2018

EPIGENETIC MODIFICATION OF VITAMIN D-INDUCED GENE EXPRESSION IN HUMAN COLORECTAL AND BREAST CANCER CELL LINES

Sharmin Hossain

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EPIGENETIC MODIFICATION OF VITAMIN D-INDUCED GENE EXPRESSION IN HUMAN COLORECTAL AND BREAST CANCER CELL LINES

A Dissertation Presented

by

SHARMIN HOSSAIN

Submitted to the Graduate School of the University of Massachusetts Amherst in partial fulfillment of the requirements for the degree of DOCTOR OF PHILOSOPHY

May 2018

Department of Nutrition
School of Public Health and Health Sciences
University of Massachusetts Amherst
EPIGENETIC MODIFICATION OF VITAMIN D-INDUCED GENE EXPRESSION IN HUMAN COLORECTAL AND BREAST CANCER CELL LINES

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Sharmin Hossain

Approved as to style and content by:

________________________
Richard J. Wood, Chair

________________________
Zhenhua Liu, Member

________________________
Kathleen Arcaro, Member

________________________
Richard J. Wood, Department Head
Department of Nutrition
DEDICATION

To my parents
ACKNOWLEDGEMENT

This research would not have been possible without the constant support of my research advisor, Dr. Richard J Wood. His expert guidance along with our shared excitement over this project have been my principal motivational force for the past six years. Thank you for believing in me and leading to me to a place I never knew I could reach. I am deeply indebted to my professors and colleagues at UMass for their invaluable support in every step of the way. I also thank my committee members, Dr. Zhenhua Liu and Dr. Kathleen Arcaro for their constant guidance and feedback.

I am eternally grateful to my family for their unconditional love and support. I remember my late father and grandfather this day, for they have been the two most important men in my life to have supported my dreams and ambitions, more than anyone else. I would not be here without their unwavering faith in me. I mourn their losses as I feel a sense of accomplishment like no other. Thank you for being there for me, in spirits, and know that you are the reason I came this far.

Finally, and with enormous pride, a sincere thanks to my dear mother. You have been my rock and will forever be.
ABSTRACT

EPIGENETIC MODIFICATION OF VITAMIN D-INDUCED GENE EXPRESSION IN HUMAN COLORECTAL AND BREAST CANCER CELL LINES

MAY 2018

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Directed by: Professor Richard J. Wood

Epigenetics is the study of the regulation of genes that is not dependent on nucleotide sequence. This may include heritable changes in gene activity and expression but also those that are not heritable. In recent years, epigenetic researchers have made great strides in understanding the molecular mechanisms behind cellular functions in response to diet, exercise, and other lifestyle modifications, like methylation, histone modification etc. It is now known that the epigenome is critical to healthy human development in addition to genetics, and dietary factors can modulate epigenetic alterations in cells.

The classic view of cancer etiology is that genetic alterations damage DNA structure and induce mutated proteins (oncogenes) that lead to disease progression. More recently, the role of epigenetic alterations during development and chronic disease development has gained increasing attention. This caused a paradigm shift in our understanding of possible mechanisms leading to disease susceptibility. Epidemiological studies have revealed an inverse correlation between the intake of cruciferous vegetables and the risk of certain types of cancer [1]. There has also been evidence, from various epidemiological studies, of higher intake or blood levels of vitamin D and its association with a reduced risk of colorectal and breast cancer [2]. Thus, there is evidence that both higher intake of cruciferous vegetable and better vitamin D status are associated with a reduced risk of cancer. The primary focus of my dissertation is the manipulation of histone modification via dietary agents like vitamin D and sulforaphane in human colorectal and breast cancer cell lines with a review of current literature focusing on vitamin D and breast cancer.
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CHAPTER 1
INTRODUCTION

1.1 Overview

Although there is some evidence that vitamin D may have a protective role in preventing colorectal cancer, existing literature marginally supports the preventive potential of vitamin D in other malignancies, like breast cancer, with the exception of possible benefits of vitamin D supported by genetic data in bladder and prostate cancer [3]. Epidemiological studies have shown that a high level of consumption of cruciferous vegetables such as broccoli, Brussel sprouts etc. is associated with a reduced risk of certain human cancers [4] and is attributable to certain bioactive phytochemicals, such as, isothiocyanates (ITCs) found at relatively high levels in these vegetables. Sulforaphane (SFN), derived from glucosinolates in cruciferous vegetables, is an ITC found to be more potent than other major glucosinolates and ITCs with proposed anticancer effects [5].

1.2 Statement of Problem

The question if vitamin D has a role in cancer incidence, progression, and mortality has been studied in detail for over two decades. Specific focus has been on colorectal, breast, and prostate cancers since these three malignancies account for approximately 35% of total cancer cases and 20% of cancer deaths in the United States. While vitamin D status has been reported to have an inverse association with colorectal cancer (CRC) incidence and mortality; findings in breast cancer (BC) patients suggested a lower risk for progression and mortality with higher serum 25- hydroxyvitamin D₃ [25(OH)D₃], a biomarker of
vitamin D status [3]. 25(OH) D$_3$ is a pro-hormone form of vitamin D produced in the liver and is considered the most reliable indicator of vitamin D status. 25(OH) D$_3$ is then converted to 1,25-dihydroxyvitamin D$_3$ [1,25(OH)$_2$D$_3$], the hormonally active form of vitamin D, in the kidneys. There are several enzymes involved in this process and will be discussed in greater detail to elucidate D action in the subsequent chapters.

Although, observational studies show positive outcomes between vitamin D and BC, randomized clinical trials have generally failed to support these associations, to date. The current literature provides conflicting findings regarding the vitamin D action in cancer. Due to these inconsistencies, it is difficult to ascertain if vitamin D (the active form) is related to a direct reduction in cancer risk, or to what extent higher concentrations of vitamin D status biomarker, serum 25(OH) D$_3$ serve as a surrogate for an overall healthier lifestyle and lower cancer risk in population studies [6].

The important role of active vitamin D compounds in various cancer cell types, including malignant breast cells, by inducing cellular differentiation, inhibition of cell growth and cell death via apoptosis is well-established [7-10]. While, dietary intake of vitamin D has been inconsistently linked to breast cancer risk, studies considering the association of breast cancer risk and sun exposure as a proxy of endogenous vitamin D synthesis, have observed inverse associations [11, 12].

Isothiocyanates (ITCs) are naturally occurring secondary metabolites of cruciferous vegetables. Glucosinolates in cruciferous vegetables are converted to ITCs upon hydrolysis by the enzyme myrosinase [13]. These ITCs, predominantly sulforaphane, have been reported to possess chemopreventive and chemotherapeutic effects both in vitro and in vivo [14]. Rigorous SFN research is now focused on its anti-carcinogenic roles considering the
growing body of evidence, although it was first identified as a potent inducer of phase 2 detoxification enzyme[15, 16].

Epigenetic changes, which can be triggered by various dietary components can cause heritable changes in the DNA structure and chromatin complex without disrupting the actual coding sequence of the DNA. In other words, epigenetics focuses on modifying the DNA (gene expression) via external agents, such as vitamins, minerals, phytochemicals etc. that do not change the primary DNA sequence. Histone protein modification in the chromatin structure and DNA methylation are two key epigenetic mechanisms studied in cancer. Epigenetic effects of SFN has been linked predominantly to post-translational histone modification processes. Histone deacetylase (HDAC) inhibition by SFN in human colon and prostate cancer cells in vitro were supported by in vivo experiments in humans, and was similar in preclinical models of gastrointestinal and prostate cancer [17]. HDACs remove acetyl groups from DNA and affect transcription which can result in silencing numerous important tumor suppressor genes. SFN has several known biochemical properties, including activation of antioxidant enzymes via Nrf2 transcription factor activation, altering phase I and II detoxification enzymes, and inhibition of HDAC activity [16, 18-21]. By inhibiting the HDAC action, sulforaphane can reverse epigenetically silenced genes in cancer cells, resulting in cell cycle arrest and/or apoptosis [18, 22].

Despite numerous studies demonstrating the anti-cancer effects of various ITCs, the exact mechanism of SFN action is still unclear and requires further attention. There is also not enough information on how sulforaphane works particularly in colorectal and breast cancers on a molecular level.
1.3 Bibliography


CHAPTER 2
LITERATURE REVIEW

Vitamin D deficiency is considered a pandemic because of its widespread presence in all age groups and in nearly all geographical regions [3]. It is a culmination of lifestyle changes in the modern societies that involve a decrease in time spent outdoors, widespread use of sunscreens, and air pollution which contribute to insufficient exposure to sunlight, and thus reduced skin synthesis of vitamin D. Other factors that notably increase the risk of vitamin D deficiency are: aging of populations and the resulting decline of vitamin D synthesis in the skin, low intake of vitamin D with the diet, and the obesity epidemic. In the absence of sufficient sun exposure to maintain adequate vitamin D status, an alternative way to get adequate vitamin D is through certain foods or vitamin D supplements.

Historically speaking, vitamin D recommendation for adults of 5 μg/d (200 IU/d) vitamin D was considered adequate to prevent osteomalacia (adult vitamin D deficiency) in the absence of sunlight. But more was needed to help prevent osteoporosis and secondary hyperparathyroidism. Later on, other health benefits of vitamin D supplementation were suggested to accrue based on observations from epidemiological studies, such as: prevention of some cancers and reduced risks of multiple sclerosis and hypertension [23]. In 2011, the US Institute of Medicine (IOM) established a Dietary Reference Intake (DRI) for vitamin D across all age groups, considering newer research findings since 1997, the previous estimate of vitamin D needs of the population. Based on this review of the evidence, the IOM has tripled the recommended daily intakes of vitamin D to 15 μg (600 IU) per day for people aged 1-70 years, and increased to 20 μg (800 IU) per day for those older than 70 years [24]. The recommendation for dietary Vitamin D is based on how much
is needed to achieve a serum 25(OH)D of 20 ng/ml (50 nmol/L) in 97.5% of the population. A serum 25(OH)D concentration of 20 ng/mL to 50 ng/mL (50nM-125nM) is considered adequate for healthy people. A level less than 20 ng/mL indicates vitamin D deficiency. However, some researchers recommend maintaining serum 25(OH)D levels of 30 ng/mL (75 nmol/L) may be needed to prevent chronic diseases like cancer, but this has not been sanctioned by the IOM due to lack of sufficient evidence. In the 2000–2004 NHANES cycle, 78% of Americans had a serum 25(OH)D3 less than 30ng/ml [25].

2.1 Synthesis and Metabolism of Vitamin D

The metabolism of vitamin D is complex and involves several different enzymes and organs. In humans with adequate sun exposure, vitamin D3 (cholecalciferol) is formed from cutaneous 7-dehydrocholesterol (7-DHC) synthesized in the basal layer of the epidermis. Initially, the β-ring of 7-DHC undergoes photolysis upon the exposure of skin to UV-B radiation, leading to the formation of pre-vitamin D3 that non-enzymatically is converted to vitamin D and then transferred into the blood[26]. Subsequent activation of vitamin D3 involves sequential 25- and 1-α-hydroxylations of vitamin D3 in the liver and kidney, respectively. In the liver, vitamin D3 is hydroxylated by 25-hydroxylase to 25-hydroxyvitamin D3 also known as 25-hydroxycholecalciferol or abbreviated as 25(OH)D3 [27]. 25(OH)D3 is then hydroxylated a second time in the kidneys by mitochondrial 1α-hydroxylase (CYP27B1) producing 1,25(OH)2D3 (calcitriol), the hormonal form of vitamin D, that can activate the vitamin D receptor [27]. Several tissues and organs, such as the intestines and skin, can also activate vitamin D through these two-sequential enzymatic hydroxylations [28], but liver and kidney are the primary sites of regulation. The activity of 25(OH)D3 and 1,25(OH)2D3 is counteracted by the enzyme 24-hydroxylase
(CYP24A1), which transforms them to the inactive vitamin D metabolites 24,25(OH)2D3 and 1,24,25(OH)3D3, respectively, prior to further oxidation and cleavage of the side chain resulting in complete inactivation and eventual elimination from the body[29]. Both 24,25(OH)2D3 and 1,24,25(OH)3D3 possess significantly lower affinity for the vitamin D receptor (VDR) than 1,25(OH)2D3 [30]. This property is important when considering molecular functions of vitamin D.

2.2 Dietary sources of Vitamin D

A well-balanced diet with a high vitamin D content can help maintain adequate vitamin D in our system in the absence of cutaneous sunlight exposure. However, only a relatively few foods naturally provide substantial amounts of vitamin D. There are a couple of dietary products that are rich in vitamin D, e.g.: fish oils, sea fish etc. Fortified products are an important dietary source of vitamin D in the US. One cup of milk contains approximately 50 IU of vitamin D, and the average American consumes about 1¼ c of milk/day, with adults consuming less[31]. It is assumed that a well-balanced diet should provide all the essential nutrients, minerals and vitamins. However, there are groups of people who are particularly vulnerable to deficiency of vitamin D, e.g. children, adolescents, pregnant and nursing women and the elderly. Vitamin D supplements should be used when sun exposure and the diet are unable to fulfill vitamin D needs.

Currently, the recommended dietary allowance (RDA) for vitamin D₃ is 600 IU/day (age 1 yr. to 69 yr.) and the upper limit tolerance is 4000 IU/day [32]. Due to the high prevalence of vitamin D deficiency and the risk of the related deficiency diseases, fortification of certain products, especially milk, orange juice, margarine, butter etc. are becoming increasingly common. Vitamin D is relatively stable and does not decompose in
heat or during long-term storage. However, it may be destroyed by ultraviolet radiation. Under aerobic conditions in aqueous medium it may undergo autoxidation and lose its functional properties.

While national surveys in both the United States and Canada show the average total intake of vitamin D among North Americans to be below the median requirement, they also show that average blood levels of 25(OH)D₃ are above 20 ng/mL, which is equivalent to 50 nmol/L, what the IOM found necessary for good bone health for all individuals [24]. The IOM interpreted the seemingly inconsistent observations of low dietary vitamin D intake but adequate vitamin D status to suggest that sun exposure currently contributes meaningful amounts of sunlight for vitamin D synthesis in North Americans and that most of the population is meeting its needs for vitamin D. Table 2.1 lists the average daily recommended amounts from the Food and Nutrition Board (a national group of experts) for different ages.

**Table 2.1: Recommended Dietary Allowances (RDAs) for Vitamin D [2]**

<table>
<thead>
<tr>
<th>Age</th>
<th>Male</th>
<th>Female</th>
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<tr>
<td>0–12 months*</td>
<td>400IU (10 mcg)</td>
<td>400IU (10 mcg)</td>
</tr>
<tr>
<td>1–70 years</td>
<td>600IU (15 mcg)</td>
<td>600IU (15 mcg)</td>
</tr>
<tr>
<td>&gt;70 years</td>
<td>800IU (20 mcg)</td>
<td>800IU (20 mcg)</td>
</tr>
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</table>

* Adequate Intake (AI). 1 mcg vitamin D= 40 IU

Since most Americans will only receive a small amount of vitamin D from dietary sources, the more logical source for vitamin D is through sun exposure. Spending approximately thirty minutes outside at noon will produce approximately 10,000 IU of vitamin D [33].
2.3 What is Sulforaphane (SFN)?

