and breast cancer risk. Cancer Epidemiol.Biomarkers Prev. 2005;14(10).

25. Raimondi S, Johansson H, Maisonneuve P, Gandini S. Review and meta-analysis on vitamin D receptor polymorphisms and cancer risk. Carcinogenesis. 2009;30(7):1170-1180.

26. Martin L, Boyd N. Mammographic density. potential mechanisms of breast cancer risk associated with mammographic density: Hypotheses based on epidemiological evidence. Breast Cancer Research. 2008;10(1):201.

# Chapter 5

# Discussion

## 5.1 Summary of Findings

This study examined the relationship between serum 25-OH-D and percent MD in postmenopausal women at northern latitudes. Potential effect modification by exemestane therapy, calcium or genetic polymorphisms (CYP24A1 rs2181874; Fok1 rs2228570) on the relationship between serum 25-OH-D and percent MD were also examined. Percent MD was measured for 568 participants with a ≥ 3 year follow-up mammogram and for 388 participants with a baseline mammogram in the same format as the follow-up. The geometric mean percent MD of the follow-up mammograms was 4.3% and few women (13.4%) had percent MD ≥ 25%. A decrease in percent MD over time between baseline and follow-up mammograms was observed as anticipated with a 0.7% decrease in percent MD per year. Unadjusted for month of blood collection, the mean serum 25-OH-D concentration was 36.5 ng/mL (SD=10.6) based on pooled baseline and year one serum samples. The majority of study participants had serum 25-OH-D levels in the sufficient range (≥20 ng/mL) with only < 5% of participants exhibiting levels that would be considered deficient. After controlling for age, month of sampling and potential confounders, serum 25-OH-D was not predictive of log transformed percent MD at ≥ 3 year follow-up ($p$=0.36) or with annual mean changes ($p$=0.33). Results from logistic regression analyses were also not statistically significant although women with lower serum 25-OH-D were observed to have higher percent MD compared with women with higher serum 25-OH-D ($p$=0.37). Statistically significant interactions with exemestane, calcium or genetic polymorphisms were not detected. Taken together, the results of this study do not support a relationship between serum 25-OH-D and percent MD at ≥ 3 year follow-up or between serum 25-OH-D and average changes in percent MD over time in this study population.

The remainder of this chapter is devoted to discussing these results in the context of the body of literature that has evaluated the association between serum 25-OH-D and percent MD to date with consideration of key criteria in Bradford Hill's framework for causality. The key strengths and limitations of this research are presented including suggestions for future research directions. This chapter concludes with the contributions this research has made and its' relevance to public health.

## 5.2 Comparison of Findings to Relevant Literature

### 5.2.1 Relationship between Serum 25-OH-D and Percent MD at ≥ 3 Year Follow-Up

This study constitutes the sixth observational study to date that has examined serum 25-OH-D in relation to MD [1-5] and the fourth study to investigate this relationship specifically among postmenopausal women [1,3,5]. While the exact biological mechanisms have not been elucidated to date, the biologic plausibility of the relationship between serum 25-OH-D and percent MD is supported by experimental evidence that has shown vitamin D to have both antiproliferative and proapoptotic properties which are hypothesized to reduce MD via paracrine and endocrine pathways [6-10]. In looking at these four studies in postmenopausal women specifically, though, there has consistently been no overall association observed between serum 25-OH-D and percent MD regardless of whether mean percent MD was evaluated across categorical measures of serum 25-OH-D [1,3,5] or with a continuous exposure measurement of serum 25-OH-D [1,5]. These associations did not change when the data were stratified by calcium intakes or season of blood draw [1,5]. Further, in three of these studies, including the current one, there was no evidence of a dose-response pattern with decreasing mean (or geometric mean) percent MD across increasing serum 25-OH-D categories after adjusting for important covariates [3,5]. However, in the study by Knight et al. [1] there was an unexpected trend of increasing percent MD with increasing serum 25-

OH-D although this was not statistically significant. Other than the current study only one other study applied a transformation to the percent MD data to improve normality [5] and the study by Knight et al. [1] included both pre- and postmenopausal women so it is difficult to directly compare the mean percent MDs across categories of serum 25-OH-D in all four studies. Only the current study also looked at serum 25-OH-D levels among women categorized as having lower (< 25%) vs. higher (≥ 25%) percent MD as it was felt that this outcome measure may better represent clinically meaningfully changes of differences in breast density that likely affect BC risk. While women with lower serum 25-OH-D levels were observed to have higher percent MD compared with women with higher serum 25-OH-D as hypothesized this relationship was not statistically significant. The current study is also the only prospective study in postmenopausal women to date which allows for a better evaluation of temporality between serum 25-OH-D levels and follow-up percent MD. The other observational studies were cross-sectional in nature and thus it cannot be assured that the exposure preceded the event.

### 5.2.2 Relationship between Serum 25-OH-D and Change in Percent MD Over Time

It is known that breast density declines with a woman's increasing age with postmenopausal women consistently observed to have lower percent MD than premenopausal women [11]. The interest in changes in percent MD over time in relation to serum 25-OH-D levels in the current project stems from literature that supports MD as modifiable beyond that observed with its natural history. MD has consistently been shown to increase in women taking combined estrogen and progestin HRT and to decrease with treatment with tamoxifen [12-15].

In the current study, associations between serum 25-OH-D and changes in percent MD over time were perplexing and contrary to study hypotheses. While it was anticipated that women with lower baseline serum 25-OH-D levels would have no or smaller decreases of percent MD on

average compared with women with higher baseline serum 25-OH-D, the results observed suggested the opposite: that women with lower serum 25-OH-D levels had larger decreases in percent MD over time compared with women with higher baseline levels. The Women's Health Initiative calcium and vitamin D trial in postmenopausal women was the only study identified in the literature that has evaluated the role of vitamin D and changes in percent MD over time [16]. As anticipated, women on the calcium and vitamin D arm of the trial experienced decreases in percent MD compared with women on the placebo arm of the trial, however, the authors did not observe a statistically significant association between calcium and vitamin D and change in percent MD after one year of supplementation (ratio of geometric means = 0.97, 95% CI: 0.81-1.17) [16]. These findings may be the result of the low mean percent MD (8.4%; SD=10.2%) among study participants or due to insufficient variation in vitamin D exposure levels between the two trial arms. Participants randomized to the treatment arm of the trial were only given 400 IU/day of vitamin D and women in the placebo group were permitted to use personal supplements. It is also noteworthy that assessment of total vitamin D status in that study was determined using self-administered food frequency questionnaires and interviews ascertaining personal supplement intake and not biomarkers of serum 25-OH-D. Assessment through questionnaires is not a comprehensive measurement tool for total vitamin D exposure since diet and supplements alone, without consideration of sun exposure, do not account for a large proportion of vitamin D levels circulating in the body.

The following section explores some potential reasons for the inconsistent results observed across the primary study objectives, particularly the unanticipated results of the analysis evaluating serum 25-OH-D and changes in percent MD over time.

### 5.2.3 Plausible Explanations for Contradictory Primary Study Results

The study population in the current project, overall, had baseline and follow-up percent MD measurements lower than anticipated as compared with population-based data obtained from the literature [17-19]. While the results of the first primary objective were not statistically significant it was observed that women with lower serum 25-OH-D had higher percent MD compared with women with higher serum 25-OH-D as expected. With respect to the second primary objective, it is possible that women with already low percent MD (who were observed to have higher serum 25-OH-D) have very little room for absolute change in percent MD compared with women in this study with higher percent MD (who were observed to have lower serum 25-OH-D). Said another way, women in this study with lower serum 25-OH-D were observed to have bigger decreases in absolute percent MD than women with higher serum 25-OH-D possibly because their breast density was more amenable to change compared with already low percent MD in the comparison group. Maskarinec and colleagues [20] conducted a longitudinal analysis of percent MD over time in a predominantly postmenopausal population (>75%) looking at predictors of changes in density. The authors observed that women with higher percent MD at baseline had a faster rate of decline in absolute percent MD over time compared with women with lower baseline percent MD [20]. Two additional studies support the finding that women with higher baseline percent MD experience greater absolute declines over time irrespective of other factors [13,21]. In the study by Kelemen and colleagues [13], for example, the authors showed that the greatest declines in percent MD occur during menopause but, interestingly, observed that this decline was greatest among women in the highest percentile distribution of percent MD. To evaluate whether the baseline percent MD measure for our study population contributed to the results observed for the relationship between serum 25-OH-D and average changes in percent MD this association was re-examined controlling for the effects of the baseline percent MD measure. Neither the

magnitude nor the direction of the effect measures were altered when average changes in percent MD accounting for the baseline measure were taken into account (results not presented).

There is very little known about the etiologically relevant time window of exposure by which serum 25-OH-D may exert its' effects on MD [1,4]. This thesis project was developed to investigate two distinct primary objectives of interest. In the first primary objective, examining the relationship between baseline serum 25-OH-D and percent MD at ≥ 3 year follow-up, the baseline measure of serum 25-OH-D was intended to represent one's typical exposure to vitamin D in the years preceding randomization to the MAP.3 parent trial. Results, while not statistically significant, were in the hypothesized direction. For the second primary objective, examining the relationship between baseline serum 25-OH-D and the average change in percent MD over the course of trial participation, evaluation of a shorter time period between serum 25-OH-D exposure and average percent change in MD was of interest. If levels of serum 25-OH-D at the time of randomization represented the relevant exposure window this objective also provided the ability to evaluate the association between serum 25-OH-D and changes in percent MD in an estrogen-suppressed group, namely those women randomized to the exemestane arm of the trial. This was of particular interest and value given the known strong association between estrogen and breast density/breast cancer (BC) risk [22-26] which may have been difficult to adequately control for in previous investigations of the vitamin D and BC relationship. The overall low percent MD among study participants may have made it difficult to detect statistically significant differences between women with lower vs. higher levels of serum 25-OH-D and these small differences in percent MD, if observed, would unlikely be of clinical significance. Further studies are warranted to evaluate the relationship between serum 25-OH-D and changes in percent MD over time in a group of women with higher baseline percent MD similar to recent intervention studies that have only included women with a baseline percent MD > 10% in order to make it possible to detect changes in percent MD over time [12,21]. In addition, it is possible that

the time frame evaluated between baseline and follow-up percent MD was insufficient to affect percent MD and, thus, future investigations that include a longer interval of follow-up are warranted. If a true association between vitamin D and percent MD exists, this may also help to further elucidate the relevant time window of exposure.

### 5.2.4 Interactions with Exemestane, Calcium and Genetic Polymorphisms

*5.2.4.1 Exemestane*

Half of the current study population (50.5%) was on aromatase inhibitor (AI) therapy with exemestane within the context of a large chemopreventive trial which has since shown that exemestane significantly reduces the incidence of invasive BC compared with placebo in postmenopausal women at moderately increased risk [27]. At the time of development of this research project there were no published studies on the relationship between AIs and, specifically, exemestane and MD. Since that time there have been nine studies that have evaluated the relationship between various AIs and MD in postmenopausal women [14,21,28-34] and investigators involved in the underlying RCT of the current study are in the midst of evaluating the relationship between exemestane and percent MD in this study cohort. In contrast to selective estrogen-receptor modulators (SERMs) such as tamoxifen, which have been shown to be effective in reducing percent MD in postmenopausal women [12], recent studies on AIs and breast density are not consistent. Overall, the results to date do not support a protective association between AIs and percent MD nor do the results support greater decreases in percent MD over time in women taking AIs compared with placebo [14,32].

There have been no studies to evaluate whether AIs may modify the vitamin D-breast density relationship which was of interest in the current study. Overall, we did not observe any significant interactions with exemestane on either the relationship between serum 25-OH-D and

percent MD at ≥ 3 year follow-up or with changes in percent MD over time. However, the finding that women on the placebo arm of the trial with lower serum 25-OH-D were more likely to have higher percent MD at ≥ 3 year follow-up compared with women with higher levels of serum 25-OH-D was very interesting. This result is consistent with the protective associations observed between high vitamin D and low percent MD primarily in premenopausal women who have higher estrogen levels. If lower serum 25-OH-D is associated with higher MD in this study population it seems that the relationship may only be evident in women who are not estrogen suppressed. Evaluation of the interaction and interpretation of the measures of effect are difficult in this analysis given he dramatic reduction in sample size and, in turn, statistical power within strata. A larger estimated sample size at study conceptualization would have facilitated this analysis and interpretation of results. Alternatively, availability of a larger group of participants on the placebo arm may have been a better target population within which to further evaluate this association. Similarly, women in the vitamin D and calcium supplementation arm of the Women's Health Initiative hormone therapy trial who were not on HRT (had lower estrogen levels) were observed to have lower percent MD [whereas women on HRT (had higher estrogen levels) had slightly higher MD although the interaction was not statistically significant (p=0.08) [16]]. Potential biological mechanisms exist in support of an interaction between vitamin D and estrogen including competitive binding for megalin, their common cellular member receptor and the down-regulation of ER expression by serum 1,25 (OH)2D which attenuates estrogen signaling in BC cells [16,35]. Alternatively, it is possible that in women without estrogen suppression small changes in percent MD related to serum 25-OH-D may be masked by the effects of estrogen itself on percent MD. Further studies are warranted to examine the potential interactions between serum 25-OH-D, estrogen and MD.

*5.2.4.2 Calcium*

Given the metabolic interrelationship between vitamin D and calcium and their inverse associations with breast density and BC in some epidemiological investigations it was of interest to evaluate the interaction between serum measures of vitamin D and calcium on percent MD in this group of postmenopausal women. Despite the hypothesis that higher blood levels of calcium would strengthen any observed protective association between serum 25-OH-D and percent MD there was no evidence of any interaction between serum 25-OH-D and calcium for either percent MD at ≥ 3 year follow-up or for changes in percent MD over time. Studies that have evaluated the relationship between vitamin D, calcium and breast density to date have largely measured dietary and/or supplemental intakes as opposed to blood levels. To our knowledge, no previous studies have evaluated both endogenous vitamin D and calcium levels with MD. There are a few plausible explanations for the results observed in the current study. First, it is possible that an interaction between vitamin D and calcium on MD is only evident in premenopausal women in the presence of higher estrogen levels and/or insulin-like growth factor (IGF) [36]. In one study, an inverse relationship between dietary intakes of vitamin D and calcium on MD was stronger in women with higher IGF levels compared with those with lower levels [36]. Second, it is possible that an interaction between serum 25-OH-D and calcium was not observed in the current study which was composed primarily of women with generally low mammographic densities. The strongest relationship between vitamin D and calcium and breast density observed to date has been in studies which included women with higher densities [37,38]. Future studies should investigate this relationship in a study population with both higher percent MD and greater variability in percent MD. It should also be noted that women in the current study not only had high baseline serum 25-OH-D levels (with <5% of study participants deficient) but the mean blood level of calcium among these women was also high with <5% of participants having below normal levels at the time of randomization. It may be that insufficient variability in both exposure and outcome measures made it difficult to detect an interaction between serum 25-OH-

D and calcium on percent MD in the current study. If the exposure levels in this study population are representative of the expected levels in North American postmenopausal women today, likely due to supplementation for bone health, future studies should include women with higher baseline mammographic densities which may be more amenable to modification by serum 25-OH-D and calcium.

*5.2.4.3 Genetic Polymorphisms*

As part of the secondary objectives of this research project, two polymorphisms related to VDR (Fok1 rs2228570) and metabolism (CYP24A1 rs2181874) genes were selected and examined for both their independent effects with percent MD and for their potential interaction with serum 25-OH-D in relation to follow-up MD in this cohort of postmenopausal women. Specifically, it was of interest to explore whether variants in these polymorphisms may exacerbate or attenuate any observed association between serum 25-OH-D and percent MD. For Fok1, the recessive model (ff vs. Ff + FF) of allele frequency was evaluated for an association with percent MD based on known functionality of this polymorphism and for CYP24A1, the rare homozygous genotype was combined with the heterozygote (GG vs. GA & AA) and evaluated for an association with percent MD. No statistically significant associations were observed between either genetic polymorphism and percent MD at ≥ 3 year follow-up nor was there any effect modification by either of these vitamin D-related SNPs on the relationship between serum 25-OH-D and percent MD. It was, however, observed that the stratum specific effect measures for the interaction between serum 25-OH-D and Fok1 for percent MD were qualitatively different. Specifically, women with the ff genotype of the Fok1 polymorphism with lower serum 25-OH-D were less likely to have higher percent MD than women with higher serum 25-OH-D levels. Conversely, women with the Ff or FF genotypes with low serum 25-OH-D were almost twice as likely to have higher percent MD compared with women with higher serum 25-OH-D levels.

While the results of the analyses examining the association between vitamin D-related genetic polymorphisms and percent MD at ≥ 3 year follow-up were not statistically significant they were consistent with the direction of effects observed in previous studies evaluating BC risk [39-42]. In the case-control study evaluating vitamin D genetic variants and BC risk by Anderson and colleagues [39] the authors observed an increased BC risk for postmenopausal women with the CYP24A1 rs2181874 GA genotype (OR=1.21; 95% CI: 1.01-1.45). Similarly, when the relationship between genotype and percent MD was evaluated among the participants in this study, adjusting for all potential confounders, it was observed that women with higher breast density were more likely to have the GA genotype (OR=1.37; 95% CI: 0.76-2.47) although this relationship was not statistically significant. In addition, the epidemiological literature supports a higher BC risk among women with the ff genotype of the Fok1 polymorphism [40-42]. In the current study, the relationship between the Fok1 genotype and percent MD was also evaluated and it was observed that women with the ff genotype were more likely to have higher percent MD (≥ 25%) compared with women with the Ff or FF genotype although this was not statistically significant. While qualitative differences in the effect measures between Fok1 genotypes were observed this is quite possibly due to chance given the limited statistical power available to evaluate interactions in this study population. For example, there were only 13 women with the ff genotype of the Fok1 polymorphism with percent MD at ≥ 3 year follow-up greater than 25% in this study cohort.

This is the first study identified that has evaluated both the independent associations of these two genetic polymorphisms with follow-up percent MD and their potential interaction with serum 25-OH-D in relation to MD. While no statistically significant results were observed, further studies with much higher sample sizes are required to adequately evaluate the potential relationship of these single vitamin D related genetic variants with MD. It has been purported that if genetic variants in the vitamin D pathway alter BC risk they may do so as effect modifiers in the case of

extreme exposure levels [43]. If this is the case, future studies which include participants with wider variability in serum 25-OH-D exposure levels will be necessary for evaluation.

Considering the available evidence, including the results from the current research project, there is insufficient evidence in support of a causal association between serum 25-OH-D and percent MD and between serum 25-OH-D and changes in percent MD over time in postmenopausal women to date.

## 5.3 Study Validity: Strengths and Limitations

### 5.3.1 Selection Bias

In considering the internal validity of this study there are several strengths and potential limitations that are noteworthy. Recall that trial participants from randomizing centres located in Canada and Buffalo, New York who had at least 3 years of prospective follow-up data including a baseline and follow-up bilateral mammogram were potentially eligible for the current study provided they did not develop BC and had provided serum and whole blood samples. It was decided *a priori* that BC cases would be excluded for a variety of reasons: (1) there was anticipated to be few cases diagnosed within our available study population and thus would not permit meaningful sensitivity analyses; (b) evaluation of percent MD in the left breast of cases diagnosed with cancer in that breast would not be possible/meaningful; and (c) BC cases diagnosed early in trial participation may have a different/more aggressive clinical course and be less amenable to modifiable factors such as serum 25-OH-D. Overall, 13 cases of BC were subsequently identified in the pool of potential study participants and excluded. The final cohort of study participants was determined based on the receipt of required mammograms at an independent hospital that was coordinating mammogram retrieval and review. Overall response

rates to requests for mammograms was quite high (77% of centres provided at least one ≥ 3 year follow-up mammogram per participant for evaluation of percent MD at follow-up; 71% of centres provided both the baseline and at least one ≥ 3 year follow-up mammogram per participant for evaluation of changes in percent MD over time). It is difficult to conceive that non-response to the request for mammograms from the few centres that were not compliant was related to both the exposure and outcome measures of interest in the current investigation. Given the prospective nature of the underlying study and data collection neither selection nor response bias is of particular concern in this study.