Sulforaphane belongs to a broad class of phytochemicals called isothiocyanates (ITCs). ITCs are a chemical class of compounds that are not naturally present in cruciferous vegetables, such as broccoli, Brussel sprouts, cabbage, radish, kale, cauliflower etc. However, it can be generated from hydrolysis of secondary metabolites, known as glucosinolates, by action of the enzyme myrosinase, released from plant cells during the process of crushing or mastication (chewing) [34]. ITCs may also be produced in the intestines where the resident microflora can promote hydrolysis of glucosinolates to isothiocyanates. Sulforaphane is especially high in broccoli and broccoli sprouts [14]. It is well known as a potent inducer of phase-2 detoxification enzymes [35] which inhibits tumorigenesis in animal models. Indeed, sulforaphane has been implicated in a variety of anticarcinogenic mechanisms including effects on cell cycle checkpoint controls and cell survival and apoptosis in various cancer cells [36-43]. The protective effects of sulforaphane in humans, however, may be influenced by individual genetic variation (polymorphisms) in the metabolism and elimination of isothiocyanates from the body [21].

2.4 Bioavailability of SFN

It is important to understand the bioavailability and function of nutrient and non-nutrient compounds found in various foods with health-promoting properties. Human intestinal perfusion studies showed that 74 ± 29% of sulforaphane from broccoli extracts can be absorbed in the jejunum [44]. Pharmacokinetic studies in both rats and humans also support the notion that sulforaphane can be distributed in the body and reach micromolar concentrations in the blood. For example, in rats, following a 50 μmol gavage, detectable sulforaphane was evident in the plasma after 1 h and peaked at ∼20 μmol/L at 4 h, with a
half-life of $\sim 2.2$ h [45]. We know that different nutrients (macro and micro) have varying degrees of absorbability that can affect their bio efficacy. Broccoli sprouts contain up to 50 times higher concentrations of the sulforaphane precursor glucoraphanin than mature broccoli. Thus, in humans, most studies have used broccoli sprouts as a particularly rich dietary source of glucoraphanin/sulforaphane. In human subjects, given single doses of 200 $\mu$mol ITC preparation from broccoli sprouts, ITC plasma concentrations peaked between 0.943 and 2.27 $\mu$mol/L 1 h after feeding (half-life =1.77 ± 0.13 h) [46]. However, it is important to note that the glucosinolate dose per unit body weight given to rats in the Hu et al. study cited above were much higher (4-fold less ITC with markedly lower body weight in the rat) than given to human subjects. The primary aim of this literature review is to present our current understanding of the role of vitamin D and SFN and breast and colon cancer - what is known and established, what is still missing and where does that lead us.

2.5 Vitamin D & CRC Epidemiological Studies

Dietary intake, dietary supplementation, latitude and sun exposure, all predictors of serum of 25(OH)D$_3$ levels, have been associated with differences in cancer incidence and mortality. This association is particularly strong for CRC [47].

2.5.1 Vitamin D & CRC Meta-Analyses and Systematic Reviews

To date, meta-analyses of the association between serum 25(OH)D$_3$ and CRC have consistently demonstrated a statistically significant inverse relationship for incidence, but not recurrence, though data for the latter outcome are comparatively sparse [48, 49]. Since
the results of these meta-analyses were reported in 2008 and 2015 respectively, a third study of adenoma recurrence was published, which again showed no statistically significant association between 25(OH)D₃ and odds of adenoma recurrence [50]. Underlying mechanisms for the observed differences in the association for 25(OH)D₃ by incident vs. recurrent adenomas are currently only speculative. Differences in methylation patterns during adenoma growth and development [51] and variation in expression of key vitamin D pathway enzymes during different stages of development [52, 53] could be two potential mechanisms through which differential effects of vitamin D are seen on CRC incidence vs. recurrence. It is also possible that individuals included in recurrent lesions studies have different risk factor profiles and the carcinogenic pathway is not affected by vitamin D the same way as individuals without recurring lesions. Taken together, these observational studies indicate that vitamin D may have a role in reducing the risk of incident colorectal adenomas, but after removal of these lesions, there is no evidence that it will prevent the formation of another.

Several meta-analyses of serum 25(OH)D₃ concentrations and CRC incidence have been conducted, and all have shown a statistically significant inverse association. The results are summarized in **Table 2.2**.

**Table 2.2: Vitamin D and CRC Meta Analyses and Systematic Review Summary**

<table>
<thead>
<tr>
<th>Serum 25 (OH)D₃</th>
<th>Outcome measure</th>
<th>Statistic</th>
<th>Effect size</th>
<th>Citations</th>
</tr>
</thead>
<tbody>
<tr>
<td>55ng/mL</td>
<td>RRₚooled</td>
<td>0.62</td>
<td>38% decrease</td>
<td>[54]</td>
</tr>
<tr>
<td>50ng/mL</td>
<td>OR</td>
<td>0.4</td>
<td>60% decrease</td>
<td>[55]</td>
</tr>
<tr>
<td>Every 25ng/mL increase</td>
<td>OR</td>
<td>0.92</td>
<td>8% decrease</td>
<td>[56]</td>
</tr>
<tr>
<td>&lt;44nM or &gt;72nM</td>
<td>RR</td>
<td>0.66</td>
<td>34% decrease</td>
<td>[3]</td>
</tr>
<tr>
<td>High vs. Low</td>
<td>OR</td>
<td>0.68</td>
<td>32% decrease</td>
<td>[57]</td>
</tr>
<tr>
<td>High vs. Low</td>
<td>RR</td>
<td>0.67 vs. 66</td>
<td>33% decrease vs. 34% decrease</td>
<td>[6]</td>
</tr>
</tbody>
</table>


<p>| | | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>High vs. Low</td>
<td>OR</td>
<td>0.66</td>
<td>34% decrease</td>
<td>[58]</td>
</tr>
<tr>
<td>High vs. Low</td>
<td>RR</td>
<td>0.96</td>
<td>4% decrease</td>
<td>[59]</td>
</tr>
<tr>
<td>Every 20ng/mL</td>
<td>OR</td>
<td>0.82</td>
<td>18% decrease</td>
<td>[48]</td>
</tr>
<tr>
<td>increase</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>High vs. Low</td>
<td>RR</td>
<td>0.67</td>
<td>33% decrease</td>
<td>[60]</td>
</tr>
<tr>
<td>Every 10ng/mL</td>
<td>RR</td>
<td>0.85</td>
<td>15% decrease</td>
<td>[61]</td>
</tr>
<tr>
<td>increase</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>High vs. Low</td>
<td>OR</td>
<td>0.70</td>
<td>30% decrease</td>
<td>[49]</td>
</tr>
<tr>
<td>≥33 ng/mL,</td>
<td>OR</td>
<td>0.49</td>
<td>50% decrease</td>
<td>[62]</td>
</tr>
</tbody>
</table>

2.5.2 Vitamin D & CRC Randomized Clinical Trials

Randomized clinical trials provide the best evidence if an exposure (e.g. vitamin D) reduces the risk of any cancer, and to date, only four have been published on vitamin D with data specific to CRC [63-66]. The Women's Health Initiative (WHI) randomized women to 400 IU vitamin D and 1000 mg of calcium vs. placebo. No differences in risk for CRC by treatment group were observed, although some limitations of the trial were noted, including the relatively healthy study population, the timing of the intervention, and the comparatively short follow-up time [65]. In addition, it has been suggested that the dose of vitamin D used in WHI was too low to elicit protective effects [64]. Another clinical trial conducted in the United Kingdom, randomized men and women to receive 100,000 IU of vitamin D every four months for five years [66]. No reduction in risk of either CRC incidence or mortality was observed. Results from the ongoing Vitamin D and Omega-3 Trial (VITAL) trial [67], will likely provide more definitive evidence regarding the efficacy of vitamin D in CRC prevention. In this study, participants are supplemented with 2000 IU/d of vitamin D with and without an omega-3 fatty acid supplement to ascertain whether the intervention can prevent the development of cancer or cardiovascular disease.
Four large-scale clinical trials on vitamin D supplementation are currently underway and summarized in Table 2.3.

**Table 2.3: Ongoing Large-Scale Randomized Trials of Vitamin D Supplementation Worldwide[67]**

<table>
<thead>
<tr>
<th>Trial, Location</th>
<th>Treatment Duration</th>
<th>Vitamin D intervention</th>
<th>Primary endpoints</th>
</tr>
</thead>
<tbody>
<tr>
<td>VITAL, USA</td>
<td>5y</td>
<td>2000IU/d</td>
<td>Cancer, CVD</td>
</tr>
<tr>
<td>D-Health, Australia(^1)</td>
<td>5y</td>
<td>60,000 IU/month (bolus)</td>
<td>Total mortality, Cancer</td>
</tr>
<tr>
<td>FIND, Finland(^2)</td>
<td>5y</td>
<td>1600 IU/d or 3200IU/d</td>
<td>Cancer CVD</td>
</tr>
<tr>
<td>VIDAL, UK(^3)</td>
<td>5y</td>
<td>100,000 IU/month (bolus)</td>
<td>Total mortality, Cancer</td>
</tr>
</tbody>
</table>

\(^1\)Neale R. [11.09.14]; D-Health Trial Australian New Zealand Clinical Trials Registry identifier: ACTRN12613000743763. Available at: [https://www.anzctr.org.au/Trial/Registration/TrialReview.aspx?id=364534&isClinical Trial=False](https://www.anzctr.org.au/Trial/Registration/TrialReview.aspx?id=364534&isClinical Trial=False)


2.5.3 Vitamin D & CRC Case-Control Studies

Numerous case-control studies analyzed the effect of vitamin D and the risk of CRC. Some of them are nested within large prospective cohorts. While some studies focused only on serum 25(OH)D$_3$, others were interested in vitamin D intake, supplementation and in some cases, sun exposure, to reach their conclusions. Majority of these studies confirm that vitamin D as measured by serum 25(OH)D$_3$ has a protective role against CRC. A summary of relevant vitamin D case-control studies is provided in Table 2.4.

Table 2.4: Vitamin D and CRC Case-Control Studies Summary

<table>
<thead>
<tr>
<th>Serum 25 (OH)D$_3$/Vitamin D intake</th>
<th>Outcome measure</th>
<th>Statistic</th>
<th>Effect size</th>
<th>Citations</th>
</tr>
</thead>
<tbody>
<tr>
<td>101.5 nM 25(OH)D$_3$</td>
<td>OR</td>
<td>0.51</td>
<td>49% decrease</td>
<td>[68]</td>
</tr>
<tr>
<td>21.9 vs. 23.9 ng/mL Of 25(OH)D$_3$</td>
<td>OR</td>
<td>0.45</td>
<td>55% decrease</td>
<td>[69]</td>
</tr>
<tr>
<td>High vs. Low</td>
<td>OR</td>
<td>0.60</td>
<td>40% decrease</td>
<td>[70]</td>
</tr>
<tr>
<td>Every 50nM increase in 25(OH)D$_3$</td>
<td>OR</td>
<td>0.57</td>
<td>43% decrease</td>
<td>[71]</td>
</tr>
<tr>
<td>&lt;20ng/mL</td>
<td>RR</td>
<td>1.80 (men)[0.22 (women)]</td>
<td>18% increase in men [78% decrease in women]</td>
<td>[57]</td>
</tr>
<tr>
<td>D intake</td>
<td>OR</td>
<td>0.77</td>
<td>23% decrease</td>
<td>[72]</td>
</tr>
<tr>
<td>High vs. Low serum 25(OH)D$_3$</td>
<td>OR</td>
<td>0.52</td>
<td>48% decrease</td>
<td>[73]</td>
</tr>
<tr>
<td>High vs. Low serum 25(OH)D$_3$</td>
<td>OR$_{pooled}$</td>
<td>0.59</td>
<td>41% decrease</td>
<td>[74]</td>
</tr>
<tr>
<td>&gt;100 nM of 25(OH)D$_3$</td>
<td>OR</td>
<td>0.77</td>
<td>23% decrease</td>
<td>[75]</td>
</tr>
<tr>
<td>High vs. Low D intake</td>
<td>OR</td>
<td>0.68</td>
<td>32% decrease</td>
<td>[1]</td>
</tr>
<tr>
<td>D intake</td>
<td>OR</td>
<td>0.69</td>
<td>31% decrease</td>
<td>[76]</td>
</tr>
<tr>
<td>D intake</td>
<td>OR</td>
<td>0.69</td>
<td>31% decrease</td>
<td>[77]</td>
</tr>
<tr>
<td>High vs. Low serum 25(OH)D$_3$</td>
<td>OR$_{pooled}$</td>
<td>0.66</td>
<td>34% decrease</td>
<td>[78]</td>
</tr>
<tr>
<td>D intake</td>
<td>RR</td>
<td>No effect</td>
<td>Reference</td>
<td></td>
</tr>
<tr>
<td>--------------------------</td>
<td>----</td>
<td>-----------</td>
<td>-----------</td>
<td></td>
</tr>
<tr>
<td>High vs. Low 25(OH)D₃</td>
<td>OR</td>
<td>0.53</td>
<td>47% decrease</td>
<td>[79]</td>
</tr>
<tr>
<td>D intake</td>
<td>OR</td>
<td>0.82</td>
<td>18% decrease</td>
<td>[80]</td>
</tr>
<tr>
<td>23- 24 ng/ml of 25(OH)D₃</td>
<td>OR</td>
<td>No effect</td>
<td>47% decrease</td>
<td>[81]</td>
</tr>
<tr>
<td>D intake</td>
<td>OR</td>
<td>0.5</td>
<td>50% decrease</td>
<td>[82]</td>
</tr>
</tbody>
</table>

### 2.5.4 Vitamin D & CRC Cohort Studies

Higher vitamin D status has been found to be protective against CRC from a few cohort studies (Table 2.5). A recent retrospective analysis looked at the association between vitamin D status of advanced CRC patients at the time of diagnosis and subsequent outcomes [84]. They measured serum 25(OH)D₃ levels in patients with new diagnosis of stage IV CRC and compared it with survival. They defined adequate level of vitamin D as >30ng/mL of serum 25(OH)D₃ and found a 61% reduced risk (HR=0.61; 95% CI 0.38-0.98). Two prospective cohort studies have specifically assessed the association between serum 25(OH)D₃ concentrations and CRC progression [85, 86]. Mezawa and colleagues measured blood levels of 25(OH)D₃ in Stage I-IV patients and found that although 25(OH)D₃ levels were significantly related to overall survival, they were not associated with disease-free survival [85]. In another study conducted among Stage IV CRC patients undergoing chemotherapy, concentrations of 25(OH)D₃ were not significantly associated with time to progression of disease [86]. Table 2.5 lists studies of interest that are prospective in design have a mean follow-up of ±10 years to incident CRC and survival.
Table 2.5: Vitamin D and CRC Prospective Cohort Studies Summary

<table>
<thead>
<tr>
<th>Serum 25 (OH)D₃</th>
<th>Outcome measure</th>
<th>Statistic</th>
<th>Effect size</th>
<th>Citations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low vs. High</td>
<td>OR</td>
<td>4.6 in men</td>
<td>460% increase</td>
<td>[87]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.7 in women</td>
<td>270% increase</td>
<td></td>
</tr>
<tr>
<td>80 nM</td>
<td>OR</td>
<td>0.38</td>
<td>72% decrease</td>
<td>[88]</td>
</tr>
<tr>
<td>Every 10 nM increase</td>
<td>HR</td>
<td>0.95</td>
<td>95% decrease</td>
<td>[89]</td>
</tr>
<tr>
<td>High vs. Low</td>
<td>OR</td>
<td>0.77</td>
<td>23% decrease</td>
<td>[90]</td>
</tr>
<tr>
<td>Every 25 nM increase</td>
<td>RR</td>
<td>0.63</td>
<td>37% decrease</td>
<td>[91]</td>
</tr>
</tbody>
</table>

2.6 Vitamin D & Breast Cancer Epidemiological Studies

Research on BC and vitamin D association have been inconsistent. A high concentration of plasma 25(OH)D₃ was associated with a significantly reduced risk of premenopausal BC [92]. Reduction in risk of BC is affected by amount of vitamin D (intake, supplementation, seasonal variation, and race) and differs by menopause status. A serum 25(OH)D₃ level of 50 ng/ml was associated with 50% reduction in incident BC, compared to a baseline of <10 ng/ml [93]. Every 1 ng/ml increment of plasma 25(OH)D₃ level decrease BC risk by 16%. However, some studies have found opposite effects of vitamin D in BC incidence, development, progression and survival. We present the most recent epidemiological evidence regarding vitamin D and BC in the following sections.
2.6.1 Vitamin D and Breast Cancer Meta Analyses and Systematic Reviews

Current breast cancer meta analyses and systematic reviews considered both intake and supplementation of vitamin D and used breast cancer prognosis, survival, recurrence and death as possible outcomes. Four meta-analyses identified a significant inverse relationship between the circulating concentrations of 25(OH)D₃ and BC [94-97]. Two meta-analyses on vitamin D and BC survival have recently been published, and both reported that higher concentrations of 25(OH)D₃ were related to better survival among women clinically diagnosed with BC [97, 98]. Low vitamin D levels were associated with a pooled hazard ratio (HR_{pooled}) of 2.13 (95 % CI 1.64–2.78) and 1.76 (95 % CIs 1.35–2.30) for recurrence and death respectively, in a recent meta-analysis[99].

Another study looked at vitamin D supplementation and BC risk [100] and found no significant association with a reduced risk of BC development in postmenopausal women. Due to several reported inconsistencies in the relationship between vitamin D and BC risk and most of these meta-analyses and systematic reviews recommended further studies i.e. clinical trials for further clarification. A summary of the most current literature is listed below.

**Table 2.6: Vitamin D and BC Meta Analyses and Systematic Review Summary**

<table>
<thead>
<tr>
<th>Serum 25 (OH)D₃</th>
<th>Outcome measure</th>
<th>Statistic</th>
<th>Effect size</th>
<th>Citations</th>
</tr>
</thead>
<tbody>
<tr>
<td>High vs. Low</td>
<td>RRₚooled</td>
<td>0.92</td>
<td>8% decrease</td>
<td>[97]</td>
</tr>
<tr>
<td>High vs. Low</td>
<td>RR</td>
<td>0.63</td>
<td>37% decrease</td>
<td>[101]</td>
</tr>
<tr>
<td>&gt;75nM</td>
<td>HRₚooled</td>
<td>0.58</td>
<td>42% decrease</td>
<td>[102]</td>
</tr>
<tr>
<td>&lt;15 to &gt;30 ng/mL</td>
<td>OR</td>
<td>0.10</td>
<td>90% decrease</td>
<td>[96]</td>
</tr>
<tr>
<td>High vs. Low</td>
<td>RR</td>
<td>0.84</td>
<td>16% decrease</td>
<td>[103]</td>
</tr>
<tr>
<td>Every 5ng/mL increase</td>
<td>RR</td>
<td>0.88</td>
<td>12% decrease</td>
<td>[104]</td>
</tr>
<tr>
<td>Every 25ng/mL increase</td>
<td>OR</td>
<td>0.92</td>
<td>8% decrease</td>
<td>[95]</td>
</tr>
<tr>
<td>40 ng/mL</td>
<td>RR</td>
<td>0.80</td>
<td>20% decrease</td>
<td>[105]</td>
</tr>
<tr>
<td>---------</td>
<td>-----</td>
<td>------</td>
<td>--------------</td>
<td>------</td>
</tr>
<tr>
<td>High vs. Low</td>
<td>HR$_{\text{pooled}}$</td>
<td>0.63</td>
<td>37% decrease</td>
<td>[106]</td>
</tr>
<tr>
<td>High vs. Low</td>
<td>RR</td>
<td>0.55</td>
<td>45% decrease</td>
<td>[107]</td>
</tr>
<tr>
<td>Every 10ng/mL increase</td>
<td>OR$_{\text{pooled}}$</td>
<td>0.99</td>
<td>1% decrease</td>
<td>[108]</td>
</tr>
<tr>
<td>Every 20ng/mL increase</td>
<td>RR</td>
<td>0.73</td>
<td>27% decrease</td>
<td>[109]</td>
</tr>
<tr>
<td>78 nM</td>
<td>OR</td>
<td>0.50</td>
<td>50% decrease</td>
<td>[110]</td>
</tr>
<tr>
<td>52 ng/ml</td>
<td>OR$_{\text{pooled}}$</td>
<td>0.50</td>
<td>50% decrease</td>
<td>[93]</td>
</tr>
</tbody>
</table>

### 2.6.2 Vitamin D and BC Clinical Trials

Randomized clinical trials for BC and vitamin D association have also presented mixed results to date, and the inconsistencies persisted though pre- and post-menopausal sub-group analyses. One study considering the Women’s Health Initiative (WHI) clinical trial of calcium and vitamin D (CaD) supplements (average intervention period of 7.0 years), found that a relationship between CaD and BC risk was only suggestive [111]. Subjects were vitamin D sufficient for all endpoint analyses. A deficiency of vitamin D, on the other hand, in BC patients, was found to be unresponsive to standard low-dose D supplementation [112].

A more recent study focused on a small group of early BC (EBC) patients treated with adjuvant or neoadjuvant chemotherapy and effects of a tailored, high-dose, oral vitamin D supplementation in a randomized phase III trial [113]. Their objective was to restore a normal serum 25(OH)D$_3$ level in this population which would reflect on the suggested beneficial role of vitamin D. At the end of the trial, 44% of these patients achieved vitamin D normalization.

Another population-based, double-blind, randomized placebo-controlled trial looked at supplemental calcium and vitamin D and associated risk of common cancers and
found that improving calcium and vitamin D nutritional status substantially reduces all-cancer risk in postmenopausal women only [64].

2.6.3 Vitamin D and BC Case-Control Studies

Case-control studies examining vitamin D and BC association have produced mixed results despite controlling for potential confounders. A Mendelian randomization study analyzing the effects of circulating concentrations of vitamin D and subsequent cancer risks found that there was a 11% decrease in BC risk with every 25ng/mL increase in circulating 25(OH)D$_3$ [56]. BC is a heterogeneous disease and it is possible that vitamin D only affects certain BC subtypes, e.g. based on tumor type, histological grade or hormone receptors. One study looked at this particular aspect and found the lowest risk of aggressive BC in patients from the second tertile compared to the lowest tertile where they used serum 25(OH)D$_3$ $\geq$75 nM to indicate vitamin D sufficiency [114].

BC is prevalent throughout the world, in varying degrees, due to the substantial differences in the overall population, landscape, exposure to sun, diet, race, age distribution, and cultural influences). Considering the rising incidence of BC and high prevalence of vitamin D deficiency in Iran, a recent case-control study assessing BC risk and vitamin D status, found three times lower the risk of BC incidence in the highest quartile of serum 25(OH)$_3$ group (OR = 0.269; 95% CI, 0.122-0.593), as well as highest intake of vitamin D (OR=0.39; 95% CI, 0.196-0.784)[115]. This association was significant only in the premenopausal women (OR = 0.25; 95% CI, 0.094-0.687) when stratified by menopause status.
Another study from India, with similar objectives, found that low serum 25(OH)D\textsubscript{3} levels were associated with a high risk of BC (OR=2.5; 95% CI, 0.9-7.4) [116]. Although seasonal variation in vitamin D association studies are important, exclusive research on this topic is rather limited. Majority of the case-control studies regarding BC and vitamin D link have been nested within large prospective cohorts and a few of them are highlighted below.

One recent nested case-control study in the Nurses' Health Study cohort found that, although not related to overall BC risk, serum 25(OH)D\textsubscript{3} significantly inversed BC risk in summer (RR = 0.66; 95% CI, 0.46-0.94) only [117]. A nested case-control study within the Multiethnic Cohort Study of five race/ethnic groups (white, African-American, Native Hawaiian, Japanese, and Latino) found BC risk was inversely associated with serum 25(OH)D among white (OR=0.43; 95% CI,0.23-0.80) but not in other race/ethnic groups [118]. However, another nested case-control study within a large prospective cohort with 10 years of follow-up, studying the same association failed to establish a connection between serum 25(OH)D levels and BC risk, although women with relatively high levels in summer months were at reduced risk [119].

A Korean study on serum 25(OH)D\textsubscript{3} and BC risk [120] showed significant association with serum 25(OH)D\textsubscript{3} highest quartile (OR=1.27; 95 %CI, 1.15-1.39) and remained significant when stratified by menopausal status: pre-menopause (OR=1.26; 95 % CI,1.09-1.45) and post-menopause (OR=1.25; 95 % CI, 1.10-1.41).

There have been several other studies regarding BC risk and serum 25(OH)D\textsubscript{3} and they’re summarized in Table 2.7.
Table 2.7: Vitamin D and BC Case-Control Studies Summary

<table>
<thead>
<tr>
<th>Serum 25 (OH)D$_3$</th>
<th>Outcome measure</th>
<th>Statistic</th>
<th>Effect size</th>
<th>Citations</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;19.8 to &gt;27 ng/mL</td>
<td>RR</td>
<td>0.80</td>
<td>20% decrease</td>
<td>[121]</td>
</tr>
<tr>
<td>18.1 to 29.5 ng/mL</td>
<td>RR</td>
<td>0.84</td>
<td>16% decrease</td>
<td>[122]</td>
</tr>
<tr>
<td>&lt;12.0 to &gt;24.0 ng/mL</td>
<td>RR</td>
<td>0.45</td>
<td>55% decrease</td>
<td>[92]</td>
</tr>
<tr>
<td>&lt;20 to &gt;40 ng/mL</td>
<td>RR</td>
<td>0.56</td>
<td>44% decrease</td>
<td>[123]</td>
</tr>
<tr>
<td>&lt;24.0 to &gt;33.7 ng/mL</td>
<td>RR</td>
<td>0.52</td>
<td>48% decrease</td>
<td>[124]</td>
</tr>
<tr>
<td>&lt;14.7 to &gt;29.2 ng/mL</td>
<td>RR</td>
<td>1.09</td>
<td>10% increase</td>
<td>[125]</td>
</tr>
<tr>
<td>&lt;12 to &gt;30 ng/mL</td>
<td>RR</td>
<td>0.31</td>
<td>69% decrease</td>
<td>[126]</td>
</tr>
<tr>
<td>9.44 to 32.8 ng/mL</td>
<td>RR</td>
<td>0.93</td>
<td>7% decrease</td>
<td>[127]</td>
</tr>
<tr>
<td>&lt;18.3 to &gt;33.7 ng/mL</td>
<td>RR</td>
<td>1.13</td>
<td>11% increase</td>
<td>[128]</td>
</tr>
<tr>
<td>&lt;22 to &gt;41.7 ng/mL</td>
<td>RR</td>
<td>0.73</td>
<td>27% decrease</td>
<td>[129]</td>
</tr>
<tr>
<td>&lt;20 to &gt;60 ng/mL</td>
<td>RR</td>
<td>0.20</td>
<td>80% decrease</td>
<td>[130]</td>
</tr>
</tbody>
</table>

2.6.4 Vitamin D and BC Cohort Studies

A recent study used a 25(OH)D$_3$ prediction model to assess the relationship between vitamin D and incident BC risk was conducted by Palmer and colleagues [131]. Participants were part of the Black Women’s Health Study who have been followed by biennial mail questionnaires since 1995. The 25(OH)D$_3$ prediction model is based on measured 25(OH)D$_3$ in plasma specimens obtained from 2856 participants (between 2013 and 2015) by using questionnaire-based variables from the same time frame. They found that, when compared to the highest quartile, women in the lowest quartile of predicted 25(OH)D$_3$ were estimated to have a 23 % increased risk of BC. The Pathways Study is a prospective cohort that looked at serum 25(OH)D$_3$ association with BC prognosis[132] and found that 25(OH)D$_3$ was significantly associated with BC-specific survival (HR=0.37; 95% CI, 0.15-0.93) in premenopausal women and supports the idea that vitamin lowers the risk of BC morbidity and mortality.
There aren’t a lot of retrospective studies in BC association with vitamin D and even less in Latin American women. A recent retrospective case-cohort study examined serum 25(OH)D₃ levels in Chilean BC patients before endocrine therapy and its association to clinical parameters at the time of diagnosis [133]. Using serum 25(OH)D₃ ≥ 30 ng/ml (75 nM) as sufficient vitamin D status [33], they found no significant association between weight, BMI, tumor stage, tumor ER expression and changes in serum 25(OH)D₃ levels. This observation may be due to a small number of subjects studied, although the results were like studies of season–dependence of 25(OH)D₃ levels.

A recent European study that analyzed the effects of serum 25(OH)D₃ on BC risk in three ongoing, large, prospective cohort studies from the CHNACES consortium presented a comprehensive finding on the association of serum 25(OH)D₃ and site-specific cancer incidence [134]. They found a 27% reduction in BC incidence (high vs. low) although this difference was not significant. Their cutoff for vitamin D sufficiency was a serum 25(OH)D₃ levels >50nM and <30 nM as deficient.

2.7 Sulforaphane and Colorectal Cancer

Sulforaphane (SFN) is an isothiocyanate (ITC) that can be formed from its precursor molecules present in cruciferous vegetables, such as broccoli, cabbage, broccoli sprouts etc. SFN was first identified as a potent inducer of phase II detoxification enzymes, and growing evidence indicates that SFN might have some chemoprotective properties[17]. Some dietary compounds have been known to de-repress epigenetically silenced genes in various cancer cells that could have important implications in cancer prevention and therapy.
One epigenetic mechanism that affects this process of de-repression is histone deacetylation inhibition. HDACs affect histone acetylation status by removing acetyl groups from the nuclear histone proteins which can cause epigenetic silencing of important genes in cancer cells. Inhibition of this process will have an opposite effect, reducing the net removal of histone acetyl groups (histone deacetylase or HDAC enzymes) and potentially de-repressing silenced genes. This could result in cell cycle arrest and/or apoptosis reducing carcinogenesis. SFN is one phytochemical that demonstrates HDAC inhibitor action [135].