### 5.3.2 Information Bias and Measurement Error

Recall that there are three sources that contribute to one's circulating vitamin D levels with sun exposure being the primary source and food and vitamin supplements contributing to a lesser extent. The large majority of studies conducted to date have evaluated dietary intake of vitamin D in association with breast density/BC using mostly self-reported questionnaires. These studies suffer from measurement error to varying degrees due to the difficulty in accurately estimating the internal dose of vitamin D with the use of more subjective measures and lack of measurement of important variables such as sun exposure and vitamin D supplements. Information bias in these studies is also of concern given the use of self-reports. In the current study, a biomarker of exposure, serum 25-OH-D, was used which provides a comprehensive measurement of vitamin D from sunlight exposure and vitamin D intake from food and supplements [44-46]. This measure of internal dose also provides a more objective and precise measure of exposure for evaluating exposure-outcome and dose response relationships provided they reflect average lifetime exposure.

In the current study, misclassification of our serum 25-OH-D levels is possible, however, as serum samples from study participants were taken without knowledge of the exposure and outcome measures of interest such misclassification would likely be non-differential which would attenuate the observed associations towards the null. The expected seasonal variation in serum 25-OH-D levels was observed lending support to the validity of the exposure measurements obtained in this study albeit not to the degree expected likely because of the very high levels of circulating vitamin D in this population. An advantage to this study over most others that have used serum 25-OH-D as the primary exposure was the use of state-of-the-art LC-MS/MS assays which provide measures of serum 25-OH-D2 and serum 25-OH-D3 leading to a more precise and accurate total serum 25-OH-D measure. As previously reported, the repeatability and validity of the samples were high with both the inter-and intra-assay % CVs less than 10%. However, the serum 25-OH-D measurements of our study population were noted to read slightly lower than the mean of other LC/MS assays. The serum-25-OH-D measures in this study were carried out by a trained biochemist who was blinded to both the treatment arm of the study participants in the original RCT as well as the outcome measures. Another advantage of the current measurement of exposure compared with previous studies is the use of two measures, one taken at baseline and the second taken approximately one year later, which should better represent one's typical exposure level. There is the potential for temporal variability with serum 25-OH-D levels which could still result in a degree of misclassification of our primary exposure measure. However, we found good correlation between baseline and year 1 samples (r=0.64 for baseline and seasonally adjusted samples; r=0.68 for baseline and year 1 samples within the same month) reducing concern for measurement error. Lastly, it is unknown whether or not serum vitamin D measures taken a few years prior to the outcome measure of interest represent the relevant time window of exposure for breast density. However, studies that have evaluated vitamin D exposures earlier in life have not observed an association between vitamin D intake and breast density [7,47].

Mammograms for study participants were carried out at various radiology facilities throughout Canada and Buffalo, New York so it is possible that utilization of different mammography equipment led to measurement error in the current study. That said, all mammography equipment in Canada, be it film-based or digital, is overseen by the Canadian Association of Radiologists who ensure certain standards are met for accreditation purposes [48] hopefully mitigating any measurement error. The mammograms for each participant in the study, with the exception of ~65 participants, also had their baseline and follow-up mammogram done at the same radiology facility thereby minimizing within subject error. For the second primary objective evaluating mean changes in breast density over time, only study participants with baseline and follow-up mammograms in the same image format (both film or both digital) were included for evaluation. While reducing the sample size and, thus, statistical power for this second objective, ensuring the change in percent MD measure was calculated from same format images reduced misclassification in this outcome measure. In sum, if there is a degree of measurement error in the mammograms collected and utilized in this study it is likely non-differential given that mammograms were conducted independent of the specific objectives and hypotheses of the current study.

A quantitative approach for the measurement of percent MD at baseline and follow-up was used in order to provide a more objective and continuous outcome measure for analysis purposes. The computer-assisted method, Cumulus, was used in this study and has been shown to have high reliability and validity in percent MD measurements [49]. However, Cumulus was developed for use with film-based images and, thus, it is possible that digital images included in this study had a degree of measurement error [49]. Measurement error with baseline mammogram images, for example, may explain why the percent MD on baseline digital images was higher than film images when the opposite was expected. It was important to look at whether the relationship between serum 25-OH-D and percent MD was modified by the format of the mammogram.

Sensitivity analyses confirmed the appropriateness of conducting the main analyses using both film-based and digital-based images together that has not been extensively reported on in the literature. Further, any misclassification in the change in percent MD outcome variable was minimized by ensuring that both the baseline and follow-up mammograms per participant were in the same format before calculating the change variable.

One study radiologist who specializes in mammography and received formal training on the use of Cumulus software independently carried out the measurements of percent MD on all mammograms included in this study. The radiologist was blinded to participant treatment assignment, serum 25-OH-D level and all other variables that may have been related to exposure or outcome assessment. In addition, the radiologist was not privy to the order of the images provided for measurement. Similar to other studies, high intra-rater reliability was observed for the 10% of repeat mammograms that the radiologist measured (r=0.95). Although the percent MD measurements in this population were lower compared with other studies of postmenopausal women and without the distribution of measurements expected, descriptive results for the baseline and follow-up images showed that percent MD decreased over time as expected and at a rate of change of similar magnitude as that reported in the literature (~1% per year of age) [50]. Thus, any measurement error in the outcome variable is likely to be small and non-differential thereby not substantially biasing observed results.

The question remains as to why there was low prevalence of high percent MD in this study population given that participants were originally recruited to a breast cancer chemoprevention trial aimed at women at moderate to high risk for BC development. Evaluation of the baseline characteristics of women in the original trial shows, however, that the majority of study participants (68%) met trial inclusion criteria based on age alone (≥ 60 years) and the Gail score observed for these women was not that much higher than that of the average North American

woman. It appears that the trial overall did not ultimately recruit a high risk population which may partially explain why the percent MD measures were lower than originally anticipated. That said, the percent MD measures in the current study were also lower compared with other population based studies that included women of average breast cancer risk. Recruitment to the RCT was primarily achieved via local media advertisements, flyers/brochures and mass mailings**.** In other words, women self-selected to this trial (provided eligibility criteria were met) and may not have represented a truly random sample. It is quite possible that women who volunteered to participate were systematically different from women who did not and those differences may have been related to both the underlying exposure and outcome under evaluation in this nested observational study. For example, women who volunteered to participate in the prevention trial may have had healthier lifestyle behaviours which may have contributed to the overall low percent MD observed. Alternatively, postmenopausal women with high MD may already be more regularly screened by their physicians and thus underrepresented in the study population. Another possible explanation for the lower percent MD observed in this study is systematic error in percent MD measurement. While one radiologist reviewed and measured all study mammograms, was blinded to exposure and demonstrated high intra-rater reliability it is possible that the measurements across study mammograms were consistently lower than that reported in other studies.

### 5.3.3 Confounding

Data collection under the auspices of the parent RCT was very comprehensive and, thus, the analyses were able to control for the majority of known and suspected risk factors for both breast density and BC, particularly those related to estrogen exposure, and those suspected of confounding the vitamin D → breast density relationship. BC risk factors that were not available for study participants included alcohol intake and physical activity. However, confounding by

physical activity may have been mitigated by having information on other factors (i.e. BMI) that are correlated with physical activity. This study was also unable to evaluate possible confounding by insulin-like growth factors on the serum 25-OH-D and breast density relationship observed in this study population which may also have been of interest [36].

Evaluation of the relationship between retained covariates and percent MD adjusting for all other variables in the model was conducted in order to see whether established relationships with breast density risk factors were observed in this study[26,51-56]. In looking at the relationship with ≥ 3 year follow-up, associations were largely as expected for the strongest known risk factors for breast density. Specifically, an inverse association was observed with BMI and positive associations were observed with parity and age at first birth and OC use[26,51,52,55,56]. Consistent with some findings, a positive association was also observed between age at menarche and breast density [53]. While not statistically significant, the relationships with age and family history of BC were in the anticipated direction of effect[26,51-54].

Overall, concern for selection bias, information bias, misclassification and confounding is minimal given that (a) the information collected on exposure and outcome measures was before evaluation of the study objectives and independent of study hypotheses; (b) objective measures were used to determine both exposure and outcome measures; (c) both serum 25-OH-D and percent MD measurements were carried out by independent, highly trained professionals without knowledge of other study characteristics which might introduce bias into their measurements; and (d) statistical analyses were able to control for a comprehensive list of covariates both known and suspected to confound the underlying vitamin D → breast density relationships. It was for these reasons, particularly the prospective nature of the underlying RCT and comprehensive BC risk factor information available, that conducting a nested observational study was a strong and

efficient approach to evaluate the association between serum 25-OH-D and percent MD. That said, there were limitations to conducting an observational study within this setting where the treatment arm of the trial demonstrated a protective effect on BC incidence and there was the potential for interaction of effects with the exemestane arm. If the effect of exemestane on BC is mediated by breast density, it may be that it is difficult for vitamin D to exert an effect on percent MD beyond that by exemestane. As previously indicated, investigators of the MAP.3 trial are currently evaluating whether exemestane is associated with breast density in this cohort of women. Further, since exemestane was shown to have a protective effect on BC incidence conducting an overall analysis on the pooled population (placebo + intervention arms) may have obscured any true biologic relationship that was being investigated if breast density was the intermediate marker by which both vitamin D and exemestane operate. As indicated early in this thesis, it was important to examine the relationship between vitamin D and breast density in both arms of the trial and if a statistically significant interaction was observed results would have been reported by trial arm. It was here that we observed some evidence that low serum 25-OH-D was associated with higher percent MD in the placebo arm, however, this was not statistically significant nor was the overall interaction term in the model.

### 5.3.4 Analytical Issues

Statistical power based on exposure and outcome distributions in the current study is the main analytic issue of concern. Post hoc detectable effect estimates were repeated for the analysis of a categorical representation of serum 25-OH-D on a continuous outcome of percent MD. Detectable effects were calculated for the contrast between participants with low levels of serum vitamin D (< 25 ng/mL) (n=55) compared with participants with high levels of serum vitamin D (≥ 45 ng/mL) (n=101). Using a standard deviation of 12.3 for the non-transformed distribution of percent MD, this study was able to detect a 5.8% difference in mean MD at ≥ 3 year follow-up

across serum 25-OH-D categories. Interestingly, this is a smaller detectable effect than that expected from the a priori sample size calculations. This is due to the smaller standard deviation of percent MD observed in the current study than that utilized from previous literature.

Post hoc detectable effect estimates were also calculated for the analysis of a categorical representation of serum 25-OH-D on a dichotomous outcome of percent MD (≥ 25% vs. < 25%). Only 13.6% of postmenopausal women in this study population of 568 had ≥ 25 percent MD. Detectable effects were based on a comparison of the lowest (n=55) versus highest (n=101) vitamin D categories and 77 events (i.e. percent MD ≥ 25%). In this contrast, we were only able to detect an OR of 4.14 for percent MD ≥ 25 in participants with low serum 25-OH-D compared to participants with high serum 25-OH-D. This high detectable effect estimate is attributed to the very low overall event rate in this population (percent MD ≥ 25%) and the low prevalence of exposure (i.e. low levels of serum 25-OH-D) among women with percent MD < 25%. These power limitations are even greater in the analysis of interactions (results not presented). Future studies with larger sample sizes and increased variability for both exposure and outcome measures will allow for smaller detectable effects that may be clinically relevant.

### 5.3.5 Use of Intermediate Endpoints

The overarching goal of this research project was to contribute to the understanding of the relationship between vitamin D and BC etiology. MD has consistently been observed to be a strong predictor of BC risk and is supported as being a potential intermediate biomarker in the vitamin D and BC pathway [19,29,51,57-61]. The examination of MD allowed for a prompt investigation of a segment of the proposed biologic pathway between vitamin D and BC development rather than awaiting the occurrence of BC events. The investigation of MD also offered the elucidation of a potentially stronger underlying relationship than that observed in

studies of vitamin D and BC to date [62]. However, one of the limitations of using an intermediate marker is that null results may mean that the intermediate marker is not in the causal pathway between the exposure and disease outcome of interest. The current body of evidence, including the results from this study, do not lend support that the vitamin D → BC relationship is mediated through the MD pathway. If vitamin D is modestly protective for BC risk it may be operating via different mechanisms.

Only one study identified in the literature has included patients with BC in their assessment of vitamin D and MD. The study by Green et al. investigated both the association between plasma 25-OH-D and MD and the potential interaction by plasma vitamin D levels on the breast density → BC association in a nested case-control study within the Nurses' Health Study [3]. While the authors found no association between plasma 25-OH-D and percent MD in postmenopausal women, they did observe a relationship between low vitamin D levels and increased BC risk among women with high MD [3]. Future prospective studies that allow for the comprehensive evaluation of each component of the vitamin D → breast density → BC pathway are needed to fully examine the importance of vitamin D. This will help to elucidate whether MD is, in fact, the right intermediate for the relationship between vitamin D and BC etiology. The biological mechanisms by which this modifiable risk factor can exert its effects on BC risk have important implications for the prevention of this disease.

### 5.3.6 External Validity

A final limitation of this thesis is that inclusion of only modestly high-risk women means that results may not be generalizable to all postmenopausal women. Participants who enroll in RCTs are also likely systematically different from the general population in many ways including their vitamin D levels and risk factors for BC. It was observed that the study population was

predominantly Caucasian, of higher education and higher BMI. However, the Gail score in the trial participants was not that much higher than the average North American woman [63] and the majority of women were eligible for the parent RCT based on age (≥ 60) alone. Despite the finding that the study cohort had higher serum vitamin D levels and lower percent MD than anticipated it was felt that this was an appropriate population for study in that it may have increased the prevalence of the outcome under investigation and was a population of particular relevance for BC prevention and/or intervention.

## 5.4 Further Research Directions

The totality of results from this study and others reviewed in this thesis do not collectively support a relationship between vitamin D and MD in postmenopausal women. However, the results from studies evaluating the relationship between vitamin D and BC are more consistent and support a modest protective effect. As previously hypothesized, it is still possible that a protective effect of vitamin D on MD exists predominantly in premenopausal women who are exposed to higher estrogen levels and have higher MD compared with postmenopausal women. Indeed we observed the strongest association between low serum 25-OH-D and high percent MD in the subset of women who were not estrogen suppressed by the treatment arm of the underlying trial. Future studies are needed to further evaluate the potential interactions between vitamin D, estrogen and insulin-like growth factors in pre- and postmenopausal women which may help to further elucidate the underlying mechanisms by which vitamin D may exert an effect on MD [36,64].

Participants in this study had unexpectedly high levels of baseline vitamin D levels, low percent MD and overall low variability in these measures. It may be that women with higher percent MD at baseline are better able to change their percent MD over time compared with women with already low baseline percent MD. Future studies may consider calculating relative, as opposed to

absolute, changes in percent MD over time which takes into account the baseline measure. This may allow for a better comparison between those women who start with higher percent MD and those who do not. That said, further studies that evaluate the relationship between serum 25-OH-D and changes in percent MD over time in a group of women with more variable serum 25-OH-D levels and higher baseline percent MD will be of value. For example, stratified sampling on MD categories that better approximate the distribution seen at a population level may also improve upon the generalizability of results [65].

It is also of recent debate whether percent density or dense area alone is more relevant in relation to BC risk [66,67]. Future studies should include amongst its objectives the examination of the relationship between vitamin D and dense area, particularly if protective associations between vitamin D and breast density are observed [67]. As percent density is influenced by the size of the fat area in the breast it is of interest to ensure that any observed statistically and clinically significant protective associations are not entirely explained by a relationship with the non-dense area in the breast [66,67]. In addition, future studies evaluating the effects of physical activity on the vitamin D and breast density/BC relationship are warranted as are studies with more ethnically diverse populations given that there are racial differences in both serum 25-OH-D levels and BC rates [68-70]. Lastly, future studies with increased sample sizes will also be important to further evaluate potential effect modification by candidate genetic polymorphisms on the underlying vitamin D and breast density relationship.

### 5.5 Conclusions and Research Contributions

In the last 7 to 8 years there has been a substantial increase in publications on the relationship between vitamin D and breast density/BC. This thesis project constitutes only the fourth study to date to investigate the relation between serum 25-OH-D and percent MD among postmenopausal

women and the first to evaluate this relationship prospectively. This thesis project addressed research objectives that have not previously been investigated and also contributed novel methodology to this field of study.

One of the novel contributions of this study was that it was the first observational investigation to evaluate serum 25-OH-D and changes in breast density over time. A more robust measurement of exposure in comparison with past studies was also incorporated by utilizing an average of two serum samples to provide a better representation of exposure. Further, this was only the second study to include digital mammograms in the outcome assessment [5] and the finding that associations between serum 25-OH-D and percent MD were similar irrespective of the type of mammography assessment was of interest given the recent change in technology. The ability to evaluate the relationship between serum 25-OH-D and percent MD at follow-up and over time in a group of higher-risk postmenopausal women who were suspected of being at higher risk for vitamin D deficiency given their residence at northern latitudes was of value. Furthermore, nesting a cohort study within this RCT provided the opportunity to look at the main association under investigation while controlling for estrogen exposure in a sub analysis of those on exemestane therapy. In addition, there was the opportunity to examine how serum 25-OH-D interacts with hormonal factors such as exemestane which it was hoped would be informative regarding clinical efficacy and biologic understanding. The results of the evaluation of potential effect modification by calcium and select genetic polymorphisms contributes to the literature in this area which is sparse to date and hopefully will stimulate future research in these areas with larger sample sizes.

The current study also overcame several methodological limitations of prior studies. This was accomplished with the use of objective measures of exposure and outcomes of interest, by better

establishing temporality using a prospective study design and reducing the possibility of residual confounding through evaluation of a comprehensive list of covariates. For example, it is known that BMI is positively correlated with the total area of the mammogram and the area of non-dense tissue and is negatively correlated with the area of dense tissue [26,51,52]. BMI is also negatively associated with percent MD [55,56]. In the current study, percent MD at ≥ 3 year follow-up and changes in percent MD over time were also inversely associated with BMI and BMI was observed to have the largest confounding effects on the underlying relationships examined although no effect modification on the primary objectives was observed. Similarly, in the study by Sprague et al. any observed associations between serum 25-OH-D and percent MD disappeared with further adjustment for BMI [5]. This illustrates the importance of controlling for the effects of BMI in the evaluation of vitamin D and percent MD relationships.

The unexpected finding that women in this study cohort had higher baseline vitamin D levels than expected and without the range of levels previously observed at a population level was surprising. As previously mentioned, it is likely that women who volunteer to participate in an RCT evaluating an outcome that they are at increased risk of developing are systematically different than women in the general population. However, it is also evident that there has been much media attention since the inception and recruitment to the MAP.3 trial advocating the benefits of supplementation with vitamin D for cancer risk reduction. A series of media releases in 2007, for example, reported on the Canadian Cancer Society's new vitamin D supplement recommendations to reduce the risk for colorectal, breast and prostate cancers [71]. The recommendations suggested that Canadian adults take 1,000 international units (IU) of vitamin D supplements per day in the fall and winter and those at risk of deficiency should take 1,000 IU/day year round [71]. Albeit not a general population sample, the current study provides some interesting new data on the prevalence of vitamin D deficiency / sufficiency in a group of North American postmenopausal women. The higher levels observed may partially be a result of

population health efforts to increase sufficiency levels in Canadian adults. This is substantiated by the finding that women in this study cohort had high levels of serum 25-OH-D2, which is generally reflective of vitamin D supplement use, in combination with crude data extracted from case report forms.