For example, one study investigated the antiproliferative and pro-apoptotic effects of SFN on the SW620 colon cancer cell line and found that SFN at concentrations of 10–50 μM reduces cell viability and inhibits cell proliferation in a time- and dose-dependent manner. DNA damage was also inhibited after 24 h and 48 h in the same cell line [37]. Another study on LS-174 and Caco-2 cells (both human colon cancer cell lines) found that a combination of SFN and mixed isothiocyanates (ITCs) reduced colon carcinogenesis by both stimulating apoptosis and enhancing intracellular defenses against genotoxic agents [136]. A more recent study analyzed 5 different colon cancer cell lines- HCT116, HT-29, KM12, SNU-1040, and DLD-1 and found that SFN (5μM and 25μM) inhibited growth via G2/M phase cell cycle arrest and increased apoptosis while exhibiting negligible toxicity in normal (nonmalignant) cells [5]. Another study isolated different hydrolysates of broccoli and found that the crude extract (80% alcohol extract) of broccoli florets had high cytotoxic activity against different human cancer cell lines, particularly, colon cancer (IC50 3.88 μg/mL)[137].
In a study examining the anti-proliferative effect of SFN on an array of human colon cancer cell lines- DLD-1, HCT116 and LoVo, SFN exhibited anti-proliferative effects demonstrated by colony formation assays. Colony formation was reduced by SFN in a dose-dependent manner, especially at 20 µM [138]. In HT-29 colon cancer cells, one study found a significant reduction in cell proliferation (≥20 µM) [139] while another found that SFN induced cell cycle arrest in a dose-dependent manner, followed by cell death [34]. SFN also showed dose-dependent cytotoxicity and proapoptotic activity in HCT-116 cells in another study[41].

A phase I placebo controlled, double-blind, randomized clinical trial to assess toxicities of either glucosinolate (300 µmol/day) or isothiocyanate (75 µmol/day) showed no toxicities or abnormal events occurred with any of the test extracts [140]. In another study comparing natural SFN vs. SFN supplements, with subjects consuming >300 µmol of glucosinolates every day for 7 days, consumption of SFN precursor (glucoraphanin) had no adverse effects, indicating that a high dose of glucosinolates was well tolerated in the subjects [141].

2.8 Sulforaphane and Breast Cancer

SFN is a chemoprotective agent used in cell culture, animal cancer models (carcinogen-induced and genetic) as well as in xenograft models of cancer. In humans, a key factor in determining the efficacy of SFN as a chemoprevention agent is gaining an understanding of the metabolism, distribution and bioavailability of SFN and the factors that alter these parameters [39]. The importance of epigenetics in the initiation and progression of breast cancer has become more appreciated and has led many investigators to incorporate epigenetics-based treatments in breast cancer drug development [142].

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One study investigating bulk histone acetylation and DNA methylation modifications in a large cohort of breast tumors found that the occurrence of certain histone marks correlates with tumor morphology and biological subtype. In other words, tumors with adverse traditional prognostic or phenotypic characteristics were found to have reduced levels of detectable histone acetylation marks and vice versa [143].

Effects of histone deacetylase inhibitor compounds have been studied both in vivo and in vitro. Most of these studies used various extracts from cruciferous vegetables and in different combinations. One study analyzed potential estrogen receptor (ER) ligands acting as agonists or antagonists, when bound to extracts from cabbage, fermented cabbage, and acidified Brussels Sprouts. These extracts were evaluated for their estrogenic and antiestrogenic activities in estrogen-dependent human breast cancer (MCF-7) cells using as endpoints proliferation and induction of estrogen-responsive pS2 gene expression. At low concentrations (5-25 ng/mL) all the extracts reduced 1 nM estradiol-induced MCF-7 cell proliferation [144]. Another study found that SFN treatment (30μM) along with autophagy inhibition in MCF-7 and MDA-MB-231 breast cancer cells might be a promising strategy for breast cancer control [145]. Sulforaphane (1-5 μmol/L) decreased aldehyde dehydrogenase–positive cell population by 65% to 80% and reduced the size and number of primary mammospheres by 8- to 125-fold and 45% to 75%, respectively in a recent breast cancer stem cell study [146]. A combination of HDAC inhibitors with demethylating agents was suggested as a promising strategy for the effective treatment of hormonal refractory BC, mainly in triple negative BC cells (TNBC) [147]. Another study also found that SFN significantly inhibits cell proliferation in multiple TNBC cell lines through inducing G2/M phase arrest and apoptosis [148].
A single oral dose of 150 μmol SFN in rats resulted in mammary tissue levels of SFN which presented with metabolites at concentrations known to alter gene expression in vitro[149]. In a follow-up study, similar results were found in human breast tissue when eight healthy women undergoing reduction mammoplasty were given a single dose of a broccoli sprout preparation containing 200 μmol of SFN [149].

2.9 Bibliography


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CHAPTER 3

PURPOSE OF THE STUDY

Colorectal cancer is the second most invasive and third most common cancer in both men and women in the US. Prevention of colorectal cancer is an ongoing challenge, part of which is attributable to the constantly changing environmental factors that influence the development of colorectal cancer like- diet, exercise, smoking, alcohol intake, obesity etc. [1]. The most significant risk factors for breast cancer are gender (being a woman) and age (growing older) while other factors include- radiation to chest before the age of 30, genetics, race, overweight/obesity, alcohol intake, smoking and lack of exercise. Research shows that women with high levels of the vitamin in their blood are more likely to survive breast cancer than patients with low levels [2].

Both higher vitamin D status [3] and cruciferous vegetable intake [4] have been linked to reduced risk of cancers. However, little is known about how these two factors may work synergistically with each other to augment the anti-proliferative activity of vitamin D. It has also been found that, enzymes involved in autocrine metabolism of vitamin D in breast tissue may provide important targets for both the prevention and treatment of breast cancer [5]. However, not much is known about the role of vitamin D in normal breast cell development and specific cell cycle events such as proliferation. This is a problem because the therapeutic window for the active vitamin D metabolite is quite small, and has an increased risk of developing toxicity from hypercalcemia and hypercalciuria at therapeutically effective, anti-proliferative doses of vitamin D. A selective downregulation of the vitamin D receptor (VDR) in colon and breast cancer cells, or alterations in the relative activity of the cellular enzymes involved in 1,25(OH)2D3
activation (CYP27B1) and inactivation (CYP24A1) can significantly alter the availability of intracellular concentrations of 1,25(OH)$_2$D$_3$. It can also alter the sensitivity of the cell to the active D metabolite by manipulating VDR activity. We hypothesized that 1,25(OH)$_2$D$_3$ treatment will decrease the rate of cell proliferation in colon and breast cancer cells by modulating D responsive genes. We also hypothesized that treatment of colon and breast cancer cells with SFN or TSA will further support vitamin D action. We anticipate the study findings will shed new light on potential anti-cancer mechanisms related to vitamin D anti-proliferative effects via increased inhibition of HDAC activity through dietary bioactive components, like SFN.

We will discuss the specific aims and hypotheses of our three papers in the following order: epidemiological evidence from a systematic review and meta-analysis on vitamin D and breast cancer followed by in vitro primary studies on human breast cancer (MCF-7 cells) and colorectal cancer (Caco-2 cells) respectively.

3.1 Paper 1: A Systematic Review and Meta-Analysis of Vitamin D and Breast Cancer

Vitamin D is well known for its role in bone health primarily. However, recent epidemiologic studies have linked vitamin D deficiency with adverse cardiovascular and cancer-related outcomes [6]. Although vitamin D can be found in a limited number of foods, the major source of vitamin D is the endogenous production. Plasma concentrations of 25(OH)D$_3$ have been used as a stable indicator of vitamin D status in humans [7]. Epidemiologic evidence for an association between plasma 25(OH)D$_3$ and BC incidence and/or prevalence is inconsistent [8].

Moreover, there aren’t enough clinical trials with vitamin D mostly due to lack of adequate power. Although, certain longitudinal studies have assessed the relationship
between plasma 25(OH)D₃ concentrations and inverse CRC risk, [9-13], there are not enough studies associated with prostate or BC incidence [11, 14-16]. Vitamin D supplementation studies with or without calcium [17-20] reported inconclusive or nonsignificant results for cancer [21]. The mixed results suggest that the relationship of vitamin D status and BC risk is still incompletely understood [22] and drove our systematic review and meta-analysis.

The primary objective of the systematic review and meta-analysis is to gather evidence over the past ~18 years linking vitamin D status and intakes (food and supplements) with the occurrence of breast cancer.

This study aims at reaching the objective by pooling data from the existing literature, interpreting and evaluating the results in both pre- and post-menopausal women. We also discussed potential biological mechanisms behind those putative associations.

The final selected 18 studies were first reviewed in a systematic way, stratifying by study design and type of exposure with measures of association compared across studies. We used the PRISMA guidelines in summarizing the article search. The study-specific RRs for each exposure of interest were examined in pooled models, after testing for heterogeneity using the I² test. As such, a summary or pooled RR was provided using forest plots and estimated using inverse variance weighting [23]. Random effects models that further incorporated between-study variability were conducted using DerSimonian and Laird’s methodology.

A pre-defined quality score (QS) was used to assess the quality of each included study and the key findings of studies for each exposure of interest were presented using a harvest plot. Finally, Begg’s funnel plots were used to examine publication bias; each OR
point estimate was plotted against corresponding standard errors (SE) for each study on a logarithmic scale [24, 25] combining all exposures (e.g. serum and dietary vitamin D exposures).

3.2 Paper 2: Histone Deacetylase activity and Vitamin D-Dependent Gene Expressions in Relation to Sulforaphane in Human Breast Cancer (MCF-7) Cells

Vitamin D has been found to be protective against BC [26]. Numerous studies tried to prove the association although the findings are not consistent. However, there are limited studies about histone deacetylation (HDAC) inhibition patterns in BC tumors. There is a strong lack of evidence regarding BC risk and vitamin D-HDAC inhibitor combination therapy.

The primary objective of this study is to determine how HDAC inhibition influences vitamin D-induced gene expression in BC. The rationale is that the chosen epigenetic modifiers (SFN and TSA) will alter the acetylation status of histone proteins, which will loosen the histone protein complex allowing increased transcription of vitamin D-inducible genes.

Hypothesis a: Expression of vitamin D-dependent genes will be increased by HDAC inhibitors

Our working hypothesis for this specific aim is that the expression of vitamin D-inducible genes will be increased by concomitant treatment of cells with histone deacetylase (HDAC) inhibitors SFN and TSA. To test this working hypothesis, we have measured, with and without HDAC inhibitors, the mRNA expressions of VDR (vitamin D receptor), CYP24A1, CYP27B1 and TRPV6 by real time quantitative polymerase chain
reaction (RT-qPCR) in MCF-7 cells. Treatments were 100 nM vitamin D with and without SFN (20µM) and TSA (1µM). Our expectation was that vitamin D treatment will increase the expression of these selected genes and that concomitant treatment with SFN or TSA will further augment the association.

**Hypothesis b. Histone protein acetylation status will be increased by SFN and TSA treatments**

To test the relative HDAC inhibition by SFN and TSA, we measured changes in histone protein acetylation following our chosen treatments of 100 nM vitamin D with and without SFN (20µM) and TSA (1µM). Cells were incubated for 24 hours once 70% confluent after being seeded onto a 96-Well plate at 10,000 cells/well. HDAC I/II reagents (HDAC I/II Assay, Promega, 2017) were added at equal volumes to each well and incubated for 1 hour at room temperature. Relative luminescence (RLu) were recorded using OptiMax Luminometer and results were analyzed.

We hypothesized that, SFN and TSA will increase histone deacetylase inhibition in MCF-7 cells and co-treatment with vitamin D would enhance D effects.

**Hypothesis c: Cell proliferation will be decreased by 1,25-dihydroxyvitamin D₃ treatment and will be further decreased by concomitant HDAC Inhibitor action**

We hypothesized that treatment of MCF-7 cells with the HDAC inhibitors will alter the acetylation status of histone proteins, thereby increase cellular vitamin D responsiveness.
We conducted colony formation (colonogenic) and migration assay to physiologically demonstrate an increase in the anti-proliferative effects of vitamin D combined with HDAC inhibitors SFN and TSA. Clonogenic assays demonstrate the effectiveness of a treatment by visually examining the number of colonies formed. Migration assay tests the ability of cells to migrate a scratch wound after treatment. Efficacy of the treatments are inversely proportional to the distance migrated i.e. if the distance migrated after the treatment is more than initial scratch, the test compound is less effective in preventing migration and vice versa. We also performed a cell viability assay that measured ATP which is directly proportional to the number of live cells in the system post-incubation (24Hrs).

Our expectation was that vitamin D will decrease cell proliferation, which would be further decreased by epigenetic modifiers affecting histone acetylation status.

3.3 Paper 3: Association Between Histone Deacetylase activity and Vitamin D-Dependent Gene Expressions in Relation to Sulforaphane in Human Colorectal Cancer Cells

Vitamin D, despite its known functions in bone metabolism, has been in the spotlight for its anti-carcinogenic properties for the past two decades. One study found that colon cancer mortality in the USA was highest in places with the least amount of sunlight [27], suggesting a protective role of Vitamin D. The development of many cancers (i.e. prostate, lungs, colon, etc.) have been associated with a reduced expression of the vitamin D receptor (VDR). Low VDR expression in cancer cells decreases cellular sensitivity to vitamin D, reducing its uptake resulting in decreased anti-proliferative action in these cells [28, 29]. Epigenomic alterations in CRC is behind the existing knowledge on gene mutations [30] and cell-specific mechanisms are yet to be explored. To the best of our
knowledge, this study is the first to demonstrate vitamin D-dependent key gene expressions in relation to histone modification in human colorectal cancer using both proliferating and differentiated Caco-2 cells as the experimental model.

The primary objective of this study is to determine to what extent treatment with HDAC inhibitors influence vitamin D-induced transcriptional activity in Caco-2 cells.

We hypothesized that treatment of cells with vitamin D would increase the sensitivity of Caco-2 cells which, in turn, will increase the action of HDAC inhibitors. We also hypothesized that, an increase in HDAC inhibition preceded by adequate vitamin D uptake will decrease progression of colorectal cancer in vitro.

**Hypothesis a: Expression of vitamin D-dependent genes will be increased by HDAC inhibitors**

Our working hypothesis for this specific aim is that the expression of vitamin D-inducible genes will be increased by concomitant treatment of cells with histone deacetylase (HDAC) inhibitors SFN and TSA. To test this working hypothesis, we have measured, with and without HDAC inhibitors, the mRNA expressions of VDR (vitamin D receptor), CYP24A1, CYP27B1 and TRPV6 by real time quantitative polymerase chain reaction (RT-qPCR) in Caco-2 cells. Treatments were 100 nM vitamin D with and without SFN (20µM) and TSA (1µM). Our expectation was that vitamin D treatment will increase the expression of these selected genes and that concomitant treatment with SFN or TSA will further augment the association.
Hypothesis b. Histone protein acetylation status will be increased by SFN and TSA treatments

To test the relative HDAC inhibition by SFN and TSA, we measured changes in histone protein acetylation following our chosen treatments of 100 nM vitamin D with and without SFN (20µM) and TSA (1µM). Cells were incubated for 24 hours once 70% confluent after being seeded onto a 96-Well plate at 10,000 cells/well. HDAC I/II reagents (HDAC I/II Assay, Promega, 2017) were added at equal volumes to each well and incubated for 1 hour at room temperature. Relative luminescence (RLu) were recorded using OptiMax Luminometer and results were analyzed.

We hypothesized that, SFN and TSA will increase histone deacetylase inhibition in Caco-2 cells and co-treatment with vitamin D would enhance D effects.

Hypothesis c: Cell proliferation will be decreased by 1,25-dihydroxyvitamin D3 treatment and will be further decreased by concomitant HDAC Inhibitor action

We hypothesized that treatment of Caco-2 cells with the HDAC inhibitors will alter the acetylation status of histone proteins, thereby increase cellular vitamin D responsiveness.

We conducted migration assay to physiologically demonstrate an increase in the anti-proliferative effects of vitamin D combined with HDAC inhibitors SFN and TSA. Migration assay tests the ability of cells to migrate a scratch wound after treatment. Efficacy of the treatments are inversely proportional to the distance migrated i.e. if the distance migrated after the treatment is more than initial scratch, the test compound is less effective in preventing migration and vice versa. We also performed a cell viability assay.
that measured ATP which is directly proportional to the number of live cells in the system post-incubation (24Hrs).

Our expectation was that vitamin D will decrease cell proliferation, which would be further decreased by epigenetic modifiers affecting histone acetylation status.

3.4 Bibliography


CHAPTER 4

VITAMIN D AND BREAST CANCER: A SYSTEMATIC REVIEW AND META-ANALYSIS

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4.1 Abstract

**Background:** In this systematic review and meta-analysis, we pooled and evaluated evidence linking serum vitamin D (both in serum and diet) with breast cancer (BC) occurrence.

**Methods:** Searching PubMed and Cochrane databases (January 1st, 2000 through August 31st, 2017), only observational studies were included. Publication bias and consistency upon replication were assessed, while harmonizing risk ratios (RR, 95% CI) of BC, per fixed increment of 5 exposures [10 ng/mL of 25(OH)D; 100 IU/d for total/dietary vitamin D intakes; vitamin D deficiency; supplement use]. RRs were pooled using random effect models.

**Results:** Pooled findings from 18 studies suggested a net direct association between 25(OH)D deficiency and BC, with RR\textsubscript{pooled}=1.73, 95% CI: 1.28-2.34, p<0.001). Total vitamin D intake was also inversely related to BC (RR\textsubscript{pooled}=0.98, 95% CI: 0.96-1.00, P=0.029, per 100 IU/d). No evidence of publication bias was found and consistency upon replication was shown for all 5 exposures of interest.

**Conclusions:** Our study indicated that serum vitamin D deficiency and total vitamin D intake were associated with BC occurrence.

**Impact:** Thus, pending future randomized controlled trials, vitamin D may be protective against BC occurrence.

**Key words:** Vitamin D, breast cancer, meta-analysis
4.2 Abbreviations

1,25(OH)2D=1,25 di-hydroxyvitamin D
25(OH)D=25-hydroxyvitamin D
BC=Breast cancer
CI=Confidence Interval
CVD=Cardiovascular Disease
HR=Hazard Ratio
IU=International Units
OR=Odds ratio
RR=Risk Ratio
SD=Standard Deviation
SE=Standard Error
QS=Quality score

4.3 Introduction

Breast cancer is the most common malignancy among women in the US that has substantial medical and economic costs associated with it [1]. In 2017, an estimated 252,710 new cases of invasive BC will be diagnosed among women (BC report 2017-18) and approximately 40,610 women and 460 men are expected to die from BC in 2017. Although there has been an overall increase in BC death rates between 1975 and 1989, a steady decline attributed to advanced healthcare and screening has been observed from 2006 to 2015. However, this decline has not been the same among different racial and ethnic groups. The relative survival rates based on the most recent data as published on the
American Cancer Society report are: 91% at 5 years after diagnosis, 86% after 10 years and 80% after 15 years BC report 2017-18. According to a recent retrospective study that looked at the stage-dependent average per capita cost of BC treatment, costs were higher in patients whose cancer were more advanced at diagnoses. For example, there is approximately a $10,000 difference in surgical costs during the first 12 months of diagnoses between a stage 0 and IV cancer screening. [1]

Vitamin D is known to play a major role in bone health and calcium homeostasis. In recent years, epidemiologic studies have linked vitamin D deficiency with several adverse outcomes, including cardiovascular and cancer-related morbidity and mortality. [2] Vitamin D is limited to certain foods (fatty fish, cod liver oil, egg yolk, some mushrooms, meat etc.) or supplements. The major contribution to vitamin D status is the endogenous production of vitamin D in the skin. Plasma concentrations of 25-hydroxyvitamin D [25(OH)D] has a half-life of 1-2 months and can reflect vitamin D production, absorption, and storage. [3] Epidemiologic evidence for an association between plasma 25-hydroxyvitamin D [25(OH)D] and breast cancer (BC) incidence and/or prevalence is inconsistent. [4]

Vitamin D deficiency is present throughout the world. [5] Increased awareness of the association of vitamin D deficiency with various health outcomes in recent years has contributed to an increase in vitamin D supplement use as well as the prescription of high dose vitamin D in treating vitamin D deficiency. [6]

The results from numerous epidemiological studies that studied circulating vitamin D concentrations and risk of various cancers have proven to be inconsistent. Moreover, there is a lack of definitive clinical trials with vitamin D supplementation and risk of cancer
mostly due to lack of adequate power. A recent Mendelian randomization study showed that a multi-single nucleotide polymorphism score for circulating 25(OH)D concentration was not directly associated with risk of several cancers including BC [7]. However, a cross sectional study in Brazilian postmenopausal women showed that low serum 25 (OH)2 vitamin D level is a risk factor for ER negative tumors, with positive axilla and a higher rate of cell proliferation. [8]

Several longitudinal studies have assessed the relationship between plasma 25-hydroxyvitamin D [25(OH)D] concentrations and cancer risk. The results have been summarized in several meta-analyses, which concluded that 25(OH)D concentrations are inversely associated with colorectal cancer incidence [9-13] but are not associated with prostate or BC incidence [11, 14-16]. Interventional studies that assessed the influence of vitamin D supplementation with or without calcium and cancer [17-20] reported inconclusive or nonsignificant results. [21] The mixed results from observational and interventional studies suggest that the relationship of vitamin D status and cancer risk is still incompletely understood.[22]

This systematic review and meta-analysis attempts to pool, interpret and evaluate research evidence over the past ~18 years linking vitamin D status and intakes (food and supplements) with the occurrence of BC among both pre- and post-menopausal women. Finally, this study discusses potential biological mechanisms behind those putative associations.
4.4 Methods

4.4.1 Search strategy

We conducted a systematic review of the literature on BC using primarily PubMed and Cochrane library as a secondary search. We focused on serum and dietary vitamin D as specific exposures. We searched systematically by combining the keywords "vitamin D" and "BC." Synonymous keywords were not included in the search to avoid heterogeneity in defining the concepts. We restricted the literature search to human studies published in English between January 1st, 2000 and August 31st, 2017. Figure 4.1 shows the search result, inclusion and exclusion criteria and the final number of studies. We considered original research published between 2000 and 2017 because the association of vitamin D with BC was studied rarely before 2000. We assessed the papers by reviewing titles and abstracts yielded by an initial search using relevant keywords combinations in abstracts (i.e. vitamin D and BC). We conducted a systematic review of the literature on BC focusing on specific exposures, namely serum and dietary vitamin D, (See Appendix C for search details). Among those that were selected for review, we retrieved further key information such as study design, contextual setting, sample size, main outcome and key findings. We used EndNote (X8.1) to create a reference database and we summarized extracted summary data in an Excel spreadsheet.
Fig. 4.1: Study Selection.*N=496 excluded based on study subject (non-human); N=1,769 excluded based on full text availability; N=26 non-English language (excluded); N=191 studies with male participants (excluded); N=164 participants younger than 19 years(excluded); N=787 studies with irrelevant parameters (excluded).


For more information, visit www.prisma-statement.org.
4.4.2 Study Identification and Selection

Two independent reviewers (Sharmin Hossain and May Beydoun) determined whether studies were selected for review and meta-analysis. We initiated study inclusion and exclusion by examining titles and abstracts. We retained only studies with direct relevance to our research question. We obtained full text for the selected papers, which we then screened for potential inclusion in the systematic review and meta-analysis. The studies that were included assessed any type of association between vitamin D status and BC incidence or prevalence. Included studies presented findings as risk ratios (RR), hazard ratios (HR) or odds ratios (OR). They used a binary outcome for BC and categorical assessment of vitamin D status (usually binary). Primary reasons for excluding studies were “No relevant data available,” “Study is a randomized controlled trial,” “Study subjects are not adult women,” “Outcome is not incident or prevalent BC,” or “Exposure is not serum 25(OH)D or dietary intake of vitamin D”. The meta-analysis was carried out on all observational study designs including cross-sectional, prospective and retrospective cohort studies.

4.4.3 Data extraction

Detailed study-level characteristics were summarized in APPENDIX B (e.g. age, gender, country), study design (e.g. case-control, cross-sectional, cohort), sample size (e.g. number of cases and controls, or total sample size), type of exposures measured, type of population and quality score (described later). Selected studies were sorted by year of publication and first author’s last time. Type of exposure was identified as serum 25(OH)D (per 10ng/mL), serum 25(OH)D (deficient vs. not), dietary vitamin D (per 100 IU/d),
supplemental vitamin D (yes vs. no), and total vitamin D intake (per 100 IU/d). Further data extraction for use in the meta-analysis was conducted using a series Excel sheets (1 per study) in which the final effect size and its 95% CI were estimated.

### 4.4.4 Qualitative review and meta-analysis

The final selected 18 studies were first reviewed in a systematic way, stratifying by study design and type of exposure. The original measures of association are presented and compared across studies. Using the same 18 studies, we conducted a meta-analysis to assess the strength of the association of BC outcomes with vitamin D exposures among pre- and post-menopausal women combined. This analysis was thus restricted to case-control, cross-sectional, and prospective cohort studies with available data that had comparable measurements for each risk/protective factor, thus allowing the estimation of a pooled measure of association across those data and studies, namely a risk ratio (RR) with its 95% CI. Modifications to the reported RR were made when measured on different scales of exposure [e.g. per 1 SD vs. quartiles vs. tertile vs. per 1-unit (e.g. 10 ng/mL) increase]. All RRs were converted into a single measure of association that closely represents the effect of a fixed incremental linear increase in the exposure on the risk of BC. After converting the RR with their 95% CI to LogeRR with its SE, both parameters (i.e. the point estimate and its SE) were divided by a conversion factor. In fact, in a normal distribution, the means of the highest and lowest tertile lie 2.18 SD apart; therefore, the log RRs were divided by 2.18 to obtain log RR per SD. Similarly, extreme quintiles effects were divided by 2.8, while extreme quartile effects were divided by 2.54. This approach was adopted elsewhere.[23] The value of SD was estimated using descriptive data from
cases and controls (or total population in the case of cross-sectional or cohort studies) or by approximating the differences between extreme quantiles and then dividing them by the conversion factors above.[24] From this, RR per 10 ng/mL for serum 25(OH)D, or per 100 IU dietary or total vitamin D was estimated by dividing the log \(_e \)RR \(_z\) with its Standard Error (SE) \(_z\) by the estimated SD value (either reported in the study or estimated from extreme quantiles) and multiplying it by 10 and 100, respectively. The log-transformed RR is then exponentiated to obtain the point estimate of RR per 10 ng/mL increase in 25(OH)D or 100 IU/d of dietary/total vitamin D intake. To obtain the 95% CI, the log \(_e \)RR point estimate is used along with its SE to obtain the lower and upper confidence limits on the log \(_e \) scale. These values are then exponentiated to obtain RR’s 95% CI. A similar approach was adopted when vitamin D exposure in a specific study was reported into non-quantile categories (i.e. exposure groups with varying sample sizes). However, in this case, each contrast with a referent category is transformed into per 10 ng/mL or 100 IU/d and then pooled into a common measure of association using random effects models within that study. Moreover, in the latter instance, median exposure value in each category is estimated and subtracted from the referent to obtain average increment in vitamin D exposure corresponding to each contrast. This is then used to ensure the measure of association corresponded to 10 ng/mL or 100 IU/d increase, for 25(OH)D and dietary/total vitamin D, respectively. Varying units and conversion factors in the measurement of serum 25(OH)D were also considered in these calculations. In studies with stratified analyses (e.g. by age group), incremental RR were estimated per strata and then pooled into one estimate with associated 95% CI, using random effects models within the study.
The study-specific RRs for each exposure of interest were then examined in pooled models, after testing for heterogeneity using the $I^2$ test. As such, a summary or pooled RR was provided using forest plots and estimated using inverse variance weighting.[25] Random effects models that further incorporated between-study variability were conducted using DerSimonian and Laird’s methodology.[25]

4.4.5 Harvest and funnel plots

A pre-defined quality score (QS) was used to assess the quality of each included study. This QS is a modified version of previously used scoring systems[26] and was applied in a previous meta-analysis.[23] In our meta-analysis, the QS scale included 4 items, namely study design, study size, outcome assessment, and adjustment for potential confounders, each of which can be scored from 0 to 2 in ascending order of quality. Thus, the total QS score could range from 0 to 8. Since only one outcome was studied (i.e. BC), only one QS was linked to each study. Three independent assessments of QS items/study were made by three co-authors (May Beydoun, Hind Beydoun and Xiaoli Chen) and the average QS was determined. A consensus was then achieved by the 3 co-authors after initial rating. To represent graphically the key findings of studies for each exposure of interest, a harvest plot was used. This plot shows the exposure-outcome associations of interest in each study, whether they were significant and in which direction (-1 = “inverse association”, 0 = “null association”, 1 = “positive association”) for each exposure against QS which is presented on the y-axis. At least 3 studies were needed to create a harvest plot per hypothesized exposure-outcome association.
Finally, in order to examine publication bias, we used Begg’s funnel plots (APPENDIX E); each OR point estimate was plotted against their corresponding standard errors (SE) for each study on a logarithmic scale,[27,28] combining all exposures (e.g. serum and dietary vitamin D exposures). This type of bias was also formally tested using the Begg-adjusted rank correlation tests[29] and the Egger’s regression asymmetry test.[30] All analyses were conducted with STATA 15.0 (StataCorp, College Station, TX).[31] Type I error was set at 0.05 for all measures of association.

4.5 Results

4.5.1 Study Selection, Characteristics and Quality Score

Out of 3,451 un-duplicated titles and abstracts between 2000 and 2017, 18 published original epidemiologic studies were considered for review and/or meta-analysis. Those selected studies were published between 2005 and 2014 (Mean±SD: 2010±3), with 11 being US studies, 1 Canadian, 4 European and 2 from Asia (APPENDIX D). Moreover, most studies had a case-control or nested case-control design (n=16), with only 2 being prospective cohort studies. Eight studies comprised adult women of varying age ranges, while 4 included only pre-menopausal women and 6 were restricted to post-menopausal women. Overall, mean age was 54.2 with a SD of 10.3y. The cumulative sample of studies included in our meta-analysis consisted of 125,291 subjects, with a Mean±SD: 6,690±17,245 subjects per study. Finally, mean QS with its SD was 4.56±1.69 (range:2-8), indicating a relatively above average quality set of studies, given that the maximum score is 8.
4.5.2 Qualitative Review of Studies

Of 16 case-control studies included in this paper, 9 studies (56.3%) showed that a reduced risk of BC was associated with high levels of 25(OH)D [19, 21, 24, 29, 32, 33] or the use of vitamin D or multivitamin supplements [34-39]. The other 7 case-control studies (43.7%), however, showed no associations of BC risk with 25(OH)D levels or vitamin D intake [30, 32, 33, 40-43].