It was also surprising to find that the large majority of study participants had percent MD measurements at follow-up < 25% with only 13.6 percent of participants with a follow-up MD measurement ≥ 25%. Further, less than 1% of participants had a follow-up MD measurement ≥ 50% and no participants had follow-up mammograms with breast densities ≥ 75%. Based on previous literature, it was expected that upwards of 40% of study participants would have mammographic densities ≥ 25% , about 17% would have mammographic densities ≥ 50% and approximately 5% of study participants would have mammographic densities ≥ 75% [17]. Aside from the likelihood of some measurement error in outcome assessment, it may be that postmenopausal women in this cohort had lower breast density given that they were off all forms of HRT for at least three months prior to trial randomization (which itself is associated with higher breast density) and perhaps were a healthier cohort than general population comparisons given trial recruitment strategies although this latter observation is difficult to substantiate in absence of data on diet, physical activity and alcohol consumption.

While the overall risk estimates from the epidemiologic research on the relationship between serum 25-OH-D and percent MD to date are modest at best, the high prevalence of deficiency in some populations would result in a large population attribute risk if a causal association does in fact exist. This would have important public health implications for prevention strategies using a readily modifiable risk factor for BC.

Overall, nesting an observational study within an underlying chemoprevention RCT of postmenopausal women at higher risk for BC development and utilizing upwards of 6 years of prospective data collection was novel and was an efficient and methodologically strong approach to research BC etiology.

## 5.6 Suitability for a PhD in Epidemiology

While this study was nested within a clinical trial, a novel epidemiologic study was designed and carried out by the student which included primary data collection and evaluation of relevant exposure and outcome measures. In recognition of limitations of past studies in the area of vitamin D and mammographic breast density, much consideration and effort was invested by the student to improve upon the vitamin D exposure measure by using an integrated measure of internal dose that takes into account all sources of vitamin D and by having the exposure measured with state of the art LC-MS/MS to minimize measurement error. Further, a great deal of effort was directed towards mammogram retrieval from participating centres/radiological clinics across Canada and Buffalo, NY to ensure the correct images were received while maximizing the amount of prospective follow-up available. This resulted in a sample size of postmenopausal women that was larger than past studies in this area and also reduced misclassification of the outcome measures. Further, this PhD dissertation required thorough consideration by the student of the etiologically relevant time window between serum 25-OH-D and percent MD, consideration of the validity of exposure and outcome measures obtained and consideration of the methodological advantages and limitations to using a cohort of postmenopausal women that were participating in a chemoprevention trial. Lastly, the statistical analyses employed by the student included the application of advanced multivariate modeling strategies with proper control of a relevant subset of covariates that could potentially obscure the exposure-outcome associations under investigation.

## 5.7 References

1. Knight JA, Vachon CM, Vierkant RA, Vieth R, Cerhan JR, Sellers TA. No association between 25-hydroxyvitamin D and mammographic density. *Cancer Epidemiol.Biomarkers Prev.* 2006;15:1988-1992.

2. Brisson J, Berube S, Diorio C, Sinotte M, Pollak M, Masse B. Synchronized seasonal variations of mammographic breast density and plasma 25-hydroxyvitamin D. *Cancer Epidemiol Biomarkers Prev.* 2007;16(5):929-933.

3. Green AK, Hankinson SE, Bertone-Johnson ER, Tamimi RM. Mammographic density, plasma vitamin D levels and risk of breast cancer in postmenopausal women. International Journal of Cancer. 2010;127(3):667-674. doi: 10.1002/ijc.25075

4. Chai, W., Maskarinec, G., Cooney, R.V. Serum 25-hydroxyvitamin D levels and mammographic density among premenopausal women in a multiethnic population. *Eur J Clin Nutr*. 2010;64(6):652-654.

5. Sprague, B.L., Trentham-Dietz, A., Gangnon, R.E., Buist, D.S.M., Burnside, E.S., Bowles, E.J.A., Stanczyk, F.Z., Sisney, G.S., Skinner , H.G. The vitamin D pathway and mammographic breast density among postmenopausal women. *Breast Cancer Res Treat*. 2012;131(1):255-265.

6. Rohan T. Epidemiological studies of vitamin D and breast cancer. *Nutrition Reviews*. 2007;65(1):80-83.

7. Cui Y, Rohan TE. Vitamin D, calcium, and breast cancer risk: A review. *Cancer Epidemiology, Biomarkers & Prevention*. 2006;15(8):1427-1437.

8. Kemmis CM, Salvador SM, Smith KM, Welsh J. Human mammary epithelial cells express CYP27B1 and are growth inhibited by 25-hydroxyvitamin D-3, the major circulating form of vitamin D-3. *J Nutr*. 2006;136(4):887-892.

9. Welsh J. Targets of vitamin D receptor signaling in the mammary gland. *J Bone Miner Res*. 2007;22(2):86.

10. Colston K, Hansen C. Mechanisms implicated in the growth regulatory effects of vitamin D in breast cancer. *Endocr Relat Cancer*. 2002;9(1):45-59.

11. Martin L, Boyd N. Mammographic density. potential mechanisms of breast cancer risk associated with mammographic density: Hypotheses based on epidemiological evidence. *Breast Cancer Research*. 2008;10(1):201.

12. Cuzick J, Warwick J, Pinney E, Warren RML, Duffy SW. Tamoxifen and breast density in women at increased risk of breast cancer. *Journal of the National Cancer Institute*. 2004;96(8):621-628. doi: 10.1093/jnci/djh106.

13. Kelemen LE, Pankratz VS, Sellers TA, et al. Age-specific trends in mammographic density: The minnesota breast cancer family study. *Am J Epidemiol*. 2008;167(9):1027-1036.

14. Vachon CM, Suman V, Brandt KR, et al. Mammographic breast density response to aromatase inhibition. *Clinical Cancer Research*. 2013. doi: 10.1158/1078-0432.CCR-12-2789.

15. Vachon C, Ghosh K, Brandt K. Mammographic density: Potential as a risk factor and surrogate marker in the clinical setting. *Current Breast Cancer Reports*. 2013;5(3):183-193. http://dx.doi.org/10.1007/s12609-013-0118-8. doi: 10.1007/s12609-013-0118-8.

16. Bertone-Johnson ER, McTiernan A, Thomson CA, et al. Vitamin D and calcium supplementation and one-year change in mammographic density in the women's health initiative calcium and vitamin D trial. *Cancer Epidemiology Biomarkers & Prevention*. 2012;21(3):462-473.

17. Boyd NF, Guo H, Martin LJ, et al. Mammographic density and the risk and detection of breast cancer. *N Engl J Med*. 2007;356(3):227-236. http://dx.doi.org/10.1056/NEJMoa062790. doi: 10.1056/NEJMoa062790.

18. Kerlikowske K, Ichikawa L, Miglioretti DL, et al. Longitudinal measurement of clinical mammographic breast density to improve estimation of breast cancer risk. *J Natl Cancer Inst*. 2007;99(5):386-395.

19. McCormack VA, dos Santos Silva I. Breast density and parenchymal patterns as markers of breast cancer risk: A meta-analysis. *Cancer Epidemiology, Biomarkers & Prevention*. 2006;15(6):1159-1169.

20. Maskarinec G, Pagano I, Lurie G, Kolonel LN. A longitudinal investigation of mammographic density: The multiethnic cohort. *Cancer Epidemiology Biomarkers & Prevention*. 2006;15(4):732-739. doi: 10.1158/1055-9965.EPI-05-0798.

21. Kim J, Han W, Moon H, et al. Breast density change as a predictive surrogate for response to adjuvant endocrine therapy in hormone receptor positive breast cancer. *Breast Cancer Research*. 2012;14(4):1-8. http://dx.doi.org/10.1186/bcr3221. doi: 10.1186/bcr3221.

22. Singletary SE. Rating the risk factors for breast cancer. *Annals of Surgery*. 2003;237(4):474.

23. Kristan J. Aronson P, Sally Campbell M, Janet Faith M, et al. Summary report: Review of lifestyle and environmental risk factors for breast cancer. Report of the Working Group on Primary Prevention of Breast Cancer. *Canadian Breast Cancer Initiative*. Minister of Public Works and Government Services Canada, 2001.

24. Phipps A, Li C. Endogenous hormones. In: Li C, ed. Springer New York; 2010:73-87. http://dx.doi.org/10.1007/978-1-4419-0685-4_4. 10.1007/978-1-4419-0685-4_4.

25. Li Yang and Kathryn H. Jacobsen. A systematic review of the association between breastfeeding and breast cancer. *Journal of Women's Health*. 2008;17(10):1635.

26. Douglas JA, Roy-Gagnon MH, Zhou C, et al. Mammographic breast density--evidence for genetic correlations with established breast cancer risk factors. *Cancer Epidemiology, Biomarkers & Prevention*. 2008;17(12):3509-3516.

27. Goss PE, Ingle JN, Alés-Martínez JE, et al. Exemestane for breast-cancer prevention in postmenopausal women. *New England Journal of Medicine*. 2011(364):2381.

28. Vachon CM, Ingle JN, Suman VJ, et al. Pilot study of the impact of letrozole vs. placebo on breast density in women completing 5 years of tamoxifen. *Breast*. 2007;16(2):204-210.

29. Fabian CJ, Kimler BF, Zalles CM, et al. Reduction in proliferation with six months of letrozole in women on hormone replacement therapy. *Breast Cancer Research & Treatment*. 2007;106(1):75-84.

30. Cigler T, Tu D, Yaffe MJ, et al. A randomized, placebo-controlled trial (NCIC CTG MAP1) examining the effects of letrozole on mammographic breast density and other end organs in postmenopausal women. *Breast Cancer Res Treat*. 2010;120(2):427-435. http://dx.doi.org/10.1007/s10549-009-0662-0. doi: 10.1007/s10549-009-0662-0.

31. Cigler T, Richardson H, Yaffe MJ, et al. A randomized, placebo-controlled trial (NCIC CTG MAP.2) examining the effects of exemestane on mammographic breast density, bone density, markers of bone metabolism and serum lipid levels in postmenopausal women. *Breast Cancer Res Treat*. 2011;126(2):453-461. http://dx.doi.org/10.1007/s10549-010-1322-0. doi: 10.1007/s10549-010-1322-0.

32. Prowell TM, Blackford AL, Byrne C, et al. Changes in breast density and circulating estrogens in postmenopausal women receiving adjuvant anastrozole. *Cancer Prevention Research*. 2011;4(12):1993-2001. doi: 10.1158/1940-6207.CAPR-11-0154.

33. Smith J, Dilawari A, Ursin G, et al. A pilot study of letrozole for one year in women at enhanced risk of developing breast cancer: Effects on mammographic density. *Anticancer Research*. 2012;32(4):1327-1331.

34. Mousa NA, Crystal P, Wolfman WL, Bedaiwy MA, Casper RF. Aromatase inhibitors and mammographic breast density in postmenopausal women receiving hormone therapy. *Menopause*. 2008;15(5):875-884.

35. Toriola A, Surcel H, Husing A, et al. Association of serum 25-hydroxyvitamin D (25-OHD) concentrations with maternal sex steroids and IGF-1 hormones during pregnancy. *Cancer Causes & Control*. 2011;22(6):925-928. http://dx.doi.org/10.1007/s10552-011-9752-5. doi: 10.1007/s10552-011-9752-5.

36. Diorio C, Berube S, Byrne C, et al. Influence of insulin-like growth factors on the strength of the relation of vitamin D and calcium intakes to mammographic breast density. *Cancer Res*. 2006;66(1):588-597.

37. Berube S, Diorio C, Verhoek-Oftedahl W, Brisson J. Vitamin D, calcium, and mammographic breast densities. *Cancer Epidemiol Biomarkers Prev*. 2004;13(9):1466-1472.

38. Tseng M, Byrne C, Evers K, Daly M. Dietary intake and breast density in high-risk women: A cross-sectional study. *Breast Cancer Research*. 2007;9(5):R72.

39. Anderson LN, Cotterchio M, Cole DEC, Knight JA. Vitamin D-related genetic variants, interactions with vitamin D exposure, and breast cancer risk among caucasian

women in ontario. *Cancer Epidemiology Biomarkers & Prevention*. 2011;20(8):1708-1717. doi: 10.1158/1055-9965.EPI-11-0300.

40. Tang C, Chen N, Wu M, etc. Fok1 polymorphism of vitamin D receptor gene contributes to breast cancer susceptibility: A meta-analysis. Breast Cancer Research (2009) 117:391–399. DOI 10.1007/s10549-008-0262-4

41. Chen WY, Bertone-Johnson ER, Hunter DJ, etc. Associations between polymorphisms in the vitamin D receptor and breast cancer risk. *Cancer Epidemiol.Biomarkers Prev.* 2005;14(10).

42. Raimondi S, Johansson H, Maisonneuve P, Gandini S. Review and meta-analysis on vitamin D receptor polymorphisms and cancer risk. *Carcinogenesis*. 2009;30(7):1170-1180.

43. McCullough ML, Bostick RM, Mayo TL. Vitamin D gene pathway polymorphisms and risk of colorectal, breast, and prostate cancer. *Annu Rev Nutr*. 2009;29(1):111-132. http://dx.doi.org/10.1146/annurev-nutr-080508-141248. doi: 10.1146/annurev-nutr-080508-141248.

44. Stroud ML, Stilgoe S, Stott VE, Alhabian O, Salman K. Vitamin D - a review. *Aust Fam Physician*. 2008;37(12):1002-1005.

45. Holick MF. Vitamin D deficiency. *N Engl J Med*. 2007;357(3):266-281.

46. Holick MF. Vitamin D status: Measurement, interpretation, and clinical application. *Ann Epidemiol*. 2009;19(2):73-78.

47. Mishra G, McCormack V, Kuh D, etc. Dietary calcium and vitamin D intakes in childhood and throughout adulthood and mammographic density in a british birth cohort. *British Journal of Cancer*. 2008;99:1539-1543.

48. Mammography Accreditation Program. Canadian Association of Radiologists. http://www.car.ca/en/accreditation/map.aspx. Published 2009.

49. Yaffe MJ. Mammographic density. measurement of mammographic density. *Breast Cancer Research*. 2008;10(3):209.

50. Boyd N, Martin L, Stone J, Little L, Minkin S, Yaffe M. A longitudinal study of the effects of menopause on mammographic features. *Cancer Epidemiology Biomarkers & Prevention*. 2002;11(10):1048-1053.

51. Boyd NF, Rommens JM, Vogt K, et al. Mammographic breast density as an intermediate phenotype for breast cancer.[erratum appears in lancet oncol. 2005 nov;6(11):826]. *Lancet Oncology*. 2005;6(10):798-808.

52. Harvey JA, Bovbjerg VE. Quantitative assessment of mammographic breast density: Relationship with breast cancer risk. *Radiology*. 2004;230(1):29-41.

53. Titus-Ernstoff L, Tosteson AN, Kasales C, et al. Breast cancer risk factors in relation to breast density (united states). *Cancer Causes & Control*. 2006;17(10):1281-1290.

54. El-Bastawissi AY, White E, Mandelson MT, Taplin SH. Reproductive and hormonal factors associated with mammographic breast density by age (united states). *Cancer Causes & Control*. 2000;11(10):955-963.

55. Boyd NF, Martin LJ, Li Q, et al. Mammographic density as a surrogate marker for the effects of hormone therapy on risk of breast cancer. *Cancer Epidemiology Biomarkers & Prevention*. 2006;15(5):961-966.

56. Boyd N. What accounts for variations in percent density. Written Communication, Nov 2010.

57. Pike MC. The role of mammographic density in evaluating changes in breast cancer risk. *Gynecol Endocrinol*. 2005;211(Suppl):1.

58. Brisson J, Berube S, Diorio C. [Breast density: A biomarker to better understand and prevent breast cancer]. *Bull Cancer*. 2006;93(9):847-855.

59. Fabian CJ, Kimler BF. Use of biomarkers for breast cancer risk assessment and prevention. *Journal of Steroid Biochemistry & Molecular Biology*. 2007;106(1-5):31-39.

60. J Cuzick, J Warwick, L Pinney, R Warren, S Cawthorn, A Howell and S Duffy. Change in breast density as a biomarker of breast cancer risk reduction; results from IBIS-1. Cancer Research. 2009: 69(2)suppl(1) doi: 10.1158/0008-5472.SABCS-61.

61. Byrne C, Spernak S. What is breast density? *Breast Cancer Online*. 2005;8(10):1-7.

62. Bonassi S, Au William W. Biomarkers in molecular epidemiology studies for health risk prediction. *Mutation Research*. 2002;511(1):73.

63. Breast Cancer Risk Assessment Tool. National Institutes of Health. http://www.cancer.gov/bcrisktool/. Published 2011.

64. Bertone-Johnson ER. Percent mammographic density readings of zero. Written communication, June 2012.

65. Yaghjyan L, Colditz G, Drake B. Vitamin D and mammographic breast density: A systematic review. *Cancer Causes & Control*. 2012;23(1):1-13. http://dx.doi.org/10.1007/s10552-011-9851-3. doi: 10.1007/s10552-011-9851-3.

66. Lokate M, Peeters PM, Peelen L, Haars G, Veldhuis W, van Gils C. Mammographic density and breast cancer risk: The role of the fat surrounding the fibroglandular tissue. *Breast Cancer Research*. 2011;13(5):1-8. http://dx.doi.org/10.1186/bcr3044. doi: 10.1186/bcr3044.

67. van Gils CH. Follow-up from AACR frontiers in cancer prevention conference. Written communication, Jan 2013.

68. Grant W. Differences in vitamin D levels may contribute to racial disparities in breast cancer. *Breast Cancer Res Treat*. 2013;138(3):967-968. http://dx.doi.org/10.1007/s10549-013-2480-7. doi: 10.1007/s10549-013-2480-7.

69. Neuhouser ML, Sorensen B, Hollis BW, et al. Vitamin D insufficiency in a multiethnic cohort of breast cancer survivors. *The American Journal of Clinical Nutrition*. 2008;88(1):133-139.

70. Yao S, Zirpoli G, Bovbjerg D, et al. Variants in the vitamin D pathway, serum levels of vitamin D, and estrogen receptor negative breast cancer among african-american

women: A case-control study. *Breast Cancer Research*. 2012;14(2):1-13. http://dx.doi.org/10.1186/bcr3162. doi: 10.1186/bcr3162.

71. Take vitamin D to reduce cancer risk, Canadian Cancer Society advises. http://www.cbc.ca/news/technology/take-vitamin-d-to-reduce-cancer-risk-canadian-cancer-society-advises-1.639301. Published 2007.

# Appendices

## Appendix 1: MAP.3 Initial Evaluation (Form 1)

NCIC Clinical Trials Group (NCIC CTG)

**FORM 1 - INITIAL EVALUATION**

To be submitted to NCIC CTG within 4 weeks of randomization

A PHASE III RANDOMIZED STUDY OF EXEMESTANE VERSUS PLACEBO
IN POSTMENOPAUSAL WOMEN AT INCREASED RISK OF DEVELOPING BREAST CANCER - MAP.3

**1. SUBJECT INFORMATION**

NCIC CTG Subject Serial No.: _____ Chart No.: _____ *(if permitted by REB/IRB)* Subject Initials: ____ ____ ____ (first-middle-last)

Institution: _____ Investigator: _____

IRB/REB Approval Date: __ __ __ __ - __ __ __ - __ __ *[initial approval or annual renewal]* yyyy mmm dd

Planned Start Date of Treatment: __ __ __ __ - __ __ __ - __ __ *[within 5 working days of randomization]* yyyy mmm dd

**EXCEPTION NUMBER (if granted):** _____

**Randomization Date:** __ __ __ __ - __ __ __ - __ __ yyyy mmm dd

**Treatment Kit Number dispensed**:_____

*Have you confirmed dispensing of this treatment via Mango?*

☐ *Yes* ☐ *No*

Did the subject consent to the optional blood collection for DNA banking?