Of two prospective cohort studies included in this current study, both showed that dietary calcium could reduce the risk of BC among postmenopausal women [44,45]. For example, in the Cancer Prevention Study II Nutrition Cohort (QS=8), McCullough et al (2005) studied dairy, calcium, and vitamin D intake and BC risk among 68,567 postmenopausal women aged 50-74 years in the US (QS=8). [46-49] Their results supported the hypothesis that dietary calcium may modestly reduce the risk of postmenopausal BC. In the Iowa Women's Health Study of 34,321 postmenopausal women aged 55-69 years with 18 years of follow-up (QS=8), [42] reported that vitamin D intake appeared to be associated with a small decrease in BC risk among postmenopausal women in the US.

4.5.3 Meta-Analysis: Findings for Serum Vitamin D

In total, measures of the association from 12 case-control studies on serum 25(OH)D in relation to BC were pooled, with a total number of 8,156 cases matched with 11,336 controls. Our pooled findings indicated that there was no detectable association between serum 25(OH)D concentration and BC occurrence (RR=0.99, 95% CI: 0.98-1.00, P=0.12, per 10 ng/mL), while based on 3 case-control studies (1,021 cases matched with 1,021
controls), serum 25(OH)D deficiency was shown to have a direct relationship with the risk of BC (RR=1.73, 95% CI: 1.28-2.34, P<0.001) (Fig 4.2).

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**Figure 4.2: Study Summary.** Heterogeneity chi-squared = 58.61 (d.f. = 11) p = 0.0001. I-squared (variation in ES attributable to heterogeneity) = 81.2%. Test of ES=1: z= 1.30 p = 0.195

4.5.4 Meta-analysis: Findings for Dietary Vitamin D (foods and supplements)

In total, measures of association from 4 studies on total dietary vitamin D (foods and supplements) relation to BC occurrence were pooled. We demonstrate a clear net inverse association between total vitamin D intake and BC, with a pooled RR=0.98, 95% CI: 0.96-1.00, P=0.029, per 100 IU/d. This small inverse association was observed for
dietary vitamin D and supplemental vitamin D usage (yes vs. no), though did not attain statistical significance.

4.5.5 Bias Assessment: Funnel and Harvest Plots

Begg’s funnel plot indicated that most of the 22 data-points fell within the expected confidence limits when plotting Loge(RR) against its SE. Begg’s test indicated no publication bias, with z=0.99 and associated p-value of 0.32, and Egger’s test indicated there was no asymmetry whereby Loge(RR) was not associated with its SE in terms of slope. In addition, there was no bias in terms of the directionality of Loge(RR) that were published in the literature.

Examining each individual exposure, harvest plots are presented in APPENDIX F, plotting qualitative findings (-1=”inverse association”, 0=”null association” and 1=”positive association”) against study-level QS.[50,51] Based on the clustering of findings, 6 of 11 studies that examined 25(OH)D in relation to BC, reported a null overall finding. Noteworthy is that those studies had a slightly higher QS compared with those that reported an inverse association, though the difference was not statistically significant (QS 4.4 vs. 4.0). In contrast, all studies with 25(OH)D deficiency, total/dietary vitamin D and supplemental vitamin D reported a finding that follows the hypothesized direction of association. The latter finding suggests consistency upon replication whereby an overall pooled finding trending towards the hypothesized direction was consistent with the overall finding of each individual study included in the meta-analysis.
4.6 Discussion

This study is to our knowledge, the most up to date among very few meta-analyses conducted to synthesize the literature on vitamin D exposure and BC occurrence. Pooled findings indicated that there was a net direct association between 25(OH)D deficiency and BC occurrence, with a pooled RR=1.73, 95% CI: 1.28-2.34, p<0.001). An inverse association was also observed for total vitamin D intake from foods and supplements (RR=0.98, 95% CI: 0.96-1.00, P=0.029, per 100 IU/d) and BC. No net association was detected between BC and serum 25(OH)D (per 10 ng/mL) or between BC and dietary/supplemental vitamin D. There was no evidence of publication bias. Harvest plots suggested consistency upon replication was found for all 5 exposures of interest.

In recent years, there has been considerable interest in whether vitamin D inhibits BC development.[42] The anticarcinogenic potential of vitamin D comes from the active, hormonal form of vitamin D, 1,25-dihydroxyvitamin D [1,25(OH)2D], which is the product of a second hydroxylation of 25-hydroxyvitamin D [25(OH)D] in the kidneys. Experimental studies indicate that 1,25(OH)2D can also be synthesized from 25(OH)D in other tissues, possibly including breast tissue. [52, 53] The vitamin D receptor is activated by 1,25-dihydroxyvitamin D [1,25(OH)D], and is found in nearly all tissues and organs in the human body. It is responsible for the transcription of numerous genes related to cell-cycle control and apoptosis. Therefore, it is of considerable interest in relation to many cancers, including BC. [54] While the circulatory levels of the biologically active metabolite 1,25-dihydroxyvitamin D [1,25(OH)2D] is tightly regulated, the precursor 25-hydroxyvitamin D [25(OH)D] serves as an indicator of vitamin D status from other sources i.e. vitamin D from cutaneous synthesis and diet. Since circulating 25(OH) is related to
1,25-dihydroxyvitamin D [1,25(OH)2D] in breast tissue and circulating 1,25-
dihydroxyvitamin D [1,25(OH)2D] is homeostatically controlled, circulating 25-
hydroxyvitamin D [25(OH)D] is thought to be potentially relevant to breast
carcinogenesis.[42]

This study has many strengths. First, this systematic review of the literature is, to
our knowledge, one of the few to have examined a wide range of vitamin D exposures and
their relationships to with BC occurrence through meta-analysis. Second, we used a
validated quality scoring system as a tool to examine heterogeneity of study results based
on quality of data. However, our study results should to be interpreted with caution
considering several limitations. First, our literature search was restricted to the PubMed
and Cochrane databases, and did not include other electronic databases such as Embase or
Web of Science. Second, we used specific key terms to perform the literature search but
did not search for cross-references or unpublished studies (abstracts, conference papers,
theses and dissertations). Third, while exposures included dietary and supplemental
vitamin D in addition to serum 25(OH)D, some meta-analyses were low-powered given
the limited number of data-points available. Fourth, evidence was mostly generated from
observational studies, namely, cross-sectional, retrospective cohort and prospective cohort
studies, which precludes our ability to confirm causality. Thus, a separate meta-analysis of
randomized controlled trials is needed, once enough trials are made available for such
analysis. Fifth, the associations reported in this study may be confounded by other micro –
and macro-nutrients, as well as lifestyle factors that have been shown to affect the risk of
BC. Finally, publication bias cannot be ruled out as an explanation for these study results.
Our review and meta-analysis indicated that serum vitamin D deficiency, as well as total vitamin D intake, were associated with BC occurrence in the general population, suggesting a putative protective effect of vitamin D pending future randomized controlled trials.

4.7 Acknowledgements

All authors read and approved the final manuscript. The authors would like to thank Gregory A. Dore and Ola S. Rostant (NIA/NIH/IRP) for their internal review of the manuscript.

4.8 Author contributions:

SH: Conceptualization; literature search and review; plan of analysis; write-up of manuscript; revision of manuscript.

MAB: Literature review, plan of analysis, data management, statistical analysis, write-up of parts of the manuscript, revision of the manuscript.

HAB: Literature review; write-up of parts of manuscript; revision of manuscript.

XC: Literature review; write-up of parts of the manuscript; revision of the manuscript.

ABZ: Plan of analysis, write-up of parts of the manuscript; revision of the manuscript.

RJW: Conceptualization; write-up of parts of the manuscript; revision of manuscript.

4.9 Conflict of interest: None.
4.10 Bibliography


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CHAPTER 5

HISTONE DEACETYLASE ACTIVITY AND VITAMIN D-DEPENDENT GENE EXPRESSIONS IN RELATION TO SULFORAPANE IN HUMAN BREAST CANCER CELLS

By

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5.1 Abstract

It is relatively unknown how dietary bioactive compound, sulforaphane, in partnership with vitamin D, regulate vitamin D-dependent gene expression in breast cancer. It has been suggested that the combination of various bioactive components with vitamins is crucial for their potential anticancer activities. METHODS: This study employed a combinatorial chemo preventive strategy to investigate the impact of dietary histone deacetylase inhibitor i.e. sulforaphane on chromatin remodeling in breast cancer. To understand the epigenetics-mediated changes in gene expression, MCF-7 cells were exposed for 24 hours to vitamin D (100nM) either alone or in combination with L-sulforaphane and TSA (20μM and 1μM respectively) at 70% confluency. Changes to VDR, CYP24A1, CYP27B1 and TRPV6 gene expressions were quantified using real-time PCR-based assays. Histone deacetylase inhibitor activity was assessed using HDAC I/II assay that measured global changes in acetylation status. Cell viability was measured using ATP and MT assays. Clonogenic and migration assays were performed to analyze the ability of single cells to grow into colonies and % closure (migration ability) upon treatments respectively. Results were expressed as ΔCT± standard error of means (SEM) from One-way ANOVA analyses for mRNA expressions and mean± SEM for all other assays. RESULTS: In MCF-7 cells, treatment with 1,25 (OH)_{2} D_{3} tended to decrease VDR (13±0.4) and CYP27B1 (12±0.96) while significantly increasing TRPV6 (p=0.02, 14±0.1) and CYP24B1 (p<0.0001, 0.38±0.12) expression. D alone and D+TSA group had the opposite effects on HDAC inhibition from SFN alone, D+SFN and TSA alone. The clonogenic assay showed a significant decrease in colony formation with no colonies for D+TSA
(p<0.03) and TSA alone group (p<0.03). Cell viability tended to decrease with D alone and in combination with TSA. CONCLUSION: These data suggest that the effects of vitamin D and sulforaphane are selective and gene-specific in MCF-7 cells.

**Key words:** HAT, histone acetyltransferase; HDAC, histone deacetylase; SFN, sulforaphane; TSA, Trichostatin A; VDRE, vitamin-D response elements.

**5.2 Introduction**

Breast cancer is the most common cancer among women worldwide and the incidence has continued to rise over time. [1] Nutrition influences cancer etiology in about 35% of cancer cases. [2] General preventive dietary advice often includes reducing intake of alcohol, red meat, and saturated fat while increasing the intake of fiber, vitamin D and phytoestrogens from various food sources. [3] Breast cancer is a heterogeneous disease and vitamin D might only affect certain breast cancer subtypes. However, a protective effect of vitamin D against breast cancer has been suggested in several studies, which has been related to sunlight, dietary intake, vitamin D supplement and ethnic background. [4] Potentially important factors associated with increased breast cancer are listed below:

**Table 5.1: Factors Influencing BC Risk [5]**

<table>
<thead>
<tr>
<th>Factors</th>
<th>Modifiable Factors</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Modifiable</strong></td>
<td>Weight gain after the age of 18 and/or being overweight or obese (for postmenopausal breast cancer)</td>
</tr>
<tr>
<td></td>
<td>Use of menopausal hormone therapy (combined estrogen and progestin)</td>
</tr>
<tr>
<td></td>
<td>Physical inactivity</td>
</tr>
<tr>
<td></td>
<td>Alcohol consumption</td>
</tr>
<tr>
<td></td>
<td>Heavy smoking</td>
</tr>
<tr>
<td></td>
<td>Shift work at night (i.e., that disrupts sleep patterns)</td>
</tr>
<tr>
<td></td>
<td>Age</td>
</tr>
<tr>
<td></td>
<td>Personal of Family History</td>
</tr>
<tr>
<td>Non-modifiable</td>
<td>Inherited Mutations in BRCA1, BRCA2 of other susceptibility genes</td>
</tr>
<tr>
<td>---------------</td>
<td>---------------------------------------------------------------</td>
</tr>
<tr>
<td></td>
<td>Atypical Hyperplasia</td>
</tr>
<tr>
<td></td>
<td>High dose radiation to the chest at a younger age (e.g., for cancer treatment)</td>
</tr>
<tr>
<td></td>
<td>High Breast Tissue density</td>
</tr>
<tr>
<td></td>
<td>High BMD (bone mineral density)</td>
</tr>
<tr>
<td>Reproductive</td>
<td>Long Menstrual History</td>
</tr>
<tr>
<td></td>
<td>Inability to have children</td>
</tr>
<tr>
<td></td>
<td>Use of Oral contraceptives</td>
</tr>
<tr>
<td></td>
<td>Having first child after age 30</td>
</tr>
<tr>
<td></td>
<td>High natural levels of sex hormones</td>
</tr>
</tbody>
</table>

Many in vitro and pre-clinical studies have examined the use of 1,25-dihydroxyvitamin D3 to treat breast cancer. Four meta-analyses identified a significant inverse relationship between the circulating concentrations of 25-hydroxyvitamin D3 and breast cancer [6-8]. By contrast, a large randomized clinical trial, Women’s Health Initiative (WHI), showed that administering 400 IU vitamin D and 1000 mg of calcium versus placebo to women did not reduce the risk of breast cancer. The finding from WHI does not support the use of vitamin D as a prophylactic agent for breast cancer. However, better survival among woman diagnosed with breast cancer has been reported to be related to vitamin D intake leading to higher concentrations of 25-hydroxyvitamin D3 [8, 9]. A high concentration of plasma 25(OH)D3 is associated with a significantly reduced risk of premenopausal breast cancer [10]. A serum 25-hydroxyvitamin D (25(OH)D3) level of 50 ng/ml was associated with 50% lower incidence of breast cancer, compared to a baseline of < 10 ng/ml [11].

It has been hypothesized that 1,25-dihydroxyvitamin D3 can reduce the risk of breast cancer. Several studies have examined the effects of 1,25-dihydroxyvitamin D3 on
mammary carcinogenesis in various cell lines and animal models and found a protective role of 1,25-dihydroxyvitamin D3 in breast cancer development.

In breast cancer, investigations carried out on histone modifications are relatively newer than DNA methylation studies. Post-translational histone modifications have a critical role in breast tumorigenesis and aggressiveness of prognosis. Since different breast cancer subtypes represent distinct gene expression profiles, it is important to clarify the effect of histone modification on gene expression levels in breast tumors. However, there are limited studies on histone modification patterns in breast cancer tumors, though the numbers of investigations are increasing. There are several bioactive compounds present naturally in food that are known to modify genetic and epigenetic profiles in various cancers. Sulforaphane is one such compound that can be extracted from its precursor molecules present in cruciferous vegetables e.g. broccoli, Brussel sprouts etc. It is known to be a potent natural source of histone deacetylase (HDAC) inhibitor activity. [12] We used Trichostatin A (TSA) as our positive control. TSA is a fungal metabolite with well-established, strong histone deacetylase inhibitor activity.

The objective of this study is to determine how histone modification using sulforaphane or Trichostatin A (TSA), influences vitamin D-induced gene transcription in breast cancer. There are not enough studies that examined this specific association and in MCF-7 cells as a model of breast cancer. The rationale is that the chosen epigenetic modifiers will alter the acetylation status of histone proteins, which will loosen the DNA-histone protein complex and allow increased transcriptional activity at the vitamin D response element (VDRE) found in the promoter region of vitamin D-inducible genes.
5.3 Methods

5.3.1 Cell Culture

Cells were treated on day 7 from the date of seeding into 6-well plates. The experiment included six different treatment groups namely- DMSO control (0.1%), 1,25(OH)2D3 (100nM), SFN (20 µM), TSA (1µM), 1,25 (OH)2D3 + SFN and 1,25 (OH)2D3 + TSA (APPENDIX G). After treatment, the cells were incubated for 24 hours before harvesting. MCF-7 cells were obtained from Arcaro lab. 1, 25 (OH)2D3 was purchased from Enzo Life Sciences, Plymouth Meeting, PA. Trichostatin A and SFN were purchased from Sigma-Aldrich, St. Louis, MO. Real-time PCR materials, including all primers and master mix were purchased from Applied Biosystems, Foster City, CA. Cell culture dishes were bought from Costar, Cambridge, MA. All other cell culture materials and materials for RNA isolation and cDNA synthesis were purchased from Life Technologies, Grand Island, NY.

5.3.2 RNA Isolation and Reverse Transcription

At the completion of the treatment period, cells were washed with saline, then treated with 1 mL Trizol per well of a 6-well plate, and cells were scraped to be harvested for subsequent RNA extraction. Cells were frozen at -80°C until RNA was ready to be isolated in collection tubes. To isolate the RNA, 200 µL chloroform was added to each homogenate for phase separation. Isopropanol (500 µL) was then used to precipitate the RNA. Washing three times with 1 mL of 75% ethanol was followed by the addition of 40 µL of Diethyl pyrocarbonate (DEPC) water. The samples were then incubated for 10
minutes at 56°C to ensure maximum RNA solubility. RNA samples were then quantified using a Nanodrop machine (λ260/280). cDNA was prepared using the SuperScript III First Strand Synthesis System for RT-PCR (Life Technologies, Grand Island, NY). Details of the protocol are listed in APPENDIX H.

5.3.3 Real Time PCR

For all experiments, mRNA expressions of GAPDH, VDR, CYP24A1, CYP27B1, and TRPV6, were measured by ViiA™ 7 Real-Time PCR System (Applied Biosystems, Foster City, CA). Reactions consisted of 10 µL of SYBR GREEN Gene Expression Master Mix (Applied Biosystems, Foster City, CA) with forward and reverse primers (10nM each) and 10 µL of DNAse/RNAse Free Water with 100 ng of cDNA. Duplicates were set up for each sample. The levels of VDR, CYP24A1, CYP27B1, and TRPV6 were measured by the comparative ΔΔCT method (ThermoFisher Scientific, 2016). For each sample, the relative abundance of GAPDH and the target genes were obtained. The difference between those two abundances (target gene- GAPDH) is the ΔCT for that sample which were the values used for statistical analysis. The mean ΔCT values were then normalized against the mean ΔCT values of the control sample, Dimethyl Sulfoxide (DMSO), resulting in a ΔΔCT value. 2 was raised to the – ΔΔCT to produce a relative quotient (RQ) for each sample, with the RQ of the control being one. RQ values were used to create the mRNA expression curves for each gene. Primer sequences are available on APPENDIX A.
5.3.4 Histone Deacetylase (HDAC) Assay

We used a luminescent HDAC-Glo™ I/II Assay (Promega, 2017). The assay uses an acetylated, live-cell-permeant, luminogenic peptide substrate that can be deacetylated by HDAC activities. Deacetylation of the peptide substrate is measured using a coupled enzymatic system in which a protease in the Developer Reagent cleaves the peptide from aminoluciferin, which is quantified in a reaction using Ultra-Glo™ Recombinant Luciferase. The HDAC-mediated luminescent signal is persistent and proportional to deacetylase activity.

Cells were harvested 24 h after treatment with SFN (20 µM) and TSA exposure (1 µM), and cell lysates were analyzed for HDAC activity, as reported [13].

5.3.5 ATP Assay

We used the CellTiter-Glo® 2.0 Assay (Promega, 2017). This assay provides a homogeneous method to determine the number of viable cells in culture by quantitating the amount of ATP present, which indicates the presence of metabolically active cells. Luminescence readout is directly proportional to the number of viable cells in culture. Cells were harvested 24 h after co-treatment with SFN (20 µM) and TSA (1 µM), and cell lysates were analyzed for ATP activity.

5.3.6 MT Assay

We used the RealTime-Glo™ MT Cell Viability Assay (Promega, 2017), and measured cell viability in MCF-7 cells continually in the same sample well up to 48 hours.
This gave us more information about the mode of action of our treatments about time dependence. The nonlytic nature and rapid response of the assay made it possible to monitor cell viability over time in the same well. The assay measures the reducing potential of viable cells, and is ATP-independent, providing an orthogonal method for viability or cytotoxicity determination.

Luminescence readout is proportional to the number of viable cells in culture. Cells were harvested 24 h after co-treatment with SFN (20 µM) and TSA (1 µM), and cell lysates were analyzed for ATP activity.

5.3.7 Caspase 3/7 Assay

We used the Caspase-Glo® 3/7 Assay (Promega, 2017) to measure apoptosis in MCF-7 cells. The assay provides a pro luminescent caspase-3/7 DEVD-aminoluciferin substrate and a proprietary thermostable luciferase in a reagent optimized for caspase-3/7 activity, luciferase activity and cell lysis. Adding the single Caspase-Glo® 3/7 Reagent in an "add-mix-measure" format results in cell lysis, followed by caspase cleavage of the substrate. This liberates free aminoluciferin, which is consumed by the luciferase, generating a "glow-type" luminescent signal that is proportional to caspase-3/7 activity in the system. Cells were harvested 24 h after co-treatment with SFN (20 µM) and TSA (1 µM), and cell lysates were analyzed for caspase 3/7 activity.

5.3.8 Clonogenic Assay

We used the “plating before treatment” setup for this assay[14]. MCF-7 cells were harvested and re-plated (500/well) in a 6-well plate and allowed overnight to attach (day
0). Cells were treated (in duplicates) the next day using DMSO as the vehicle control, D (100nM), SFN (20µM) and TSA (1µM) as primary treatments and in combinations (D+SFN) and (D+TSA) (day 1). The plates were then incubated for three weeks at 37°C. After the desired incubation period, the cells were fixed using 70% EtOH followed by Crystal violet (0.5%) staining. Colonies were counted using a standard colony counter. Plating efficiency (PE) and Surviving fractions (SF) were calculated as per protocol described by Franken et al. [14]. This entire experiment was repeated two more times for an N=3.

5.3.9 Migration Assay

A simple scratch test was used to determine the rates of closure following treatments to assess their efficacy. MCF-7 cells were seeded in 6-well plates at a density of 325,000 cells/well and allowed to attach overnight. Once the cells were 70% confluent, wounds were created using the etching at the bottom of the wells as guide, using a p-200 pipet tip. The wells were then washed with 1X sterile PBS and treated as designed. Photographs were taken on day 0 at four reference points (4 quadrants) and marked as the “0Hr”. Each wound was then followed over the course of 24 hours to determine the rate of closure in response to the treatments. Photographs were taken again and marked as “24Hr”. This entire experiment was repeated for an N=3. Percent closure is calculated as below:

\[
\text{% closure}= \frac{(0Hr \text{ avg. wound size} - 24Hr \text{ avg. wound size})}{0Hr \text{ avg. wound size}}
\]
5.3.10 Statistical Analyses

Results from real-time PCR were analyzed using one-way ANOVA with a p-value of <0.05 used as the cut-off for statistical significance. The analysis was setup as a randomized block design with each experiment as a block. Dunnett’s Multiple Comparison Tests was also used to find which means of the treatment groups differed significantly from the control (DMSO) and used a P-value of <0.05 as a cut-off for statistical significance. All statistical analyses and figures were generated using GraphPad Prism (v 7.04). Tukey’s post-test was conducted to determine significant multiple comparison differences.

5.4 Results

5.4.1 D-responsive gene expression

We hypothesized that the expression of vitamin D-inducible genes will be increased by concomitant treatment of MCF-7 cells with HDAC inhibitors (SFN and TSA). We measured, with and without the HDAC inhibitors, the mRNA expressions of genes known to be involved in vitamin D action: namely, VDR (vitamin D receptor), CYP24A1 (24 hydroxylase), CYP27B1 (1α hydroxylase) and TRPV6 (transient receptor potential, vanilloid family, member 6 calcium-specific channel protein) by real time quantitative polymerase chain reaction (RT-PCR); results are expressed as ΔCT±SEM and the graphs represent the RQ values ($2^{\Delta\Delta CT}$). Although, VDR expression (N=4) in MCF-7 cells was not statistically significant from the one-way ANOVA test, Browne- Forsythe test showed a significant association (p=0.0009). When compared to the control, D alone group (-0.47±0.11), D+SFN group (-0.28±0.32), and D+TSA group (-0.20±0.29) tended to
decrease its expression while SFN alone (0.003±0.23) and TSA alone (0.05±0.41) showed minimal increase. There was no statistical difference observed from the Wilcoxon Signed Rank test.

TRPV6 (N=3) expression, was overall significant (p=0.04) from the One-way ANOVA test. D alone (3.56±2.5), D+TSA (1.21±0.69) and SFN alone (4.34±2.3) groups tended to increase TRPV6 expression. TSA alone (19.14±9.4, p=0.04) showed a moderate increase after D+SFN group (47.88±35.00, p=0.02) and were statistically significant. There was no statistical difference observed from the Wilcoxon Signed Rank test.

CYP24A1 expression (N=2) was statistically significant (p<0.0001) from both the One-way ANOVA and the Brown-Forsythe test (p<0.0001). As expected, D alone group (36.16±8.66) demonstrated statistically significant response. SFN alone (2.69±0.90, p<0.0001), D+SFN group (2.00±2.8, p=0.0009) and TSA alone (0.29±0.34, p=0.0002) showed smaller change in CYP24A1. When combined with D, TSA showed the second highest response in CYP24A1 (13.24±2.31, p<0.0001) and was statistically significant.

No treatment showed statistical significance in CYP27B1 (N=3) expression. Results summary compared to control: D alone (-0.16±0.41), SFN alone (-0.16±0.34), D+SFN (-0.67±0.06) and D+TSA (-0.67±0.06) decreased CYP27B1 expression while TSA alone (0.66±0.60) tended to increase its expression. There was no statistical difference observed from the Wilcoxon Signed Rank test. The results for RT-PCR experiments are presented in Fig 5.1.
Fig 5.1: mRNA Expressions of Vitamin D Responsive Genes in MCF-7 Cells. (a) Cyp24a1 showed the highest response in D only group; (b) TRPV6 showed the highest response in D+SFN group. Treatments had no effect on (c) Vitamin D receptor (VDR) and (d) Cyp27b1 when compared to control. The data were analyzed by One-way ANOVA with *p <0.05, **p<0.01, ***p<0.001 and ****p<0.0001 as significant.
5.4.2 Histone Acetylation Status

Both epigenetic modifiers used in our experiments are known to have well-demonstrated HDAC inhibitor properties. However, SFN has a more diverse biochemical effect, including triggering increased expression of many cytoprotective proteins in the cell than TSA (positive control). To test the relative HDAC inhibition by SFN and TSA, we measured nuclear HDAC I/II enzyme activity and changes in histone protein acetylation to measure the extent of alteration in histone acetylation status. MCF-7 cells were seeded at 10,000 cells/well in a 96-well, opaque walled plate. Cells were treated at 70% confluency with the desired treatments and incubated for 24 hours before the assay was conducted. Our expectation was that the selected dosages of SFN (20 μM) and TSA (1 μM), are sufficient to provide significant changes in histone acetylation in MCF-7 cells. The HDAC assay results are presented in Fig 5.2.
Fig 5.2: HDAC Activity in MCF-7 cells. *No significant difference was observed among the treatments. Each data point represents mean±SEM (N=3).*

The overall HDAC activity (N=3) was insignificant from One-way ANOVA analysis and the treatments showed minimal to no HDAC inhibitor activity. TSA alone group (-107.31±43.3) and SFN alone (-45.67±29.46) demonstrated the expected response to HDAC inhibition, albeit insignificant. D alone group (194±194) showed no inhibition and so did D+TSA group (260±185.6). D+SFN group (-45.67±66.11) showed similar effects as SFN alone group.
5.4.3 ATP-dependent Cell Viability Assay

To determine cell viability, we measured cellular ATP activity. MCF-7 cells were seeded at 10,000 cells/well in a 96-well, opaque walled plate. Cells were treated at 70% confluency with the desired treatments and incubated for 24 hours before the assay was conducted. Our expectation was that the selected dosages of SFN (20 μM) and TSA (1 μM) with vitamin D (100 nM), are sufficient to decrease viability in MCF-7 cells. The results are presented as percent (%) control in Fig 5.3.

Fig 5.3: ATP-dependent Cell Viability in MCF-7 cells. No effect of treatments was observed on ATP-dependent viability. Values are representative of three independent experiments (N=3) and are expressed as percent (%) control.
5.4.4 ATP-independent Cell Viability Assay

To determine cell viability in an ATP-independent manner, we measured cellular MT Cell Viability Substrate activity. MCF-7 cells were seeded at 10,000 cells/well in a 96-well, opaque walled plate. Cells were treated at 70% confluency with the desired treatments and incubated for 24 hours before the assay was conducted. Our expectation was that the selected dosages of SFN (20 μM) and TSA (1 μM) with vitamin D (100 nM) would be sufficient to decrease viability in MCF-7 cells. Two-way ANOVA test was performed to test for time X treatment interaction.

Compared to control, all treatments were significant different at all time points (p<0.0001) with an overall significant interaction(p<0.0001). Tukey’s post test showed significant difference in D+SFN (p<0.0001) and D+TSA (p<0.0001) at 0 and 48 hours, with an increase in cytotoxicity by SFN and TSA at 48 hours. There was no significant difference among the treatments at 6, 12 and 24 hours. The results are presented as relative luminescence in Fig 5.4.
5.4.5 Apoptosis in MCF-7 cells

We used Caspase-Glo® 3/7 lytic assay and measured the degree of caspase cleavage of the substrate. The cleavage liberates free aminoluciferin, which is consumed by the luciferase enzyme, generating signals proportional to caspase-3/7 activity (used as a proxy measure of apoptosis). In other words, the higher the activity of the enzyme, the higher the luminescent signal. MCF-7 cells were seeded at 10,000 cells/well in a 96-well, opaque walled plate. Cells were treated at 70% confluency with the desired treatments and incubated for 24 hours before the assay was conducted. Our expectation was that the selected dosages of SFN (20 μM) and TSA (1 μM) with vitamin D (100 nM) would be
sufficient to induce apoptosis in MCF-7 cells. The results are presented as relative luminescence in **Fig 5.5**.