☐ No ☐ Yes

*If yes, specify* ☐ Yes ☐ No for genetic testing

☐ Yes ☐ No possible future testing

Did the subject consent to the optional tissue banking?

☐ No ☐ Yes

*If yes, specify* ☐ Yes ☐ No for genetic testing

☐ Yes ☐ No possible future testing

*Note: a subject may consent to none, one or all of the optional components*

Was the subject enrolled on a companion study at the time of randomization? ☐ No ☐ Yes

*If yes, specify* ☐ *Bone Mineral Density (MAP.3B)*

**NOTE: Socio-demographic Information and Reproductive History are part of a stand-alone form [Form 1A]. Please attach.**

**NCIC CTG use only**

Logged: _____ Study Coord: _____ RA: _____ Data Ent'd: Verified: PC: _____ Monitor: _____

_____ - ___ - ___ _____ - ___ - ___ _____ - ___ - ___ _____ _____ _____ - ___ - ___ _____ - ___ - ___

**2. MEDICAL HISTORY / PHYSICAL FINDINGS (to be done within 8 weeks prior to randomization)**

**Date of Evaluation:** __ __ __ __ - __ __ __ - __ __
yyyy mmm dd

**Verification of postmenopausal status** *check one*:

☐ > 50 years of age with no spontaneous menses for at least 12 months;

☐ ≤ 50 years of age with no spontaneous menses (amenorrheic) within the past 12 months (i.e. spontaneous or secondary to hysterectomy)

AND with a FSH level within institution's postmenopausal range

☐ bilateral oophorectomy

**Verification of breast cancer risk factors** *check one [if #3 please indicate which one]*:

☐ A Gail score of >1.66 Score: ___.___

☐ Age ≥ 60 years

☐ One of the following (*please check one*)

☐ Prior Atypical Ductal Hyperplasia ☐ lobular hyperplasia ☐ lobular carcinoma in situ (LCIS) on breast biopsy.

☐ Prior DCIS treated with mastectomy

*(Path report required for ADH, LH, LCIS, DCIS)*

**Gail Score *(as calculated by the Gail Model Risk Assessment Tool):*** Score: ___.___ *(enter score of 0.0 if LCIS/DCIS checked)*

**Clinical Skeletal Fractures**

Has the subject experienced a bone fracture in the last 10 years?

☐ No ☐ Yes → If yes, please complete the entire table below

| Bone Fracture Site | *Check (✓)* | | | Date of Fracture (yyyy-mmm-dd) |
|---|---|---|---|---|
| | No | Yes | Unknown | |
| Spine | | | | |
| Wrist | | | | |
| Pelvis | | | | |
| Hip | | | | |
| Femur | | | | |
| Tibia | | | | |
| Ankle | | | | |
| Other *(specify)* | | | | |

If other specified, indicate site: _____

**Osteoporosis**

Has the subject had a diagnosis of osteoporosis (not osteopenia)? ☐ No ☐ Yes ☐ Unknown

Date of first diagnosis of osteoporosis: __ __ __ __ - __ __ __ - __ __
yyyy mmm dd

**2. MEDICAL HISTORY / PHYSICAL FINDINGS cont'd**

**Cardiovascular Morbidity**

Has the subject suffered cardiovascular disease?

☐ No ☐ Yes → If yes, please complete the entire table below

| Cardiovascular Event | *Check (✓)* | | | Date of onset |
|---|---|---|---|---|
| | No | Yes | Unknown | |
| Myocardial infarction | | | | |
| Stroke/transient ischemic attack (TIA) | | | | |
| On-going angina (no surgical intervention) | | | | |
| Angina requiring percutaneous transluminal coronary angioplasty (PTCA) | | | | |
| Angina requiring coronary artery bypass graft (CABG) | | | | |
| Thromboembolic event | | | | |
| Other *(specify)* | | | | |

If other specified, please describe event: _____

_____

**Cigarette Smoking History**

1. Please record the smoking history check one:

   ☐ fewer than 100 cigarettes in entire lifetime.

   ☐ ≥ 100 cigarettes in entire lifetime. also complete questions 2 to 4.

   ☐ Unknown

2. If the subject smoked > 100 cigarettes:

   What age did she start? _____ years old

3. Does the subject still smoke?

   ☐ YES

   ☐ NO → Age when subject quit _____ years old ☐ Unknown

4. On average, how many cigarettes do/did the subject smoke a day?

   Number of cigarettes per day: _____

## 2. MEDICAL HISTORY / PHYSICAL FINDINGS cont'd

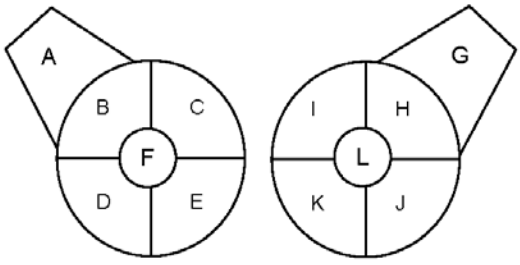
**Vital Signs**

| Height (without shoes) | Weight (without shoes) | Sitting Blood Pressure (mmHg) | Pulse(bpm) |
|---|---|---|---|
| ___ ___ ___ . ___ ☐cm **OR** ☐in | ___ ___ ___ . ___ ☐kg **OR** ☐lbs | ___ ___ ___ / ___ ___ ___ *systolic* *diastolic* | ___ ___ ___ |

**Breast Examination**

Date of Breast Examination: __ __ __ __ - __ __ __ - __ __ (yyyy - mmm - dd)

Breast Site Codes




Findings: ☐ Normal ☐ Abnormal

Breast Site Code(s): ☐ ☐ ☐

Comment(s) on finding(s):

**Are there any current (on-going) major medical conditions**? No ☐ Yes ☐ ⇐ specify below

| Body System Code* NCIC CTG Use Only | Date First Documented yyyy-mmm-dd | CONDITIONS | ✓STATUS Stable | ✓STATUS Unstable | TREATMENTS / COMMENTS |
|---|---|---|---|---|---|
| ___ ___ | | | ☐ S | ☐ U | |
| ___ ___ | | | ☐ S | ☐ U | |
| ___ ___ | | | ☐ S | ☐ U | |
| ___ ___ | | | ☐ S | ☐ U | |
| ___ ___ | | | ☐ S | ☐ U | |
| ___ ___ | | | ☐ S | ☐ U | |
| ___ ___ | | | ☐ S | ☐ U | |
| ___ ___ | | | ☐ S | ☐ U | |
| ___ ___ | | | ☐ S | ☐ U | |

*Body System Code Key:

| | | | |
|---|---|---|---|
| 01 = Abdomen | 06 = Chest / Chest Wall | 11 = Genitourinary / Renal | 16 = Musculoskeletal |
| 02 = Allergies / Immunology | 07 = Dermatologic / Skin | 12 = Gynecological | 17 = Neurologic |
| 03 = Breast | 08 = Endocrinologic / Metabolic | 13 = HEENT | 18 = Psychiatric |
| 04 = Blood / Bone Marrow | 09 = Extremities (incl Peripheral Vascular) | 14 = Hepatic | 19 = Respiratory / Pulmonary |
| 05 = Cardiovascular | 10 = Gastrointestinal | 15 = Lymphatic | 20 = Systemic Infectious Disease |
| | | | 21 = Other |

**3. LABORATORY INVESTIGATIONS** (to be done within 8 weeks prior to randomization)

**HEMATOLOGY** Specify collection date: __ __ __ __ - __ __ __ - __ __
yyyy mmm dd

| | *Value* | Lab Unit of Measure *(circle or specify)* |
|---|---|---|
| White Blood Count | __ __ __ | $x10^9$/L or 1000/uL or _____ |
| Granulocytes (neutrophil) | __ __ __ | $x10^9$/L or 1000/uL or _____ |
| Platelet Count | __ __ __ | $x10^9$/L or 1000/uL or _____ |
| Hemoglobin | __ __ __ __ __ | $x10^9$/L or 1000/uL or _____ |

**BIOCHEMISTRY** Specify collection date: __ __ __ __ - __ __ __ - __ __
yyyy mmm dd

| | *Value* | Lab Unit of Measure *(circle or specify)* | Range |
|---|---|---|---|
| Alkaline Phosphatase | __ __ __ | U/L or _____ | Alkaline Phosphatase UNL __ __ __ |
| Calcium | __ __ __ | mmol/L or mg/dL or _____ | Calcium LNL __ __ __ UNL __ __ __ |
| Creatinine | __ __ __ | µmol/L or mg/dL or _____ | Creatinine UNL __ __ __ |
| ALT (*SGPT*) | __ __ __ | U/L or _____ | ALT UNL __ __ __ |
| AST (*SGOT*) | __ __ __ | U/L or _____ | AST UNL __ __ __ |

**Other**

| | | | |
|---|---|---|---|
| Follicle Stimulating Hormone(FSH)* | __ __ __ | IU/L | Postmenopause LNL __ __ __UNL __ __ __ |

* *Required only if necessary to confirm postmenopausal status.*

**4. PRIOR MEDICATION USE**

Has the subject ever taken a SERM [tamoxifen, toremifene, raloxifene]? ☐ No ☐ Yes → If yes, please record below

| Agent | Start Date (yyyy-mmm-dd) | Stop Date (yyyy-mmm-dd) | Reason |
|---|---|---|---|
| | | | |
| | | | |

**5. CONCOMITANT MEDICATIONS**

Has the subject taken any medication within 7 days prior to randomization? ☐ No ☐ Yes →If yes, please record below.
*(If you answered "yes" to this question, please complete entire table below)*

| Agent Classification | Trade Name | Indication | Continuing? *(after randomization)* | |
|---|---|---|---|---|
| | | | No | Yes |
| Aspirin (*chronic low dose <100 mg)* | | prophylaxis | | |
| Bisphosphonate | | | | |
| Calcium | | | | |
| Lipid Lowering Drug | | | | |
| Cardiovascular Medication | | | | |
| NSAIDs | | | | |
| *Other:* | | | | |
| | | | | |
| | | | | |
| | | | | |
| | | | | |
| | | | | |
| | | | | |
| | | | | |
| | | | | |
| | | | | |

Is the subject using vaginal estrogen? ☐ No ☐ Yes →If yes, please record below

| Trade Name | Unit Dose | Frequency of use *(times per week)* | Continuing? *(after randomization)* | |
|---|---|---|---|---|
| | | | No | Yes |
| | | | | |
| | | | | |
| | | | | |

| NCIC CTG –MAP.3- FORM 1 - INITIAL EVALUATION | Subject. Serial #: _____ Initials: _____ _____ _____ |
|---|---|

**6. BASELINE SYMPTOMS/ADVERSE EVENTS (to be done within 7 days prior to randomization)**

Did the subject have any symptoms present on the day of baseline evaluation? ☐ No ☐ Yes If yes, enter details below:

| Baseline Symptoms/Adverse Events[1and 2] | A blank grade will be interpreted to mean the adverse event is absent. | |
|---|---|---|
| Short Name[3] | select sub-term[4] *(if applicable choose one option listed under "select" from the CTCAEv3)* | Grade[5] |
| Anorexia | | |
| Constipation | | |
| Cough | | |
| Dizziness | | |
| Edema: limb | | |
| Fatigue | | |
| Heartburn | | |
| Hemorrhage, GU | vagina | |
| Hot flashes | | |
| Hypertension | | |
| Mood alteration | | |
| Nausea | | |
| Pain | | |
| Rash | | |
| Sexual-Other *(specify)* | vaginal atrophy | |
| Sexual-Other *(specify)* | vaginal itch | |
| Sexual-Other *(specify)* | | |
| Sweating | | |
| Ulcer, GI | | |
| Vaginal discharge | | |
| Vaginal dryness | | |
| Vomiting | | |
| other CTC Adverse Event Term(s) not listed: | | |
| | | |
| | | |
| | | |
| | | |
| | | |

[1] Include all symptoms/findings present at baseline. All symptoms listed here by NCIC CTG must be followed on subsequent forms.

[2] On this page, it is not necessary to code and enter hematologic or biochemical adverse events for which actual dates and values are entered on page 8

[3] The **short name** describes the Adverse Event found in the NCI Common Terminology Criteria for Adverse Events (CTCAE) Version 3.0. On the case report form record, exactly, the **short name(s)** used in this criteria (e.g. perforation, GI).

[4] The **select sub-term** column is to be used to further define the short name. Allowable sub-terms must be selected from the "Adverse Event" column in the CTCAE Version 3.0 (e.g. colon).

[5] Grades can be found in the CTCAE Version 3.0.

| NCIC CTG –MAP.3- FORM 1 - INITIAL EVALUATION | Subject. Serial #: _____ Initials: _____ _____ _____ |
|---|---|

**7. BASELINE RADIOLOGY**

**DUAL ENERGY X-RAY ABSORPTIOMETRY (DEXA) of the $L_1$-$L_4$ Postero-Anterior Spine and Hip** — **To be done within 12 months prior to randomization**

Type of scanner (check 3 one):
☐ Hologic ☐ Lunar ☐ Other specify: _____

Date of DEXA: __ __ __ __ - __ __ __ - __ __ (yyyy mmm dd)

Location: ☐ Total Hip DEXA __. __ __ __ g/cm$^2$ T Score + __ . __ __ T Score - __ . __ __

☐ $L_1$-$L_4$ PA Spine DEXA __. __ __ __ g/cm$^2$ T Score + __ . __ __ T Score - __ . __ __

**BILATERAL MAMMOGRAM to be done within 12 months prior to randomization**

| | Not Done | Date (yyyy-mmm-dd) | Normal | Abnormal | |
|---|---|---|---|---|---|
| | | | | Not Malignant | Malignant |
| Mammogram | | | | | |
| Comments | | | | | |
| | | | | | |
| | | | | | |

**8. QUALITY OF LIFE ASSESSMENT**

Were the QoL questionnaires completed? SF-36 ☐ Yes ⇐ language ☐ English ☐ French ☐ Spanish ☐ **No**

MENQOL ☐ Yes ⇐ language ☐ English ☐ French ☐ Spanish ☐ **No**

**Please complete a QoL cover sheet to document reason(s)**

☐ **Not Required QoL waived for this subject due to inability (illiteracy, loss of sight, other equivalent reason)**

**9. COMMENTS**

**10. SUPPORTING DOCUMENTATION Have you attached copies of: (please3)**

*All documentation must have trial code and NCIC CTG subject serial number*

| | | | |
|---|---|---|---|
| Signed informed consent form | ☐ Yes ☐ No | DEXA | ☐ Yes ☐ No |
| Genetic testing consent form | ☐ Yes ☐ No | Mammogram Report | ☐ Yes ☐ No |
| Form 1a | ☐ Yes ☐ No | Quality of Life questionnaires | ☐ Yes ☐ No |
| Serum/DNA Banking Submission Report | ☐ Yes ☐ No | Laboratory reports | ☐ Yes ☐ No |

**Note: Please remove identifying information from supporting documentation**

**17. INVESTIGATOR SIGNATURE**

Investigator Signature: _____

Person Completing Form, First and Last Name: _____

Form Completion Date: __ __ __ __ - __ __ __ - __ __ (yyyy mmm dd)

*When completed please mail to:*
*National Cancer Institute of Canada, Clinical Trials Group, Queen's University, 10 Stuart Street, Kingston, Ontario, K7L 3N6*

**NCIC CTG use only**
STRATIFICATION: low dose aspirin: ☐ Yes ☐ No Gail Score: ☐ ≤2 ☐ >2 Correct at randomization: ☐ Yes ☐ No

## Appendix 2: MAP.3 Socio-Demographic Information (Form 1A)

National Cancer Institute of Canada
Clinical Trials Group (NCIC CTG)

**FORM 1A - SOCIO-DEMOGRAPHIC AND REPRODUCTIVE HISTORY**

To be completed on all subjects, attached to the Form 1 – Initial Evaluation, and submitted within 4 weeks of randomization

A PHASE III RANDOMIZED STUDY OF EXEMESTANE VERSUS PLACEBO
IN POSTMENOPAUSAL WOMEN AT INCREASED RISK OF DEVELOPING BREAST CANCER - MAP.3

**1. SUBJECT INFORMATION**

NCIC CTG Subject Serial No.: _____ Chart No.: _____ *(if permitted by REB/IRB)* Subject Initials: ____ ____ ____ (first-middle-last)

Institution: _____ Investigator: _____

**2. SOCIO-DEMOGRAPHIC INFORMATION (to be done within 8 weeks prior to randomization)**

**Date of evaluation**: __ __ __ __ - __ __ __ - __ __ (yyyy mmm dd) DOB: __ __ __ __ - __ __ __ - __ __ (yyyy mmm dd) Zip/Postal Code _____

Subject Race (check **all that apply**) ***Subject is encouraged to self designate***

- ☐ White
- ☐ Black or African American
- ☐ Native Hawaiian or other Pacific Islander
- ☐ American Indian or Alaska Native
- ☐ Asian
- ☐ Unknown

Subject Ethnicity (check **one only**)

- ☐ Hispanic or Latino
- ☐ Non-Hispanic
- ☐ Unknown

***(CRA Interview with Subject)***
***Please read the instructions and questions to the subject exactly as they are written. Check all the appropriate answers.***

*The next few questions are about you and your life situation. This information will help us determine whether quality of life of all the women in this trial is affected in the same way, or whether some groups of women are affected differently.*
*The questions will also help us compare your quality of life to that of other Canadian, American and Spanish women like you*

Presently, are you:
- ☐ Single [never legally married]
- ☐ Legally married
- ☐ Separated [but still legally married]
- ☐ Divorced
- ☐ Widowed

With whom do you currently live? (***Please check all that apply***)
- ☐ On your own
- ☐ With partner/spouse
- ☐ With children/grandchildren
- ☐ With parent(s)
- ☐ With other relatives
- ☐ With friends
- ☐ Other [please specify] _____

Among the people you know, is there someone you can confide in or discuss problems with?
- ☐ No
- ☐ Yes → If yes: How many people like this do you have? _____ people

What is the highest level of formal education you have completed
- ☐ Elementary school
- ☐ Some high school
- ☐ High school diploma
- ☐ Technical/Community College or CEGEP
- ☐ Some university
- ☐ Bachelor's Degree at University [BA, BSc, LLB]
- ☐ University degree above a Bachelor's degree

Do you currently have a paid job or are you self-employed?
- ☐ No
- ☐ Yes → If yes: On average, do you work
  - ☐ 30 or more hours per week
  - ☐ less than 30 hours per week

**NCIC CTG use only**

Logged: _____ Study Coord: _____ RA: _____ Data Ent'd: Verified: PC: _____ Monitor: _____
_____-___-__ _____-___-__ _____-___-__ _____ _____ _____-___-__ _____-___-__

| NCIC CTG - MAP.3 FORM 1A | SOCIO-DEMOGRAPHIC AND REPRODUCTIVE HISTORY | Subject ID: _____ Subject Initials: _____ _____ _____ *(first - middle - last)* |
|---|---|---|

***(CRA Interview with Subject - CONTINUED)***

| | | |
|---|---|---|
| *In this trial, the questionnaires about your quality of life are only available in English, French or Spanish. For this reason, we would like to ask you a couple of questions about the language or languages you understand and use in your everyday life.* | | |
| What is the first language you learned and are still able to speak? | ☐ English<br>☐ French | ☐ Spanish<br>☐ other (specify) _____ |
| What language do you yourself usually speak at home (if you speak more than one, which one do you speak most often)? | ☐ English<br>☐ French | ☐ Spanish<br>☐ other (specify) _____ |
| *Finally, it is known that income is still an important contributor to a person's overall health. For this reason, we are asking you to give us your best estimate of your family's income level last year* | | |
| What was your combined family income from all sources before taxes last year? | ☐ Below $20,000<br>☐ $20,000 to $39,999<br>☐ $40,000 to $59,999 | ☐ $60,000 to $79,999<br>☐ $80,000 or more<br>☐ Don't know / refuse to answer |

**3. REPRODUCTIVE HISTORY *(CRA Interview with Subject)* – (to be done within 8 weeks of randomization)**

1. At what age did you first start your menstrual period? _____ years old
   Date of last menstrual period: __ __ __ __ - __ __ __ - __ __ (yyyy - mmm - dd)
   Usual length of menstrual cycle (from the start of one period to the start of the next) : _____ days
   Menstrual cycles were usually: regular ☐ irregular ☐

2. Have you ever used birth control pills? No ☐ Yes ☐
   a) At what age did you stop taking birth control pills? _____ years old
   b) How long in total did you take birth control pills? _____ months

3. Have you ever been on hormone replacement therapy? No ☐ Yes ☐
   *please indicate reason:* Menopausal symptoms ☐
   Bone disease prevention ☐
   Other (specify) : _____
   At what age did you start? _____ years How long did you take it? _____ months
   Did you take: tablets No ☐ Yes ☐
   patches No ☐ Yes ☐
   cream/Estring No ☐ Yes ☐
   Was it? Opposed ☐ unopposed ☐

| NCIC CTG - MAP.3 FORM 1A | SOCIO-DEMOGRAPHIC AND REPRODUCTIVE HISTORY | Subject ID: _____ Subject Initials: _____ _____ _____ *(first - middle - last)* |
|---|---|---|

***(CRA Interview with Subject- CONTINUED)***

4 Have you ever given birth? No ☐ Yes ☐ → number of pregnancies _____
How old were you when you gave birth for the first time? _____ years old

5. Have you ever had any pregnancies that did not go to full term?
No ☐ Yes ☐ → how many? _____ How old were you the first time? _____ years old

6. Have you ever breastfed? No ☐ Yes ☐ → how many months in total: _____ months

7. Have you ever had a breast biopsy? No ☐ Yes ☐ → reason: _____
↓
Was it core ☐ FNA ☐ Excision ☐

8. Have you ever had breast surgery? No ☐ Yes ☐ → reason: _____

9. Do you have a history of benign breast disease? i.e. nodular hyperplasia, fibrocystic disease, LCIS, DCIS?
No ☐ Yes ☐ → specify: _____

10. Is there a history of benign breast disease in your family? No ☐ Yes ☐

| Please specify disease, if known, e.g. ADH, LCIS, DCIS, fibrocystic | Relationship to you |
|---|---|
| | ☐ mother ☐ daughter ☐ sister ☐ grandmother ☐ aunt |
| | ☐ mother ☐ daughter ☐ sister ☐ grandmother ☐ aunt |
| | ☐ mother ☐ daughter ☐ sister ☐ grandmother ☐ aunt |
| | ☐ mother ☐ daughter ☐ sister ☐ grandmother ☐ aunt |

11. Is there a history of malignant breast disease in your family? No ☐ Yes ☐

| Please specify disease, if known, e.g. invasive, inflammatory | Relationship to you |
|---|---|
| | ☐ mother ☐ daughter ☐ sister ☐ grandmother ☐ aunt |
| | ☐ mother ☐ daughter ☐ sister ☐ grandmother ☐ aunt |
| | ☐ mother ☐ daughter ☐ sister ☐ grandmother ☐ aunt |
| | ☐ mother ☐ daughter ☐ sister ☐ grandmother ☐ aunt |

| NCIC CTG - MAP.3 FORM 1A | SOCIO-DEMOGRAPHIC AND REPRODUCTIVE HISTORY | Subject ID: _____ Subject Initials: _____ _____ _____ *(first - middle - last)* |
|---|---|---|

| 4. HISTORY OF CANCER | | | | |
|---|---|---|---|---|
| Have you or any member of your family, been diagnosed with cancer? *(check all that apply)* | No | Yes | Don't know | Relationship (mother/father/sibling etc) |
| Ovarian | ☐ | ☐ | ☐ | |
| Colon | ☐ | ☐ | ☐ | |
| Prostate | ☐ | ☐ | ☐ | |
| Lung | ☐ | ☐ | ☐ | |
| Other: _____ | ☐ | ☐ | ☐ | |
| Other: _____ | ☐ | ☐ | ☐ | |

**This must be signed and dated by the interviewer. When completed, please attach a copy to the Form 1.**

**Name of Interviewer:** _____

**Signature of Interviewer:** _____

**Interview Date:** __ __ __ __ - __ __ __ - __ __
yyyy mmm dd

*When completed please attach to the Form 1 – Initial Evaluation and mail to:*

*National Cancer Institute of Canada, Clinical Trials Group, Queen's University, 10 Stuart Street, Kingston, Ontario, K7L 3N6*

*When signed and dated by the interviewer, this stand alone form may be considered source documentation for the purpose of monitoring visits.*

## Appendix 3: Letter to MAP.3 Centres for Requests for Mammograms

NCIC Clinical Trials Group
NCIC Groupe des essais cliniques

P 613.533.6430 Cancer Clinical Trials Division
F 613.533.2941 Cancer Research Institute
F 613.533.2411 Queen's University
10 Stuart Street
www.ctg.queensu.ca Kingston ON Canada K7L 3N6

ExCe
Working together to prevent breast

Date: August 3, 2011

To: MAP.3 Canadian Principal Investigators and Principal Clinical Research Associates

From: Harriet Richardson, Project Coordinator

Re: A Phase III Randomized Study of Exemestane vs. Placebo in Postmenopausal Women at Increased Risk of Developing Breast Cancer MAP.3 / ExCel

***** MAP.3 Request for Mammograms*****

As part of MAP.3, participants were required to have a baseline bilateral mammogram (2 view screening) within 12 months prior to randomization and then every 12 months from the time of the initial mammogram. *[Please ensure you have forwarded all mammogram reports to NCIC CTG as required per protocol.]* As of amendment #4, the main MAP.3 consent form informs participants that researchers will be borrowing their mammograms to measure breast density in order to learn about the role that breast density has on breast cancer risk and how changes in density may influence changes in breast cancer risk. For example, correlative sciences projects to examine exemestane and breast density and vitamin D and breast density will be conducted. Participating Canadian centres have received research ethics board approval for amendment #4 and participants were to be re-consented to study.

At this time, we would like to request the retrieval of mammograms (baseline + follow-up mammograms done at the same radiology clinic) for each participant who has greater than 3 years of follow-up on the MAP.3 trial to date. At least two mammograms are required in order to evaluate the change in breast density over time. Centre-specific spreadsheets identifying these participants at your individual sites will follow under separate cover.

Once you receive the spreadsheet, please do the following:

(1) Contact the radiology departments listed in the spreadsheet and, for each participant, request the mammograms specified. Contact information for all relevant radiology departments is provided in the spreadsheet.
(2) We have provided template letters to relevant radiology clinics for your use. Review the accompanying template letters and complete any required information. Please take note of the specifications within and append any relevant documents (i.e. signed consent forms).
(3) If film mammograms are available please request the original films [not copies].
(4) Have all mammograms sent to your site for forwarding to the Hotel Dieu Hospital in Kingston, ON. Address below and courier instructions to follow.

The Hotel Dieu Hospital in Kingston Ontario is a representative of NCIC CTG for the measurement of mammographic breast density in MAP.3 participants who have been followed for > 3 years. The mammogram images, including any identifying information, will be kept behind locked doors and stored confidentially in files maintained at the Hotel Dieu Hospital in Kingston ON. Film based mammograms will be digitized at the Hotel Dieu Hospital. All personal identifying information from either film-based or digital mammograms will be removed and annotated with the MAP.3 NCIC CTG subject serial number prior to breast density measurement.

As per above, please send the mammograms (each labelled with the MAP.3 NCIC CTG subject serial number) to the Hotel Dieu Hospital at the following address:

Hotel Dieu Hospital
Radiology department
Attn: Kim Attwood
Re: NCIC CTG MAP.3 Trial
166 Brock St.
Kingston ON
K7L 5 G2

The Hotel Dieu Hospital will strive to return all film based mammograms to the originating radiology clinics within 2-3 weeks of receipt via courier. Centres will receive a stipend of $100 per participant for the retrieval of these mammograms.

If you have any questions regarding the above process please do not hesitate to contact Melanie Walker at 613-533-6430 who will be coordinating the retrieval of mammograms.

Thank you for all of your hard work and continued support of the MAP.3 trial.

Yours sincerely,

Harriet Richardson Ph.D
MAP.3 Project Coordinator

## Appendix 4: Information Package Sent to Centres for Mammogram Retrieval

NCIC Clinical Trials Group
NCIC Groupe des essais cliniques

P 613.533.6430 Cancer Clinical Trials Division
F 613.533.2941 Cancer Research Institute
F 613.533.2411 Queen's University
10 Stuart Street
www.ctg.queensu.ca Kingston ON Canada K7L 3N6

ExCel
Working together to prevent breast cancer

Date: August 4, 2011

To: MAP.3 Canadian Principal Clinical Research Associates

From: Melanie Walker, Study Coordinator

Re: A Phase III Randomized Study of Exemestane vs. Placebo in Postmenopausal Women at Increased Risk of Developing Breast Cancer MAP.3 / ExCel

***** MAP.3: Courier Information for Transfer of Mammograms *****

Dear Collaborators,

It was communicated to you recently that we will be retrieving mammograms from each participant who has greater than 3 years of follow-up on the MAP.3 trial to date at your centres.

As was also previously indicated, once mammograms from the originating radiology departments / clinics have been received at your centre please send all mammograms to the Hotel Dieu Hospital in Kingston, ON via **FedEx**. The Hotel Dieu Hospital is a representative of NCIC CTG for the measurement of mammographic breast density in these MAP.3 participants. As we will be covering the cost of sending and returning mammograms it is imperative that you include the specific information below on the FedEx form at the time of shipping:

**Queen's University FedEx Information:**

| Accounts | Account Numbers | Instructions |
|---|---|---|
| FedEx Account Number | 153166757 | Put in the area of the form where you note it is being sent |
| Queen's Chartfield Account Number | 30000 13206 608002 369552 | This MUST be put in the part of the form where it says "Reference" |
| *** *Please scan and .pdf a copy of the bill of lading and send via email to Lee Watkins at lmw@queensu.ca and Melanie Walker at mwalker@ctg.queensu.ca* *** | | |

In an effort to keep track of all shipments and ensure that we accommodate the shipping budget allotted for this study, please take note of the following:

- Include the 'Transfer of Mammograms' form appended to this letter with each shipment sent to the Hotel Dieu Hospital.
- Ensure the mammogram (film or digital) for a given participant is labelled with a note that includes the NCIC CTG unique participant identifier, the date the mammogram was taken and the visit (i.e. baseline or year 3, 4 or 5 follow-up).
- Ship mammograms once / week (i.e. Friday mornings). Please note that you need to wait for the baseline and follow-up mammograms for a given participant before sending (i.e. send as a package).
- If radiology clinics/departments will not release mammograms without coverage for courier costs the FedEx information above may be provided. This information should only be provided if requested and radiology clinics using our FedEx account must scan and send a .pdf copy of the bill of lading to the individuals noted above.
- Send the mammograms to the Hotel Dieu Hospital at the following address:

  Hotel Dieu Hospital
  Radiology department
  **Attn: Marie Pitcher**
  Re: NCIC CTG MAP.3 trial
  166 Brock St.
  Kingston ON
  K7L 5G2

The Hotel Dieu Hospital will strive to return all film based mammograms to the originating radiology clinic within 2-3 weeks of receipt via courier. Digital images received on CD will be destroyed after the study is complete.

If you have any questions regarding the above process please do not hesitate to contact me at 613-533-6430. I look forward to working with you.

Yours sincerely,

Melanie Walker (PhD candidate)
Study Coordinator

## "Transfer of Mammograms" Form

*Checklist:*

- ☐ *The 'transfer of mammograms' form is complete and a copy is included with this shipment of participant mammograms*
- ☐ *All mammograms included in this shipment are identified with the NCIC CTG participant ID, the date the mammogram was taken and the follow-up visit (i.e. baseline or year 3, 4 or 5 follow-up)*
- ☐ *All mammograms received this week have been bundled and are being sent as one shipment (Friday mornings preferable)*
- ☐ *All mammograms for a given participant have been received and are included in this shipment*
- ☐ *Mammograms have been sent via FedEx with all required account information*
- ☐ *Mammograms have been sent to the Hotel Dieu Hospital in Kingston, ON to the attention of Marie Pitcher*
- ☐ *A .pdf copy of the FedEx bill of lading has been emailed to Lee Watkins and Melanie Walker*

**Baseline Mammograms:**

| MAP.3 Participant Identifier | Date of Baseline Mammogram (yyyy/mmm/dd) | Film or Digital? | Date of Shipment to Hotel Dieu Hospital (yyyy/mmm/dd) | Address of Radiology Dept where mammogram should be returned |
|---|---|---|---|---|
| | | | | |
| | | | | |
| | | | | |
| | | | | |
| | | | | |

**Follow-Up Mammograms:**

| MAP.3 Participant Identifier | Date of Follow-Up Mammogram (yyyy/mmm/dd) | Film or Digital? | # Years of Follow-Up (3, 4, 5, etc.) | Date of Shipment to Hotel Dieu Hospital (yyyy/mmm/dd) | Address of Radiology Dept where mammogram should be returned |
|---|---|---|---|---|---|
| | | | | | |
| | | | | | |
| | | | | | |
| | | | | | |
| | | | | | |
| | | | | | |

**Winnipeg Health Sciences Centre**

*<please insert on hospital/institution letterhead>*

19 August 2011

To: Department of Diagnostic Imaging
Attn: Film Library

From: *<insert PI/Clinical Research Associate names>*

Re: Mammogram Retrieval for Clinical Trial Participants

## *** Request for Mammograms***

Our centre is participating in a breast cancer chemoprevention clinical trial sponsored by the NCIC Clinical Trials Group (NCIC CTG) in Kingston, Ontario. Participation in this clinical trial requires that women have a baseline bilateral mammogram within 12 months prior to trial randomization and every 12 months thereafter. Some of the women our centre has participating in this trial have had mammograms done at your radiology clinic. At this time, we would like to request the retrieval of select mammograms for some of these women identified below. This clinical trial has received ethics approval from our overseeing REB and women who have voluntarily joined this study have already provided consent to lend their mammograms for the measurement of breast density. Please find appended to this letter a copy of the signed participant consent forms for the relevant individuals.

One of the objectives of the clinical trial is to learn about the role that breast density has on breast cancer risk and how changes in density may influence changes in breast cancer risk. At least two mammograms for each participant will be requested in order to evaluate the change in breast density over time.

We would greatly appreciate your help in retrieving the mammograms for each participant appended to this memo keeping in mind the following:

1. **If the format of the mammograms you are retrieving are <u>film/analog</u>:**

   - Please <u>do not</u> send copies; we must receive the original films for the measurement of breast density within the research protocol. As well, copies that have been digitized are not acceptable.
   - Please only send mammograms – no other films done for these women at your radiology department should be sent to us.
   - Send films with the <u>craniocaudal</u> view only.

- If your centre is not permitted to release original films and original digitized film mammograms are being sent to us please specify the digitizer that was used and what resolution the film was scanned at (please note that a minimum of 250 microns / pixel is required for the measurement of breast density in this study). Only digitized images that are in DICOM [Digital Imaging and Communications in Medicine] (.dcm) format are acceptable.

  Note, we prefer to have our collaborating hospital digitize all original film based mammograms for research purposes. These mammograms will be digitized by a mammographic quality digitizer at the Hotel Dieu Hospital in Kingston ON. The iCad digitizer will be used to produce a dicom digital image for all film based mammograms, with the GE RA600 and PACS Cube used to remove patient demographics, annotate with the participant study identifier and/or burn images to CD, as required, for all images.

- Hotel Dieu Hospital (Kingston, ON) will strive to return original films to your centre within 2-3 weeks of receipt via courier.

2. **If the format of the mammograms you are retrieving are digital:**

   - Please ensure they are in universally accepted DICOM image format
   - Send digital images with the craniocaudal view only.
   - Please note digital images should be sent on CD and will not be returned to the centre.

Please send the mammograms (film or digital) directly to us for forwarding to the radiology department of the Hotel Dieu Hospital in Kingston ON. Please ensure the mammogram is appropriately identified with the woman's name and is tagged with the date so the baseline and follow-up mammograms for a given individual are easily distinguishable.

We greatly appreciate your timely attention to this request.

Yours sincerely,

<*insert name*>

**Winnipeg Health Sciences Centre**

Participant Identifier: **CARM0001**

Patient Name: Click here to enter text. Date of Birth: Click here to enter text. Health Card: Click here to enter text.

| Baseline Mammogram Date | *Film or Digital? | Mammogram #1 Date | *Film or Digital? | Mammogram #2 Date | *Film or Digital? | Mammogram #3 Date | *Film or Digital? | Mammogram #4 Date | *Film or Digital | Mammogram #5 Date | *Film or Digital | Mammogram #6 Date | Film or Digital? |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 5/30/2005 | | 6/20/2008 | | 6/5/2009 | | 6/18/2010 | | | | | | | |

Participant Identifier: **CARM0005**

Patient Name: Click here to enter text. Date of Birth: Click here to enter text. Health Card: Click here to enter text.

| Baseline Mammogram Date | *Film or Digital? | Mammogram #1 Date | *Film or Digital? | Mammogram #2 Date | *Film or Digital? | Mammogram #3 Date | *Film or Digital? | Mammogram #4 Date | *Film or Digital | Mammogram #5 Date | *Film or Digital | Mammogram #6 Date | Film or Digital? |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 9/23/2005 | | 10/24/2008 | | 10/23/2009 | | 10/22/2010 | | | | | | | |

Participant Identifier: **CARM0008**

Patient Name: Click here to enter text. Date of Birth: Click here to enter text. Health Card: Click here to enter text.

| Baseline Mammogram Date | *Film or Digital? | Mammogram #1 Date | *Film or Digital? | Mammogram #2 Date | *Film or Digital? | Mammogram #3 Date | *Film or Digital? | Mammogram #4 Date | *Film or Digital | Mammogram #5 Date | *Film or Digital | Mammogram #6 Date | Film or Digital? |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 10/28/2005 | | 10/19/2009 | | 10/5/2010 | | | | | | | | | |

Participant Identifier: **CARM0021**

Patient Name: Click here to enter text. Date of Birth: Click here to enter text. Health Card: Click here to enter text.

| Baseline Mammogram Date | *Film or Digital? | Mammogram #1 Date | *Film or Digital? | Mammogram #2 Date | *Film or Digital? | Mammogram #3 Date | *Film or Digital? | Mammogram #4 Date | *Film or Digital | Mammogram #5 Date | *Film or Digital | Mammogram #6 Date | Film or Digital? |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 4/4/2006 | | 3/18/2010 | | 3/29/2011 | | | | | | | | | |

Participant Identifier: **CARM0024**

Patient Name: Click here to enter text. Date of Birth: Click here to enter text. Health Card: Click here to enter text.

| Baseline Mammogram Date | *Film or Digital? | Mammogram #1 Date | *Film or Digital? | Mammogram #2 Date | *Film or Digital? | Mammogram #3 Date | *Film or Digital? | Mammogram #4 Date | *Film or Digital | Mammogram #5 Date | *Film or Digital | Mammogram #6 Date | Film or Digital? |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 8/23/2006 | | 9/18/2009 | | 9/28/2010 | | | | | | | | | |

Participant Identifier: **CARM0029**

Patient Name: Click here to enter text. Date of Birth: Click here to enter text. Health Card: Click here to enter text.

| Baseline Mammogram Date | *Film or Digital? | Mammogram #1 Date | *Film or Digital? | Mammogram #2 Date | *Film or Digital? | Mammogram #3 Date | *Film or Digital? | Mammogram #4 Date | *Film or Digital | Mammogram #5 Date | *Film or Digital | Mammogram #6 Date | Film or Digital? |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 10/3/2006 | | 10/30/2009 | | 10/8/2010 | | | | | | | | | |

Participant Identifier: **CARM0031**

Patient Name: Click here to enter text. Date of Birth: Click here to enter text. Health Card: Click here to enter text.

| Baseline Mammogram Date | *Film or Digital? | Mammogram #1 Date | *Film or Digital? | Mammogram #2 Date | *Film or Digital? | Mammogram #3 Date | *Film or Digital? | Mammogram #4 Date | *Film or Digital | Mammogram #5 Date | *Film or Digital | Mammogram #6 Date | Film or Digital? |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 9/26/2006 | | 11/20/2009 | | 11/5/2010 | | | | | | | | | |

Participant Identifier: **CARM0032**

Patient Name: Click here to enter text. Date of Birth: Click here to enter text. Health Card: Click here to enter text.

| Baseline Mammogram Date | *Film or Digital? | Mammogram #1 Date | *Film or Digital? | Mammogram #2 Date | *Film or Digital? | Mammogram #3 Date | *Film or Digital? | Mammogram #4 Date | *Film or Digital | Mammogram #5 Date | *Film or Digital | Mammogram #6 Date | Film or Digital? |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 10/27/2006 | | 10/27/2009 | | 10/6/2010 | | | | | | | | | |

Participant Identifier: **CARM0038**

Patient Name: Click here to enter text. Date of Birth: Click here to enter text. Health Card: Click here to enter text.

| Baseline Mammogram Date | *Film or Digital? | Mammogram #1 Date | *Film or Digital? | Mammogram #2 Date | *Film or Digital? | Mammogram #3 Date | *Film or Digital? | Mammogram #4 Date | *Film or Digital | Mammogram #5 Date | *Film or Digital | Mammogram #6 Date | Film or Digital? |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 2/2/2007 | | 5/4/2010 | | 5/11/2025 | | | | | | | | | |

## Appendix 5: Service Agreement between NCIC Clinical Trials Group, Queen's University and Hotel Dieu Hospital, Kingston Ontario

**Agreement for Research Services**

Effective Date: July 21, 2011

To:
Religious Hospitallers of Saint Joseph
of the Hotel Dieu of Kingston ("**Hotel Dieu**")
166 Brock Street
Kingston ON K7L 5G2

From:
Queen's University at Kingston ("**Queen's**")
NCIC Clinical Trials Group
10 Stuart Street
Kingston ON K7L 3N6

**RE: COORDINATION, DIGITIZATION AND DE-IDENTIFICATION SERVICES FOR MAP.3 PARTICIPANT MAMMOGRAMS**

By signing this Agreement for Research Services, Hotel Dieu and Queen's agree to the following terms:

1. Hotel Dieu shall provide the research services set out in the attached Project Specifications, on behalf of Queen's, for the study entitled *"A Phase III Randomized Study of Exemestane Versus Placebo in Postmenopausal Women at Increased Risk of Developing Breast Cancer" (MAP.3).*

2. Queen's shall pay for the research services in the amounts specified, inclusive of all applicable taxes, in the attached cost estimation as provided by Hotel Dieu as well as reimbursement for courier services.

3. In providing the Services, Hotel Dieu shall be responsible for ensuring its relevant personnel are aware of and abide by the requirement to keep strictly confidential all personal information and personal health information provided by Queen's to Hotel Dieu under this Agreement.

Queen's University at Kingston

Susan Wood
Signature of Authorized Officer
Name: Susan Wood Ph.D
Title: Director, Research Services

RALPH M. MEYER MD FRCPC
DIRECTOR
NCIC-CTG
Aug 4/11

Religious Hospitallers of Saint Joseph of the Hotel Dieu of Kingston

Marie Pitcher
Signature of Authorized Officer
Name: MARIE PITCHER
Title: PROGRAM MANAGER

Karen Pearson
Signature of Authorized Officer
Name: KAREN PEARSON
Title: DIRECTOR of IMAGING SERVICES

Hotel Dieu shall provide the following research services for the MAP.3 trial:

- Receipt of mammogram images from MAP.3 participating centres in Canada and in Buffalo, New York for ~500 trial participants.
- Confidential storage of mammogram images in files maintained at Hotel Dieu with access by limited personnel.
- Digitization of film based mammograms by a mammographic quality digitizer (the iCad digitizer)
- De-identification of mammograms, annotation with the NCIC CTG unique identifier and the burning of images to CD, as required, for all images with the GE RA600 and PACS Cube.
- Maintenance of a tracking system (developed by Queen's) for all mammograms received including the NCIC CTG unique identifier, date of receipt at Hotel Dieu, date of the mammogram, date of digitization (if applicable), date of return or destruction.
- Once digitized, Hotel Dieu will return the films to the originating mammography radiology department/clinic via courier.
- Destruction of digital images when no longer required by Queen's.
- All required set-up of mammograms in the Cumulus software for breast density measurement by the study radiologist (Dr. D Jabs).
- At study completion, transfer of the mammogram tracking system directly to Queen's (NCIC CTG) which will include the breast density measurements for each participant. No identifying information is to be sent to Queen's.
- Hotel Dieu will not use the data without subsequent agreement between Queen's (NCIC CTG) and faculty at Hotel Dieu hospital.

Religious Hospitallers
of Saint Joseph
of the Hotel Dieu of Kingston

HOTEL DIEU HOSPITAL
166 BROCK STREET
KINGSTON, ONTARIO K7L 5G2

Telephone (613) 544-3310
auto attendant (613) 544 3400
website www.hotelDieu.com

**RESEARCH PROPOSAL**
**QUEEN'S UNIVERSITY**

**Vitamin D and Breast Cancer Risk in Postmenopausal Women**
**Principle Investigators:**
**Dr. Will King**
**Dr. Harriet Richardson**

Dear Drs. King and Richardson,

It is estimated that this proposal requires the digitizing/importing of 750 mammograms, anonymisation of patient demographics, the annotation of unique study identifiers and the burning of CDs.

Proposal requires the use of a mammographic quality digitizer, the use of the RA 600 and PACS Cube, all of which currently reside in the imaging department of Hotel Dieu.

Proposal also requires some software integration in a specific reading station (yet to be determined) that would allow for the measurement of tissue density.

A project of this magnitude must be done outside of regular hours of operation. Cost of equipment rental will be paid to directly Hotel Dieu. Payment of IT and administrative/technical support will be direct to individuals yet to be determined.

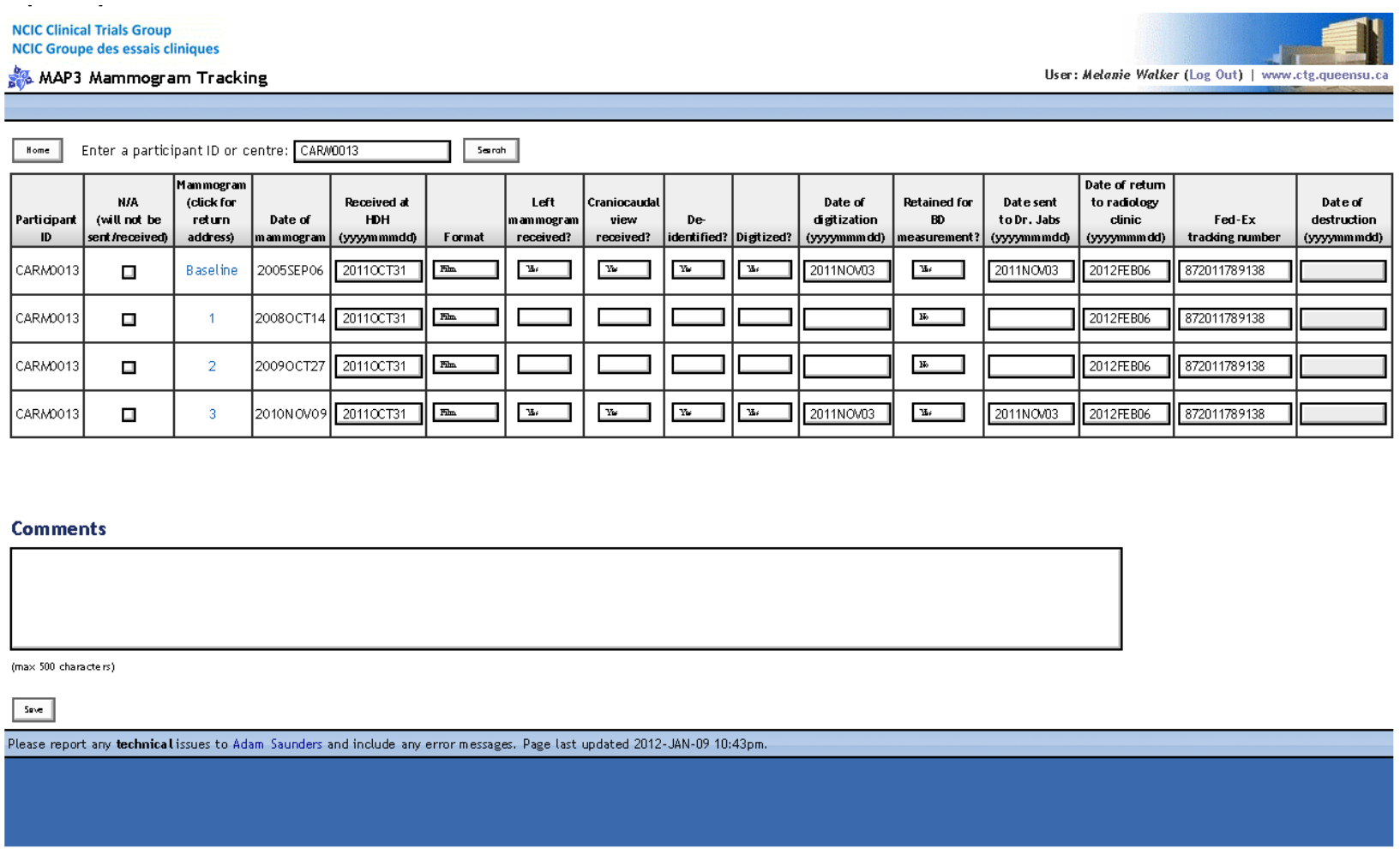
**Projected costs:**

| | |
|---|---|
| Equipment rental ( 12.50/mammogram)<br>( Digitizer, Pacscube, RA600) | 9,375 |
| Associated network costs ( 10 hours @ $60/hr) | 600 |
| Administrative/technical costs/mammogram ( 12.50/mammogram) | 9, 375 |
| Project set-up and wrap-up meetings (3 @ $200/meeting) | 600 |
| **Total Cost** | 19, 950 |

M. Pitcher
Diagnostic Imaging
613 544-3400 EXT 2641

*a partner in the Southeastern Ontario Health Sciences Centre*

## Appendix 6: Web Based Mammogram Tracking System



NCIC Clinical Trials Group
NCIC Groupe des essais cliniques

MAP3 Mammogram Tracking

User: *Melanie Walker* (Log Out) | www.ctg.queensu.ca

Home | Enter a participant ID or centre: CARM0013 | Search

| Participant ID | N/A (will not be sent/received) | Mammogram (click for return address) | Date of mammogram | Received at HDH (yyyymmmdd) | Format | Left mammogram received? | Craniocaudal view received? | De-identified? | Digitized? | Date of digitization (yyyymmmdd) | Retained for BD measurement? | Date sent to Dr. Jabs (yyyymmmdd) | Date of return to radiology clinic (yyyymmmdd) | Fed-Ex tracking number | Date of destruction (yyyymmmdd) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| CARM0013 | ☐ | Baseline | 2005SEP06 | 2011OCT31 | Film | Yes | Yes | Yes | Yes | 2011NOV03 | Yes | 2011NOV03 | 2012FEB06 | 872011789138 | |
| CARM0013 | ☐ | 1 | 2008OCT14 | 2011OCT31 | Film | | | | | | No | | 2012FEB06 | 872011789138 | |
| CARM0013 | ☐ | 2 | 2009OCT27 | 2011OCT31 | Film | | | | | | No | | 2012FEB06 | 872011789138 | |
| CARM0013 | ☐ | 3 | 2010NOV09 | 2011OCT31 | Film | Yes | Yes | Yes | Yes | 2011NOV03 | Yes | 2011NOV03 | 2012FEB06 | 872011789138 | |

**Comments**

(max 500 characters)

Save

Please report any **technical** issues to Adam Saunders and include any error messages. Page last updated 2012-JAN-09 10:43pm.

## Appendix 7: MAP.3 Template Informed Consent Document

Current Version:
**Amendment #5**
**2009-APR-07**

PROTOCOL DATE: 2003-OCT-29
NCIC CTG TRIAL: MAP.3

Amendment #2: 2004-MAY-25 ; Amendment #3: 2005-JAN-24; Amendment #4: 2006-JUL-20
Consent Amendment #1: 2007-JAN-17

ENGLISH Sample Consent Form

A PHASE III RANDOMIZED STUDY OF EXEMESTANE VERSUS PLACEBO IN POSTMENOPAUSAL WOMEN AT INCREASED RISK OF DEVELOPING BREAST CANCER

NCIC CTG MAP.3

Le formulaire de consentement est disponible en français sur demande.

**Note: If REB-approved French language consent form is NOT used at your institution, you should remove this statement.*

This is a clinical trial (a type of research study). Clinical trials include only subjects who choose to take part. Please take your time to make your decision. Discuss it with your friends and family.

You are being asked if you would like to take part in this study because you have an increased risk for developing breast cancer.

Health Canada and the FDA have not approved the sale or use of exemestane for breast cancer prevention, although they have approved its use in this clinical trial.

BACKGROUND

Breast cancer is the most common cancer in women, affecting one woman in nine during her lifetime. The risk of developing breast cancer increases with age.

There are certain factors that affect a woman's chance of getting breast cancer. Some of these are: age, family history of breast cancer, number of previous breast biopsies, age at first menstrual period, age at time of first live birth of a child. These factors can be put together to calculate a woman's risk of developing breast cancer. This is called the Gail score. A high Gail score means a woman has a greater risk of getting breast cancer.

In addition to an increase in breast cancer risk, as women get older their risk of cardiovascular diseases, such as heart attacks and strokes, increases. This is partly due to the loss of the protective effects of estrogen in women who are past menopause The incidence of osteoporosis, a disease that thins and weakens the bones, also increases placing older women at an increased risk of fractures, especially of the hip, spine and wrist.

Before you agree to take part in this study, your Doctor will calculate your Gail score and explain what it means. If your Gail score is high (greater than or equal to 1.66) your doctor will talk to you about the use of tamoxifen and raloxifene along with the pro's and con's of considering these treatments.

Three other breast cancer prevention studies have already been done. These studies all compared tamoxifen to placebo. All three of these studies have shown that certain groups of women who took tamoxifen greatly lowered their chance of getting breast cancer. These groups were women 35-59 years old who had an above average risk of developing breast cancer and women older than 60 years who had an average or high risk of developing breast cancer.

Because of its side effects, tamoxifen has only been approved by the U.S. Food and Drug Administration (FDA) for preventing breast cancer in women who are at "high risk". For example, tamoxifen caused endometrial cancer, stroke, blood clots and eye problems (cataracts) especially in older women who were past menopause. It also caused increased symptoms in women in menopause such as hot flashes, vaginal discharge and genital itch.

Another study, called STAR, has been done that compared tamoxifen to raloxifene. Based on the STAR results, tamoxifen and raloxifene seem to be equally effective at reducing the incidence of invasive breast cancer. However, raloxifene did not significantly reduce the incidence of pre-invasive breast cancer (LCIS or DCIS). Raloxifene can cause blood clots, although apparently less frequently than tamoxifen, but does not appear to cause endometrial cancer while tamoxifen does. The frequency of cardiovascular events (like stroke or heart attack) was the same for both drugs.

Your doctor will talk to you about the use of tamoxifen and raloxifene and explain the benefits of both drugs, together with their side effects. If you decide not to take tamoxifen or raloxifene, you may choose to go on this study. If you choose to take tamoxifen or raloxifene you may not take part in this study.

Following discussion with your doctor, you understand that tamoxifen is the approved drug in the U.S. for the reduction in short term incidence of invasive breast cancer and you have decided that you do not want to take it. You also understand that raloxifene is another option for the reduction in short term incidence of invasive breast cancer and you have decided that you do not want to take it.

## WHY IS THIS STUDY BEING DONE?

After menopause when the ovaries stop making estrogen the body continues to make estrogen from skin, muscle and fat. Even though it is only present at low levels it continues to be very important in the development of breast cancer.

A new drug, called exemestane, stops the supply of estrogen to pre-cancerous and cancerous cells and helps to prevent them from growing.

Tamoxifen and raloxifene work by stopping the effects of estrogen on breast cancer cells but they have a number of serious side effects. By stopping estrogen from being made, exemestane may be better at lowering the risk of breast cancer than tamoxifen or raloxifene. It may also offer protection against other health problems linked to menopause. It may not only be better than tamoxifen or raloxifene at preventing breast cancer but may also be a better choice for women's health overall

In this study we are testing whether exemestane will reduce the rate of breast cancer in women at high risk for developing the disease. Exemestane has not been approved for this use by Health Canada.

Reason for Using a Placebo

In this study, there is a 1 in 2 chance that you will receive only placebo (a substance that does not do anything). You may have concerns about taking part in the study because of this.

However, this is the best way to see if a new therapy is effective and to clearly see the potential side effects and impact on quality of life

The researchers also believe that using a placebo is appropriate because tamoxifen has not been widely used for preventing breast cancer. This is because of the risk of endometrial cancer and blood clots. The benefit of tamoxifen is best seen in young women before menopause . It is less helpful to women who have past menopause.

The American Society of Clinical Oncology has noted the side effects of tamoxifen. They also noted the fact that the trials have not clearly shown that this drug has a good effect on overall health and helps to lower the rate of death from breast cancer. Because of this, they have recommended the use of a placebo in trials for breast cancer prevention.

Raloxifene is another option that women may consider for breast cancer risk reduction. However, while the risk profile for raloxifene may be better than tamoxifen, it is still associated with increased risk of thromboemoblic events (blood clots) and decreased sexual function.

## HOW MANY PEOPLE WILL TAKE PART IN THE STUDY?

About 4,560 women from the United States, Canada and other countries will take part in this study.

The study will take 5 years to complete and the results should be known soon thereafter.

WHAT IS INVOLVED IN THE STUDY?

Randomization (assignment to a group):

If you decide to participate you will be "randomized" into one of the study groups described below. Randomization means that you are put into a group by chance. It is like flipping a coin. A central statistical office will be called which will assign one of the treatments to you. Neither you nor your doctor can choose what group you will be in. You will have a one in two chance of being placed in either group.

This study is a double-blind study, which means that neither you nor your doctor will know if you are taking exemestane alone or placebo. In an emergency, if the treatment needs to be identified it will be.

Treatment:

If you agree to take part in this study, you will get one of the following:

Group 1: exemestane
If you are randomized to Group 1 you will get 1 pill. It will be exemestane. You will take this pill by mouth after your morning meal for 5 years.

Group 2: placebo
If you are randomized to Group 2 you will get 1 pill. It will be a placebo. You will take this pill, by mouth after your morning meal, every day for 5 years.

Procedures and Medical Tests:

Before beginning any treatment you will have some tests done. None of these tests are experimental. They are routine. These include:

- measuring your blood pressure
- taking your pulse
- height and weight
- checking your breasts, lungs, heart and abdomen
- routine blood tests. The total blood needed for these tests is approximately two tablespoons.
- mammogram - If you have not had one done within the past 12 months you will also have a mammogram
- a bone mineral density x-ray test to measure the thickness of your bones, if you have not had one in the past year

Many of these tests will also be repeated during the study. Some of these tests may be done more frequently than if you were not taking part in this research study.

You will have one screening visit (to see if you are eligible for the trial) and one baseline visit (to do the necessary tests) at the start of the study. You will be seen by your doctor every 6 months during the first year and then every 12 months thereafter for clinical evaluation, to have routine blood tests and a mammogram.

During the study you will be asked about any side effects you may be having and also any medications that you may be taking.

Amend#3: 2005-JAN-24; #4: 2006-JUL-20; Consent Amend #1: 2007-JAN-17; Amendment #5: 2009-APR-07
All procedures described above are felt to have very low risks. The needles used to take blood might be uncomfortable. You might get a bruise or rarely an infection at the site of the needle puncture When you have a mammogram you will receive a very low dose of radiation. The chance of this causing a cancer is very small.

You have already had a mammogram so that your doctor can see whether or not there is cancer in your breasts. Your doctor can also see, by looking at these x-rays, how dense your breast tissue is. The "breast density" can also be measured, from the x-ray, with the help of a computer. The researchers doing this study are interested in learning about the role that breast density, bone density and hormone levels have on breast cancer risk and how changes in breast density might influence changes in risk of breast cancer.

In order to do this, the researchers will be borrowing your mammograms, from the hospital where you had it done, so that the computer-assisted measurement can be done.

Blood Collection

It is known that sex hormones such as estrogen and testosterone have been linked with breast cancer risk.

Women who have finished menopause and who have high levels of estrogen and testosterone have about twice the risk of developing breast cancer as women with relatively low levels of these hormones.

By allowing researchers to study the blood, they may learn more about what causes cancer and other diseases, how to prevent and to treat these diseases, and perhaps eventually to cure them.

A small tube of blood will be collected by needle from your vein at the beginning of the study, again at one year and also at the end of treatment. This extra blood sample will be stored for an unlimited time at a laboratory located at Queen's University in Kingston, Ontario, Canada. The samples will be kept until they are used up.

The only identification that will be on your samples that is kept in the laboratory will be a study specific code and your initials. Blood used for research is identified only by a special code to protect your identify and privacy.

Reports about any research done with your samples will not be given to you or your doctor. These reports will not be put in your medical records. The research using your samples will not affect your care.

Tissue Collection

Should you develop invasive or non-invasive breast cancer while on study, we would like to take small tissue samples from the breast cancer once removed by surgery. No further surgeries or biopsies would be required of you for this purpose. This will allow the researchers to compare the pathology results with those from the hospital where your surgery was performed. All women on this study who develop breast cancer will have their pathology material reviewed by the same pathologist at the laboratory at Queen's University in Kingston, Ontario, Canada. This is called central pathology review and provides consistency of results.

Restrictions

There may be some drugs such as hormones or steroids and some over the counter preparations that you are not allowed to take during the course of the study. You should discuss your use of all drugs with the study doctor.

Quality of Life Questionnaires

There is a growing agreement that the goal of medical care today for most people is well-being in everyday life. The researchers are very interested in your 'overall health'. Information about your quality of life is essential and only you can tell us about these very important details. It is also important to know how this drug affects your quality of life too.

The *purpose* of looking at your quality of life is to measure how each treatment affects many areas of your life. The *value* of collecting such information is to try to have the best possible results for women at risk for breast cancer.

Quality of life is a very important part of this study and will be followed closely and in detail through two quality of life questionnaires. You will be asked to fill out these questionnaires before going on study and at each visit. The questionnaires ask about how you are feeling and take about 15-20 minutes to complete. Some of the questions are personal and you may refuse to answer these if you wish. The information you provide is for research purposes only and will remain strictly confidential. The individuals (e.g. doctors, nurses, etc.) directly involved in your care will not usually see your responses to these questions - if you wish them to know this information please bring it to their attention.

HOW LONG WILL YOU BE IN THE STUDY?

Your treatment will last for 5 years and you will be followed for a minimum of 5 years after randomization.

The researchers can take you off the study treatment early for reasons such as:

- the side effects of this treatment become too severe
- new information shows this treatment is not in your best interest
- you did not follow the treatment instructions properly
- it is discovered at a later time that you were not eligible to enter the study
- the study is cancelled for any reason
- you develop cancer
- you develop certain breast abnormalities
- you develop a problem, for example a bone fracture or a heart attack, that might be related to the study drug
- you take any medication that is not allowed (see Restrictions above)
- the sponsor decides to stop the trial

If new side effects or information about your breast cancer risk or treatment are discovered during the study, your doctor will tell you about them.

You can refuse to participate in this study or stop participating at any time. If you decide to stop participating in the study, we encourage you to talk to your doctor first.

WHAT ARE THE RISKS OF THE STUDY?

While on the study, you are at risk for the side effects listed below. You should discuss these with your doctor. As with any experimental drug additional unexpected and sometimes serious side effects are a possibility.

Treatment of mice with exemestane at doses resulting in blood levels many times higher than are seen in women caused an increase in the incidence of benign (not cancerous) kidney tumors in male mice but not female mice. However, this finding in animal studies is not felt to indicate that humans have an increased risk of developing kidney or other cancers.

Your doctor will watch you closely to see if you have side effects. Side effects from long-term treatment with exemestane are unknown.

Risks and side effects related to EXEMESTANE

Please note that the list below is taken from women receiving exemestane for advanced breast cancer. Some of the side effects suggested below are therefore likely due to their disease and may not be seen in healthy women. However, we have reported them all below for your information.

Very likely (> 20% of patients)

- fatigue
- hot flashes (a sensation of warmth and flushing along with sweating in the shoulders, neck, and head)

Less likely: (5-20% of patients)

- gastrointestinal effects
  - abdominal pain
  - loss of appetite
  - constipation
  - nausea
  - vomiting
- general effects
  - increased sweating
  - flu-like symptoms such as fatigue, fever, headaches
  - nervous effects
  - depression
  - insomnia
  - anxiety
- dizziness
- headache
- respiratory effects
  - shortness of breath
  - coughing
- vaginal dryness
- joint pain

Rarely: (1-4% of patients)

- diarrhea
- increased appetite
- high blood pressure (hypertension)
- increased cholesterol
- osteoporosis (thinning of your bones, which may lead to bone fractures)
- osteoarthritis (arthritis that affects the joints in the body)
- visual disturbances
- vaginal bleeding (spotting)
- hepatitis (inflammation of the liver)
- carpal tunnel syndrome (numbness and weakness in the hand due to nerve pressure in the wrist)

Other rare side effects include: bone, chest, muscle or back pain, itching, hair loss, rash (rarely severe), swelling, runny nose, heartburn, numbness or tingling in hands and/or feet, dulled sensitivity to touch, urinary tract infection, upper respiratory tract infection, sore throat, sinusitis, confusion, gastric ulcer, increase of liver enzymes in the blood or decrease in certain white blood cells (lymphocytes).

Although unlikely, unforeseeable or unexpected risk(s) may be involved. Exemestane will decrease your body's estrogen levels. The effect of this lowering of estrogen may affect your ability to think clearly and may increase certain blood lipids (which may increase your chances of having rare but serious heart or circulation problems). Male hormone effects (hoarseness, hirsutism [excess hairiness in females] or acne) may very rarely occur.

Exemestane may cause blood clots rarely. Blood clots can be serious but can be treated with blood thinners. Please tell your doctor immediately if you have any new swelling in a leg or arm or have sudden breathing problems. These may be signs of a clot forming or a clot moving to your lungs, heart or brain.

In one large study in patients with early breast cancer, more women receiving exemestane were reported to have heart attacks or angina (chest pain) than women receiving tamoxifen. In both groups, the rate of heart attacks and angina were uncommon (less than 3%). The difference between the exemestane and tamoxifen groups was small and it could have been due to chance. It is also not currently known whether the frequency of these side effects is higher than would occur in similar women not taking exemestane.

Reproductive Risks:

Only women who are in menopause are allowed to participate in this study.

ARE THERE BENEFITS TO TAKING PART IN THE STUDY?

If you agree to take part in this study, there may or may not be direct benefit to you.
A reduction in your risk of developing breast cancer may occur. We hope the information learned from this study will benefit other women in the future.

WHAT OTHER OPTIONS ARE THERE?

If you decide not to take part in this study, your doctor will discuss other treatment options with you. These may include:

Tamoxifen: Tamoxifen is an approved therapy for the reduction in short term incidence of invasive breast cancer and could be an alternative therapy. Your doctor will discuss its benefits and risks with you.

Raloxifene: Raloxifene is another option that women may consider for breast cancer risk reduction. Your doctor will discuss its benefits and risks with you

Please refer to the background section of this consent and talk to your doctor about this and other options. As with any treatment, there are possible benefits and risks. Your doctor will be able to provide you with information about any known benefits and risks of these other treatment options. Your doctor can also discuss with you what will happen if you decide not to undertake any treatment at this time.

WHAT ABOUT CONFIDENTIALITY?

Every effort will be made to keep your personal information confidential.

Qualified representatives of the following organizations may inspect your medical/study records and receive information from your medical/study records for quality assurance and data analysis:

- NCIC Clinical Trials Group (NCIC CTG), the research group coordinating this study
- The research ethics committee who oversees the ethical conduct of this study in your hospital/clinic
- Health Canada (because they oversee the use of drugs in Canada)
- U.S. Food and Drug Administration (because they oversee the use of drugs in the U.S.)
- Pfizer, Inc the company that makes exemestane
- Other regulatory authorities (because they oversee the use of drugs in other countries)

Central laboratories or central review centres may also receive information from your medical/ study records for confirmation of local findings if you develop certain breast abnormalities while on study.

This may contain information that could potentially identify you, and includes:

- test results
- reports of operations
- x-rays or other body scan reports
- reports about your treatment and side effects

The organizations listed above will keep the information they see or receive about you confidential, to the extent permitted by applicable laws. Identifying information will be kept behind locked doors. Identifying information will never be included in a publication of the research.

The information collected during this study will be used in analyses and will be published/ presented to the scientific community at meetings and in journals. This information may also be used as part of a submission to regulatory authorities around the world to support the approval of drugs used in this research. It is expected that the study results will be published 2 to 3 years after we have completed accrual. Your study doctor will be informed of the results of the study once they are known.

## WHAT ARE THE COSTS?

The study drug exemestane or placebo will be given to you free of charge as long as you receive treatment on the study.

You will not be paid for taking part in this study. Taking part in this study may result in added costs to you.

In the case of research-related side effects or injury, medical care will be provided by your doctor or you will be referred for appropriate medical care. Although no funds have been set aside to compensate you in the event of injury or illness related to the study treatment or procedures, you do not waive any of your legal rights for compensation by signing this form.

## WHAT ARE YOUR RIGHTS AS A PARTICIPANT?

Taking part in this study is voluntary. You may choose not to take part or may leave the study at any time. Deciding not to take part or deciding to leave the study later will not result in any penalty or any loss of benefits to which you are entitled. Your doctor will discuss further treatments with you.

A Data Safety Monitoring Board, an independent group of experts, will be reviewing the data from this research throughout the study.

You will be told, in a timely manner, about new information that may affect your health, welfare, or willingness to stay in this study.

You will be given a copy of this signed and dated consent form.

## CONFLICT OF INTEREST

Note to centres: Please include details of any actual or potential conflict of interest concerning this study.

This centre is receiving funds from the NCIC Clinical Trials Group to help offset the costs of conducting this research. NCIC CTG is a non-profit research group.

## WHO DO YOU CALL IF YOU HAVE QUESTIONS OR PROBLEMS?

If you have questions about taking part in this study, or if you suffer a research-related injury, you can talk to your doctor. Or, you can meet with the doctor who is in charge of the study at this institution. That person is:

_____ _____
Name Telephone

If you would like advice regarding your rights as a participant or about ethical issues related to this study, you can talk to someone who is not involved in the study at all. That person is:

_____ _____
Name Telephone

| Version date and/or REB approval date of this form: _____ | NCIC CTG Pt. Serial #: _____ |
|---|---|

Amendment #3: 2005-JAN-24; Amendment #4: 2006-JUL-20; Consent Amendment #1: 2007-JAN-17

SIGNATURES

My signature on this consent form means the following:

- The study has been fully explained to me and all of my questions have been answered.
- I understand the requirements and the risks of the study.
- I authorize access to my medical records as explained in this consent form and
- I agree to take part in this study.

_____ _____

Signature of Participant Date

_____ _____

Signature of Investigator Date

Was the participant assisted during the consent process in one of the ways listed below?

☐ Yes ☐ No

If yes, please check the relevant box and complete the signature space below:

☐ The consent form was read to the participant, and the person signing below attests that the study was accurately explained to, and apparently understood by, the patient.

☐ The person signing below acted as a translator for the participant, during the consent process.

_____ _____

Signature of Person Assisting in the Consent Discussion Date

*Please note: More information regarding the assistance provided during the consent process should be noted in the medical record for the patient if applicable.*

# A PHASE III RANDOMIZED STUDY OF EXEMESTANE VERSUS PLACEBO IN POSTMENOPAUSAL WOMEN AT INCREASED RISK OF DEVELOPING BREAST CANCER

NCIC CTG MAP.3

Le formulaire de consentement est disponible en français sur demande.

**Note: If REB-approved French language consent form is NOT used at your institution, you should remove this statement.*

## BLOOD COLLECTION AND TISSUE BANKING

Sometimes blood is used to better understand inherited genetic changes that are passed on in families and are markers of breast cancer risk. This is often called "genetic testing".

The researchers doing this study are interested in doing research studies on blood samples from you now and in the future to better understand the nature of the inherited genetic changes that are markers of breast cancer risk. The researchers are also interested in doing future research on these blood samples. These samples may help us understand who will benefit the most from this type of treatment. The collection of these blood samples is an optional part of this study. You may refuse to have your blood collected and still may take part in the study.

If you agree to donate your sample, it will be taken at the same time as your study related tests at the beginning of the study. This means 2 extra 5 ml (1 teaspoon) blood samples, taken with a needle from a vein in your arm, will be collected in addition to the study-related blood samples. The needles used to take blood might be uncomfortable. You might get a bruise, or rarely, an infection at the site of the needle puncture.

The researchers doing this study are also interested in doing research tests on tumour tissue samples should you develop breast cancer while on study. We would like to take small tissue samples from the breast cancer once removed by surgery. No further surgeries or biopsies would be required of you for this purpose. This may allow researchers to better understand your disease and to learn more about the various characteristics of the cancer. You may refuse to have your tissue stored and still take part in the main study.

Any blood and/or tissue samples collected will be stored at a central tissue bank (a facility where tissues, including tumours, blood and urine, are stored for future research) located at Queen's University in Kingston, Ontario. The samples will be kept until they are used up. The samples will be used for research purposes only and will not be sold. The research done with your samples may or may not help develop commercial products or tests. There are no plans to provide payment to you if this happens.

WHAT ARE YOUR RIGHTS AS A PARTICIPANT?

Taking part in the blood and/or tissue banking part of this study is voluntary. You may choose not to take part, or may at any time withdraw your consent for this portion of the study and ask that the collected blood samples not be used. Deciding not to take part, or deciding to withdraw your consent for this portion of the study later, will not result in any penalty or loss of benefits to which you are entitled. If you decide to stop participating, and no longer want your blood and/or tissue samples to be used in this research, you should tell your doctor. Your doctor will notify the NCIC Clinical Trials Group (NCIC CTG) who will ensure the blood samples are destroyed and/or the tissue samples are returned to the hospital where you had your surgery or biopsy.

All the information provided in the main study consent form about confidentiality, costs, your rights as a participant, risks, and who to contact with questions, applies to this blood collection and tissue banking consent form as well.

The identification that will be on your blood and/or tissue samples kept in the bank may include your study code, study serial number, initials and tumour bank code. Samples will be given only to researchers whose proposals have been approved by the NCIC Clinical Trials Group. Any research done on your blood or tissue will have been approved by a research ethics board. A research ethics board oversees the ethical conduct of a study, including protection of patient rights, confidentiality and safety. Use of your samples will not affect your privacy as the researchers will not be able to identify you in any way. Your samples will be identified to these researchers by a code number only.

Reports about any research done with your samples will not be given to you or your doctor. These reports will not be put in your medical records. The research using your samples will not affect your care. Every effort will be made to protect your privacy and the confidentiality of these results. If you do not wish to have your samples used for this purpose you may indicate your wish at the end of this consent form.

In the future, people who do research with your samples may need to know more about your health. While the researchers coordinating this study may give them reports about your health, they will not give them your name, address, phone number or any other information that will let them know who you are.

In the future, researchers may want to do genetic testing on your tissue to see whether your cancer may be hereditary (run in your family). Reports about the results of genetic tests will not be put into your medical records. Every effort will be made to protect your privacy and the confidentiality of these results. If you do not wish to have your samples used for this purpose you may indicate your wish at the end of this consent form.

SIGNATURES

*For genetic testing:*

☐ I agree to allow blood samples to be used for genetic testing as described above.

☐ I do not agree to allow blood samples to be used for genetic testing as described above.

☐ I agree to allow tumour tissue samples to be used for genetic testing as described above.

☐ I do not agree to allow tumour tissue samples to be used for genetic testing as described above.

*For possible future testing:*

☐ I agree to allow blood samples to be used for possible future testing as described above.

☐ I do not agree to allow blood samples to be used for possible future testing as described above.

☐ I agree to allow tumour tissue samples to be used for possible future testing as described above.

☐ I do not agree to allow tumour tissue samples to be used for possible future testing as described above.

PLEASE CHECK OFF THE APPROPRIATE BOX ABOVE BEFORE SIGNING BELOW.

My signature on this consent form means the following:

- The study has been fully explained to me and all of my questions have been answered
- I understand the requirements and the risks of the study
- I authorize access to my medical records as explained in this consent form

_____ _____
Signature of Participant Date

_____ _____
Signature of Investigator Date

Was the participant assisted during the consent process in one of the ways listed below?

☐ Yes ☐ No

If yes, please check the relevant box and complete the signature space below:

☐ The consent form was read to the participant, and the person signing below attests that the study was accurately explained to, and apparently understood by, the patient.

☐ The person signing below acted as a translator for the participant, during the consent process.

_____ _____

Signature of Person Assisting in the Consent Discussion

Date

*Please note: More information regarding the assistance provided during the consent process should be noted in the medical record for the patient if applicable.*

## Appendix 8: Initial Health Sciences Research Ethics Board Approval for Study

**QUEEN'S UNIVERSITY HEALTH SCIENCES & AFFILIATED TEACHING HOSPITALS RESEARCH ETHICS BOARD**

October 22, 2010

This Ethics Application was subject to:

☐ Full Board Review
Meeting Date:
☒ Expedited Review

Dr. Will King
Department of Community Health and Epidemiology
Room 211, Carruthers Hall
Queen's University

Dear Dr. King,

**Study Title:** **Vitamin D and mammographic density in postmenopausal women: A cohort study nested within a chemoprevention trial**
**Co-Investigators:** **Dr. H. Richardson, Dr. R. Meyer, Dr. D. Tu, Dr. P. Goss, Ms. M. Walker**

I am writing to acknowledge receipt of your recent ethics submission. We have examined the protocol for your project (as stated above) and consider it to be ethically acceptable. This approval is valid for one year from the date of the Chair's signature below. This approval will be reported to the Research Ethics Board. Please attend carefully to the following list of ethics requirements you must fulfill over the course of your study:

- **Reporting of Amendments**: If there are any changes to your study (e.g. consent, protocol, study procedures, etc.), you must submit an amendment to the Research Ethics Board for approval. (see http://www.queensu.ca/vpr/reb.htm).

- **Reporting of Serious Adverse Events**: Any unexpected serious adverse event occurring locally must be reported within 2 working days or earlier if required by the study sponsor. All other serious adverse events must be reported within 15 days after becoming aware of the information.

- **Reporting of Complaints**: Any complaints made by participants or persons acting on behalf of participants must be reported to the Research Ethics Board within 7 days of becoming aware of the complaint. Note: All documents supplied to participants must have the contact information for the Research Ethics Board.

- **Annual Renewal**: Prior to the expiration of your approval (which is one year from the date of the Chair's signature below), you will be reminded to submit your renewal form along with any new changes or amendments you wish to make to your study. If there have been no major changes to your protocol, your approval may be renewed for another year.

Yours sincerely,

Albert Clark
Chair, Research Ethics Board

Oct 22, 2010
Date

ORIGINAL TO INVESTIGATOR - COPY TO DEPARTMENT HEAD- COPY TO HOSPITAL – BINDER COPY - FILE COPY

**Study Code: EPID-325-10**

- **Investigators please note that if your trial is registered by the sponsor, you must take responsibility to ensure that the registration information is accurate and complete**

QUEEN'S UNIVERSITY HEALTH SCIENCES & AFFILIATED TEACHING HOSPITALS RESEARCH ETHICS BOARD

The membership of this Research Ethics Board complies with the membership requirements for Research Ethics Boards as defined by the Tri-Council Policy Statement; Part C Division 5 of the Food and Drug Regulations, OHRP, and U.S DHHS Code of Federal Regulations Title 45, Part 46 and carries out its functions in a manner consistent with Good Clinical Practices.

Federalwide Assurance Number : #FWA00004184
#IRB00001173

Current 2010 membership of the Queen's University Health Sciences & Affiliated Teaching Hospitals Research Ethics Board

| | |
|---|---|
| Dr. A.F. Clark | Emeritus Professor, Department of Biochemistry, Faculty of Health Sciences, Queen's University (Chair) |
| Dr. H. Abdollah | Professor, Department of Medicine, Queen's University |
| Dr. M. Evans | Community Member |
| Dr. S. Horgan | Manager, Program Evaluation & Health Services Development, Geriatric Psychiatry Service, Providence Care, Mental Health Services<br>Assistant Professor, Department of Psychiatry |
| Dr. L. Keeping-Burke | Assistant Professor, School of Nursing, Queen's University |
| Ms. D. Morales | Community Member |
| Dr. W. Racz | Emeritus Professor, Department of Pharmacology & Toxicology, Queen's |
| Dr. B. Simchison | Assistant Professor, Department of Anesthesiology, Queen's University |
| Dr. A.N. Singh | WHO Professor in Psychosomatic Medicine and Psychopharmacology<br>Professor of Psychiatry and Pharmacology<br>Chair and Head, Division of Psychopharmacology, Queen's University<br>Director & Chief of Psychiatry, Academic Unit, Quinte Health Care, Belleville General Hospital |
| Dr. E. Tsai | Associate Professor, Department of Paediatrics and Office of Bioethics, Queen's University |
| Rev. J. Warren | Community Member |
| Ms. K. Weisbaum | LL.B. and Adjunct Instructor, Department of Family Medicine (Bioethics) |
| Dr. S. Wood | Director, Office of Research Services (Ex-Officio) |

## Appendix 9: Service Agreement between NCIC Clinical Trials Group, Queen's University and Dr. G Jones for Serum 25-OH-D Measurement

**Agreement for Research Services**

Between
Dr. GlenvilleJones("G Jones")
Department of Biochemistry
Queen's University at Kingston
Kingston ON Canada K7L 3N6

And
Dr. Will King ("W King")
Department of Community Health and Epidemiology
Queen's University at Kingston
Kingston ON Canada K7L 3N6

Effective Date: August 25, 2011

**RE: MEASUREMENT OF THE PRIMARY VITAMIN D METABOLITE, TOTAL 25(OH)D FOR MAP.3 SUBSTUDY**

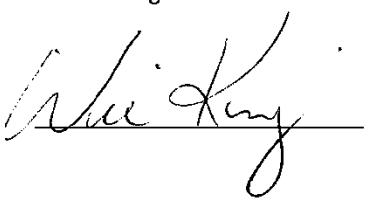
By signing this Agreement for Research Services, G Jones and W King agree to the following terms:

1. G Jones shall provide the research services set out in the attached project specifications, on behalf of W King, for the study entitled "Vitamin D and mammographic density of postmenopausal women – a cohort study nested within a chemoprevention trial: MAP.3".

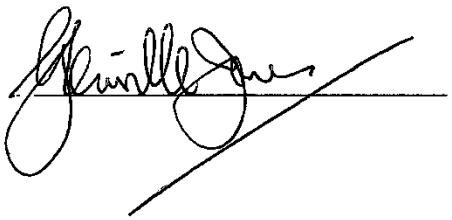
2. W King shall pay for the research services in the amounts specified, inclusive of all taxes, in the attached cost estimation as provided by G Jones.

3. In providing the Services, G Jones shall be responsible for ensuring its relevant personnel are aware of and abide by the requirement of the TTDR Access Agreement between NCIC Clinical Trials Group and W King. TTDR Access Agreement attached as appendix A.

Dr. Will King



Dr. Glenville Jones



**Research Services**

The laboratory of Dr. Jones(Biochemistry Dept. Queen's University) shall provide the following research services for the study "Vitamin D and mammographic density in postmenopausal women: A cohort study nested within a chemoprevention trial: MAP3"

1. Receipt of baseline and year 1 serum samples for ~500 MAP.3 study participants from the NCIC CTG Tumour Bank located in the Department of Pathology, Queen's University. Samples will be annotated with the NCIC CTG tumour bank identifier only.

2. Confidential storage of these serum samples with access by limited personnel.

3. Maintenance of a tracking system for all serum samples received including the NCIC CTG tumour bank identifier, date of receipt, date of the serum sample (baseline date and year 1 date), date of vitamin D measurement for each sample, date of return of any residual unused samples to the NCIC CTG Tumour Bank (if applicable)

4. Quality assurance for the new Waters/ Micromass LC-MS/MS instrument.

5. Measurement of the primary vitamin D metabolite, total 25(OH)D, on approximately 0.25-0.50 µL of the available serum collected at baseline and year 1 using LC-MS/MS

6. Return of any unused serum samples to the NCIC CTG Tumour Bank, if applicable.

7. At study completion, transfer of the tracking system for serum samples to Queen's which will include the vitamin D measurements for each participant at baseline and year 1. Specific values for 25(OH)D levels at baseline and year 1 for each participant will be provided in ng per millilitre.

Queen's University

DEPARTMENT OF BIOCHEMISTRY
Botterell Hall, Room 650, 18 Stuart Street
Queen's University
Kingston, Ontario, Canada K7L 3N6
Tel 613 533-2900
Fax 613 533-2497

Will King, PhD.
Harriet Richardson, PhD.
Queen's University
Community Health and Epidemiology
Abramsky Hall
Kingston, Ontario
K7L 3N6

September 2, 2009

**RE: 'Vitamin D and Breast Cancer Risk in Postmenopausal Women' grant proposal**

Dear Drs. King & Richardson,

This letter is provide a written quote for 25-OH-D assay to support the above grant proposal:-

Total 25-OH-D (25-OH-D2 + 25-OH-D3) analysis on ~ 1000 samples of blood (500 baseline samples + 500 year 1 samples to give an average 25-OH-D for each of ~500 participants to be included in this study) @ $25.00/sample -- **Total Cost $25,000.**

The assay cost will include technician time, LC-MS/MS operation and maintenance, internal standards, columns and HPLC-grade solvents. We will employ a published LC-MS/MS method based upon DMEQ-TAD derivatization [Aronov et al. Anal Bioanal Chem 391:1917.2008] & which utilizes our CIHR-funded Waters/ Micromass LC-MS/MS instrument and the method has been validated in our laboratory and is supported by DEQAS accreditation.

I look forward to our collaboration on this project.

Yours sincerely,

Glenville Jones
Craine Professor and Head, Department of Biochemistry
& Professor of Medicine

## Appendix 10: Service Agreement between NCIC Clinical Trials Group, Queen's University and Dr. H Feilotter for DNA Extraction and Genotyping

**Agreement for Research Services**

Between
Dr. Harriet Feilotter (**"H Feilotter"**)
Department of Pathology and Molecular Medicine
Queen's University at Kingston
Kingston ON Canada K7L 3N6

And
Dr. Will King (**"W King"**)
Department of Community Health and Epidemiology
Queen's University at Kingston
Kingston ON Canada K7L 3N6

Effective Date: December 4, 2012

**RE: DNA EXTRACTION AND GENOTYPING SERVICES FOR MAP.3 PARTICIPANT WHOLE BLOOD SAMPLES**

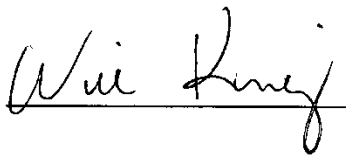
By signing this Agreement for Research Services, H Feilotter and W King agree to the following terms:

1. H Feilotter shall provide the research services set out in the attached project specifications, on behalf of W King, for the study entitled "Vitamin D and mammographic density of postmenopausal women – a cohort study nested within a chemoprevention trial: MAP.3".

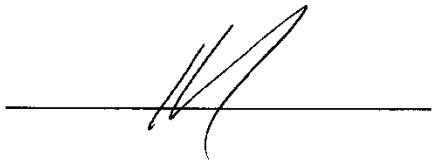
2. W King shall pay for the research services in the amounts specified, inclusive of all taxes, in the attached cost estimation as provided by H Feilotter.

3. In providing the Services, H Feilotter shall be responsible for ensuring its relevant personnel are aware of and abide by the requirement of the TTDR Access Agreement between NCIC Clinical Trials Group and W King. TTDR Access Agreement attached as appendix A.

Dr. Will King



Dr. Harriet Feilotter



The Queen's Laboratory for Molecular Pathology (H Feilotter) shall provide the following research services for the study "Vitamin D and mammographic density in postmenopausal women: A cohort study nested within a chemoprevention trial: MAP3."

- Receipt of baseline whole blood samples for 568 MAP.3 study participants from the NCIC CTG Tumour Bank located in the Department of Pathology, Queen's University. Samples will be annotated with the NCIC CTG tumour bank identifier only.
- Confidential storage of these whole blood samples with access by limited personnel.
- Maintenance of a tracking system for all whole blood samples received including the NCIC CTG tumour bank identifier, date of receipt, date of the whole blood sample, date of DNA extraction and date of genotyping
- DNA extractions using an automated platform with 200 µL of whole blood
- Genotyping for 2 single nucleotide polymorphisms related to vitamin D receptor and metabolism (*fok1* and *CYP22A1*)
- Quality control measures for the above processes including blinded quality control samples to provide a measure of validation of the genotyping procedures
- At study completion, transfer of the tracking system for whole blood samples to Queen's University (W King) which will include the genotyping of each participant for each of the two SNPs

*Harriet Feilotter, Ph.D., FCCMG, FACMG*
*Assistant Professor*
*Dept. of Pathology & Molecular Medicine*
*Richardson Laboratory*
*Queen's University, Kingston, ON K7L 3N6*
*(613) 548-1302 FAX (613) 548-1356*
*Email: feilotth@kgh.kari.net*

Pathology and Molecular Medicine
QUEEN'S UNIVERSITY

Will King, PhD.
Harriet Richardson, PhD.
Queen's University
Community Health and Epidemiology
Abramsky Hall
Kingston, Ontario
K7L 3N6

**RE: 'Vitamin D and Breast Cancer Risk in Postmenopausal Women' grant proposal**

Dear Drs. King & Richardson,

This letter is to confirm my intention to collaborate with you and other investigators at Queen's University in a study investigating the interactions of two vitamin D receptor polymorphisms on the relationship between circulating levels of vitamin D in postmenopausal women at increased risk for breast cancer and mammographic density. As director of the Queen's Microarray facility, which functions as part of the Queen's Laboratory for Molecular Pathology, I will be happy to assist you with the details of this work.

Yours sincerely,

Harriet Feilotter, PhD, FCCMG
Director, Queen's Microarray Facility
Assistant Prof, Dept of Path and Mol Med, Queen's University
Chief of Service, Division of Laboratory Genetics, KGH

Revised Budget Dated Nov 2012

This quotation is prepared based on information provided by Melanie Walker from the laboratory of Dr Will King. Services include DNA extraction and quantification using the BioMek robot, and SNP genotyping for a total of 568 samples

| | |
|---|---|
| Development:<br>*Use of the BioMek robot for sample extractions will require development and software.*<br>*Software*<br>*Consumables*<br>*Labour costs* | <br><br>$3000<br>$500<br>$1000 |
| **Total method development cost** | **$4500** |
| Experimental:<br>DNA extractions using robot @$12/sample<br>DNA Quantitation using PicoGreen<br>Plastics<br>Each SNP assay (includes consumables, plastics, plates, gloves, tips and labour) | <br>$6816<br>$1250<br>$500<br>$2700 **Note this is a per SNP cost**<br>Total cost $11266 |
| **Total Study Cost** | **$15,766** Add $2700 per additional SNP required** |

Terms and Conditions:

1. All prices are in Canadian Dollars unless otherwise specified.
2. This work order is subject to additional taxes/shipping/brokerage charges/currency exchange rates where applicable.

NCIC Clinical Trials Group Tumor Tissue Data Repository (TTDR)

INVESTIGATOR AGREEMENT

TTDR Access Agreement

Trial #: NCIC CTG MAP 3

Title: A phase III randomized study of exemestane vs placebo in postmenopausal women at increased risk of developing breast cancer.

This agreement is made as of Oct 3, 2011 between

NCIC Clinical Trials Group (NCIC CTG) located at 10 Stuart Street, Kingston, Ontario Canada K7L 3N6

and

Community Health and Epidemiology (CHE) located at 62 Fifth Field Company Lane, Carruthers Hall, Queen's University, Kingston, ON, Canada K7L 3N6

And

Dr. Will King (Investigator) located at 62 Fifth Field Company Lane, Room 211, Carruthers Hall, Queen's University, Kingston, ON, Canada K7L 3N6

Whereas the study above has resulted in the creation of mammogram images maintained at participating centers in Canada, a clinical database held by NCIC CTG as well as tissue samples collected for research purposes; and

Whereas the Investigator has applied to the TTDR to conduct a substudyentitled : "Vitamin D and mammographic density of postmenopausal women – a cohort study nested within a chemopreventive trial: MAP.3" (hereinafter referred to as the "Substudy") using data in the clinical database held by NCIC CTG.

In Consideration of the mutual covenants made in this Agreement, the parties agree as follows:

1. **Submission and approval of Substudy:**

   The Investigator and CHE submitted a Substudy proposal to NCIC CTG for approval. Attached as appendix A.

2. **Conduct of Substudy:**

   In the conduct of the Substudy the Investigator and CHE will ensure that he/it will

i) Not exceed the scope of the Substudy approved as outlined above without the expressed written consent of the CSTB Committee of the NCIC CTG.
ii) Investigator must agree to keep the individual patient data confidential. The data may only be shared within the team conducting the analysis project. Requests from other individuals for access to the data shall be referred to the NCIC CTG.
iii) Carry out research in accordance with all applicable laws and regulation including but not limited to section 44(5) of the *Personal Health Information Protection Act*, 2004, S.O. 2004, c. 3 ("PHIPA"). Not commence without the prior written approval of the institutional research ethics board (REB) where the research is being conducted. A copy of this approval must be provided to the NCIC CTG.
iv) Keep the data received from NCIC CTG in an appropriate and secured environment.
v) Ensure that only authorized users have access to the data.
vi) The project is to be approved by the applicable Research Ethics Board (REB) of the institution where the work will be performed. NCIC CTG is to be provided with a copy of the REB approval letter.
vii) Return all data originally transferred or generated to NCIC CTG at the end of the Substudy.
viii) Upon completion of the stated project, all copies of the transferred data are to be destroyed.

3. **Analysing and correlating Substudy results with clinical data:**

The parties agree that NCIC CTG will prepare and furnish, to investigator and CHE, the data necessary for the analysis and correlations with the clinical data for the Substudy and that the analysis will be performed with oversight from the NCIC CTG. The Investigator shall pay for the services performed in preparing and furnishing the data to a maximum set out in the budget attached.

4. **Publication:**

The parties will collaborate to ensure that the results arising out of the analysis of the data can be published as soon as possible. Copies of all abstracts and manuscripts arising from the project must be sent to NCIC CTG with an opportunity for NCIC CTG to provide commentary prior to abstract / manuscript submission. Any publication will acknowledge appropriately the contribution of NCIC CTG following the norms for academic standard, including where applicable, with authorship. The NCIC CTG and its TTDR shall be acknowledged as the resource where specimens were acquired.

5. **Compliance:**

The investigator and CHE will ensure that any internal personnel and/or agents who need access to the data for the purposes herein will be bound by the terms of this agreement.

6. **General Terms and Conditions:**

i) No party shall be entitled to assign or transfer the Agreement or the rights and obligation hereunder to any third party without prior written approval of the other parties.
ii) This agreement represents the entire understanding among the parties related to the Substudy.
iii) This agreement shall not be amended, modified, varied or supplemented except in writing signed by each of the parties.
iv) No failure to delay on the part of any party hereto to exercise any right of remedy under this Agreement shall be construed or operate as a waiver thereof.
v) The parties hereto are independent contractors. Nothing contained herein shall be deemed or construed to create among the parties hereto a partnership or joint venture or employment or principal-agent relationship. No party shall have the authority to act on behalf of any other party or to bind another party in any manner.
vi) Each party to this Agreement assumes responsibility for its own obligations under this Agreement.
vii) No party shall use, or authorize others to use, the name, symbols, or marks of another party hereto or its staff for any endorsement purposes without prior written approval from the party whose name, symbols or marks are to be used. This Agreement shall be governed by and constructed in accordance with the laws of the Province of Ontario and the federal laws of Canada applicable therein.

**Acknowledged and agreed by:**

**For NCIC Clinical Trials Group**

Signature: _____

Name: **Dr. Ralph Meyer**
Title: **Director, NCIC Clinical Trials Group**

**For Community Health and Epidemiology**

Signature: _____

Name: WJ MacKillop
Title: Head, Dept Community Health & Epid

**For Investigator**

Signature: _____

Name: **Dr. Will King**
Title: **Associate Professor, Community Health and Epidemiology**

**Appendix 11: Crude Relationship between Serum 25-OH-D and ≥ 25 Percent Mammographic Density at ≥ 3 Year Follow-Up in Participants with and without Missing Data**

| Serum 25-OH-D (ng/mL) | Mammographic Density (N=568) | | Crude Analysis (N=568) | | Mammographic Density (N=564) | | Crude Analysis (N=564) | |
|---|---|---|---|---|---|---|---|---|
| | < 25% | ≥ 25% | Odds Ratio | 95% CI | < 25% | ≥ 25% | Odds Ratio | 95% CI |
| 0-24.9 | 48 | 7 | 0.59 | (0.23–1.50) | 48 | 7 | 0.59 | (0.23-1.50) |
| 25-34.9 | 174 | 22 | 0.51 | (0.27-0.99) | 173 | 21 | 0.49 | (0.25-0.96) |
| 35-44.9 | 188 | 28 | 0.60 | (0.32-1.13) | 186 | 28 | 0.61 | (0.33-1.15) |
| ≥ 45 | 81 | 20 | 1.00 | (referent) | 81 | 20 | 1.00 | (referent) |
| Total N | 491 | 77 | *p-value = 0.23* | | 488 | 76 | *p-value=0.20* | |