**Fig 5.5: Apoptosis in MCF-7 cells.** *All treatments demonstrated significant decrease in caspase 3/7 activity in MCF-7 cells. Values are representative of four independent experiments (N=3) and represented as percent (%) control (Mean±SEM). Tukey’s post test showed no significant difference between D+SFN when compared to D alone.*

### 5.4.6 Clonogenic Assay

Clonogenic assays, first tested in 1956 and later modified to fit several mammalian cell types, including stem cells, essentially test every cell in a population for its ability to undergo “unlimited” cell division [14]. This assay is especially suitable to determine cell reproductive death post-treatment with ionizing radiation as well as other choice of cytotoxic compounds. The general principle is, only a fraction of the cells will retain the
capacity to produce colonies. We treated MCF-7 cells as per protocol (described in the methods section) for three weeks with desired treatments. We then counted the cell colonies (Fig 5.6) formed and the calculated the surviving fraction (SF). There seems to be a trend of decreased colony formation when compared to control after three weeks (N=3).

Summary of treatment vs control: DMSO (0.15±0.07), D alone (0.02±0.005), SFN alone (0.024±0.005), D+SFN group (0.024±0.0005), D+TSA (p=0.03, 0.0015±0.0015) and TSA (p=0.03). Data is represented in the form of a surviving fraction (SF) curve in Fig.5.7.

![Clonogenic Assay in MCF-7 Cells](image)

**Fig 5.6: Clonogenic Assay in MCF-7 Cells.** Untreated controls (DMSO) with 60 and 56 clones, respectively, formed after seeding 1000 cells per well. D treatment group had 30 and 26 clones per well, respectively, while SFN group had 11 and 30. D+SFN group had 33 and 22 clones respectively, while neither D+TSA or TSA showed any colony formation.
Fig 5.7: (A) Surviving Fraction Curve from Clonogenic Assay in MCF-7 Cells. (A)

D+TSA group (p< 0.03) and TSA group (p<0.03) significantly reduced colony formation in MCF-7 cells. (B) Sample control (DMSO) cell picture on day 0.

5.4.7 Migration Assay

MCF-7 cells were plated and treated as per protocol described above. We then calculated the % closure for each treatment compared to the control per the formula shown in the methods section. Compared to control (N=3) the combination groups D+SFN (0.87±0.09) and D+TSA (0.86±0.07), showed no difference in migration (Fig 5.8). D alone (0.89±0.09) and TSA alone (0.90±0.10) groups showed moderate reduction in migration while SFN alone (0.80±0.07) demonstrated the opposite effect in MCF-7 cells.
Fig 5.8: Percent (%) Closure in MCF-7 cells. No significant difference was observed in MCF-7 cells when compared to control.

5.5 Discussion

Breast cancer is the most frequently diagnosed cancer in women [15]. Breast cancer ranks second as a cause of cancer death in women. Overall, breast cancer death rates declined by 36% from 1989 to 2012 due to improvements in early detection and treatment, translating to the avoidance of approximately 249,000 breast cancer deaths [5].

A large proportion of breast cancer cells contain the VDR protein. However, the level of expression is variable within individual cells and, therefore, the biological response to 1,25-dihydroxyvitamin D3 varies for an individual patient’s cells as well as between patients. One of the key factors regarding the activity of 1,25-dihydroxyvitamin D3 is its availability in the breast cancer environment. This is maintained by the balance between synthesis and catabolism of vitamin D. Many epidemiological studies have reported
positive associations between gene polymorphisms and risk of breast cancer; these include vitamin D related genes such as polymorphisms in VDR, vitamin D-binding protein, as well as the CYP24A1 gene [3]. In the present study, our chosen treatments had no effect on VDR expression in MCF-7 cells.

Intracellular calcium plays a critical role in many fundamental cellular processes such as proliferation, apoptosis, and secretion. Therefore, calcium levels are tightly controlled in cells and any disruptions in intracellular calcium homeostasis play a critical role in tumor progression in all cancer types. Studies have been conducted to analyze how intracellular calcium homeostasis and the associated calcium signaling pathways are altered in breast cancer cells compared with normal mammary gland cells. Calcium channels are either upregulated or downregulated depending on the types of cancer. One such example is the altered expression of TRPV6 in prostate, pancreatic, thyroid, colon, ovary, and breast cancer [16]. TRPV6 mRNA was first shown to be regulated by 1,25(OH)2D3 [17] in Caco-2 cells, a human intestinal cell line. The epithelial calcium channel TRPV6 is upregulated in breast carcinoma compared with normal mammary gland tissue [18] and TRPV6 overexpression is strongly associated with the invasive status. It regulates both breast cancer cell migration and invasion [19]. Although studies have shown TRPV6 to be increased in breast carcinoma [20], one study found TRPV6 mRNA to be expressed at very low levels in MCF-7 cells [18]. Although we did not find any significant association of vitamin D with TRPV6 expression, D+SFN significantly increased its expression, which is along the line of what we hypothesized.

The primary effect of exposure of breast cancer cells to 1,25-dihydroxyvitamin D3 is cell cycle arrest due to changes in the functional status of the proteins that regulate cell
cycle [21]. 1,25(OH)2D can also induce morphological changes associated with apoptosis in breast cancer cells [22]. 1,25-dihydroxyvitamin D3 can inhibit the invasion and metastasis of cancer cell 1,25(OH)2D and has potent anti-angiogenic properties that can inhibit tumor cell invasion [23]. Effect of 1,25-dihydroxyvitamin D3 on cell proliferation can also be mediated in an indirect way, via interference to the function of estrogen receptors (ER) [24]. 1,25-dihydroxyvitamin D3 and its analogues reduce the expression of ERα, which in turn reduces the level of mitogenic signals to breast cancer cells from estrogens.

CYP27B1 is present in some breast cancer cells and may lead to the autocrine synthesis of 1,25-dihydroxyvitamin D3 from 25(OH)D3. It has been shown that breast adipocytes also express CYP27B1 and could bio-activate 25(OH)D3 to 1,25-dihydroxyvitamin D3 and in a paracrine fashion deliver 1,25-dihydroxyvitamin D3 to the breast epithelium. The expression of CYP27B1 can be considered a central mechanism in the association between active vitamin D and its antitumor effects [25]. In a recent study, a targeted ablation of CYP27B1 was accompanied by significant acceleration in initiation of spontaneous mammary tumorigenesis where cell proliferation, angiogenesis, cell cycle progression, and survival markers were up-regulated in tumors, and apoptosis was down-regulated [26]. In the present study, we found that CYP27B1 expression was not affected by the treatment groups.

The availability of 1,25-dihydroxyvitamin D3 is also determined by its degradation, along with its activation. This process is initiated by the 24-hydroxylation of 1,25-dihydroxyvitamin D3 by the enzyme, CYP24A1. In normal tissues, this enzyme is induced in response to 1,25-dihydroxyvitamin D3 exposure, providing a regulatory mechanism that
maintains the concentration of 1,25-dihydroxyvitamin D3 at a non-toxic level. Genome hybridization studies have revealed that in certain human breast cancers the CYP24A1 gene is amplified and this may cause a reduction in the level of 25(OH)D3 and 1,25-dihydroxyvitamin D3 level and could allow cells to proliferate unduly [27]. We found that CYP24A1 expression was significantly decreased when added SFN to vitamin D, which is the opposite of what we hypothesized. However, in advanced cancers, this role might prove to be very beneficial.

Phytochemicals have been studied extensively for the treatment of various diseases and disorders [28]. They exhibit a wide range of safety and target multiple pathways and targets in breast cancer cells [29]. Current evidence suggests that naturally occurring phytochemicals can target breast cancer stem cells (CSCs) [1, 30]. Hence, phytochemicals are proposed to be useful in the treatment of cancer. Consumption of cruciferous vegetables such as broccoli, correlates with decreased risk of cancer induction. This protective effect has been shown to be in part due to the presence of an isothiocyanate (ITC)-glucoraphanin [31, 32]. The four important ITCs formed from glucosinolates by the activity of myrosinase are benzyl-ITC, allyl-ITC, phenylethyl-ITC (PEITC) and methylsulphinylbutyl-ITC (sulforaphane). PEITC [33] and sulforaphane [34] are two well-studied ITCs for their anticancer activities. Sulforaphane has been found to inhibit proliferation, angiogenesis, and metastasis as well as induce cell cycle arrest and apoptosis in breast cancer cells. It reactivated expression of ER-α in MDA-MB-231 cells, whereas, in combination with tamoxifen, reduced proliferation in MDA-MB-231 cells. Sulforaphane treatment caused cell cycle arrest and decreased cyclin A, cyclin B1 and CDC2 expression in breast cancer (MDA-MB-231) cells [35]. Finally, sulforaphane decreased the production of IL-1β, IL-6,
TNF-α, interferon-γ, IL-4, platelet-derived growth factor and VEGF in MDA-MB-231 cells [36]. Although more cited for its antioxidative and anti-inflammatory defense mechanisms, the induction of proliferation arrest and apoptotic cell death primarily contribute to its anticancer functions. [37-39] However, the precise molecular mechanisms of sulforaphane-elicited anticancer effect are not thoroughly elucidated. [40]

Modified histones have gained importance as biomarkers of breast cancer prognosis. The investigations carried out on mechanisms of histone modifications are also promising for the development of efficient HDAC inhibitor therapies.

It is not clear from the results as to how the HDAC inhibitors work when combined with vitamin D. We did observe some predicted response to D in genes such as CYP24A1, and TRPV6. However, SFN and TSA had opposing effects in most cases. Clonogenic assay findings confirmed that D+SFN group was more effective than D alone group even though we did not reach statistical significance. Overall, we demonstrated significant association between vitamin D and the HDAC inhibitors as we hypothesized but merits further exploration due to the opposing effects of SFN and TSA.

Insights into the biology of breast cancers have been gained from the identification of genes commonly mutated in these cells signaling pathways and have led to paradigms that have informed the study of epigenetic alterations in cancer. These insights are also currently being used to develop new diagnostic and prognostic assays and potential therapies for breast cancer.

In summary, we state that the components of the vitamin D pathway can potentially be used both as treatment and preventive strategies for breast cancer. In cancers with low VDR expression, it may be possible that future treatments could target the genomic and
epigenomic level alterations to increase VDR expression by modulating expression of transcription factors or utilizing HDAC inhibitors, respectively.

5.6 Bibliography


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CHAPTER 6

EFFECT OF HISTONE DEACETYLASE INHIBITORS ON AND VITAMIN D-DEPENDENT GENE EXPRESSIONS IN CACO-2 HUMAN COLORECTAL CANCER CELLS

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6.1 Abstract

It is relatively unknown how dietary bioactive compound, sulforaphane, in partnership with vitamin D, regulate gene expression linked to colorectal cancer. It has been suggested that the combination of various bioactive components with vitamins is crucial for their potential anticancer activities. METHODS: This study employed a combinatorial chemo preventive strategy to investigate the impact of dietary histone deacetylase inhibitor i.e. sulforaphane on chromatin remodeling in human colorectal carcinoma. To understand the epigenetics-mediated changes in gene expression in response to sulforaphane and vitamin D, Caco-2 cells were exposed for 24 hours to vitamin D (100nM) either alone or in combination with L-sulforaphane and TSA (20μM and 1μM respectively) at 70% confluence (proliferating) and after 13 days post confluency (fully differentiated) and Changes to VDR, CYP24A1, CYP27B1 and TRPV6 gene expressions were quantified using real-time PCR-based assays. Histone deacetylase inhibitor activity was assessed using HDAC I/II assay that measured global changes in acetylation status. RESULTS: In proliferating Caco-2 cells, D+SFN (p<0.04) increased VDR expression and decreased CYP27B1 (p<0.01) more than D alone (p=0.38 and p=0.07 respectively). However, in differentiated Caco-2 cells, none of the genes had significant changes from D alone group. D+SFN (p=0.99) demonstrated an opposing effect from D alone and decreased VDR expression. Although statistically significant, D+SFN (p=0.01) effects on HDAC inhibitor activity was lower than TSA alone (p<0.0004) or SFN alone group (p<0.0014). CONCLUSION: These data suggest that colon cancer cells respond to dietary components differently under different culture conditions. The effect of vitamin D and
sulforaphane is selective and gene-specific in the complex multistep process of colorectal carcinogenesis in vitro.

**Key words:** HAT, histone acetyltransferase; HDAC, histone deacetylase; SFN, sulforaphane; TSA, Trichostatin A; VDRE, vitamin-D response elements.

### 6.2 Introduction

Colorectal cancer is one of the most invasive and the third most common cancer in both men and women in the United States. Among men and women combined, colorectal malignancies are the second most common cause of cancer mortality in the United States, with approximately 50,000 deaths each year [1]. Although, a serum vitamin D level of 20 ng/mL to 50 ng/mL is considered adequate for healthy people, it is more reasonable to say serum 25(OH)D₃ levels of 30 ng/mL is physiologically beneficial in preventing chronic diseases like cancer. Per a study using data from NHANES 2000–2004, up to 78% of Americans have a serum levels less than 30ng/ml of 25(OH)D₃ [2].

Vitamin D has been known to play an important role in bone and mineral homeostasis. However, it has been studied for its anti-neoplastic roles ever since it was discovered that colon cancer mortality rates were the highest in places with the least amount of sunlight in the US [3], suggesting a protective role of Vitamin D. The active, hormonal form of vitamin D, 1α,25-dihydroxyvitamin D [1α, 25(OH)2D] mediates its actions through activation of the Vitamin D Receptor (VDR), a ligand-dependent transcription factor.[4] The development of many cancers is often associated with a reduced expression of the vitamin D receptor protein resulting in decreased cellular sensitivity to 1α,25-dihydroxyvitamin D [1α, 25(OH)2D] and a possible loss of the anti-
proliferative action provided by vitamin D [5, 6]. Our understanding of epigenomic alterations in colorectal cancer, is less advanced than that of our understanding of gene mutations, but substantial progress has recently been made in this area [7]. However, the exact mechanisms are yet to be explored.

Epigenetic effects, including changes in histone acetylation status, influence chromatin remodeling and gene expression in cancer cells. To date, no other study has looked at these specific vitamin D-dependent gene expressions in relation to histone modification in colorectal cancer cells. We have used Caco-2 cells as our model as opposed to HCT116 or other frequently used colon cancer cell lines. This is because Caco-2 cells are more commonly used as fully differentiated cells that mimic intestinal mucosal cells and therefore, are a preferred choice in studies involving intestinal uptake e.g. drug interventions. This study will also address the unique question as to how Caco-2 cells behave as colorectal cancer cells for the first time.

Many bioactive compounds have been shown to alter genetic and epigenetic profiles in various cancers. Of these compounds, sulforaphane (SFN), found in cruciferous vegetables such as kale, cabbage, broccoli sprouts etc., has been present as one of the most potent, natural (histone deacetylase) HDAC inhibitors to date [8]. Trichostatin A (TSA) is a fungal metabolite with well-known histone deacetylase inhibitor activity as well and often used as positive controls in experiments with natural HDAC inhibitors such as sulforaphane.

The objective of this study is to determine to what extent treatment with the histone deacetylation inhibitors (HDACs) sulforaphane or Trichostatin A (TSA), influences vitamin D-induced transcriptional activity. The rationale for this study is that treatment of
cells with these epigenetic modifiers will alter the acetylation status of histone proteins. This, in turn, will loosen the association of DNA with the histone protein complex of the allowing increased access of the vitamin D receptor - RXR heterodimer to the vitamin D response element (VDRE) found in the promoter of vitamin D-inducible genes.

6.3 Methods

6.3.1 Cell Culture

Cells were treated on day 7 (proliferating) 14 from the date of seeding into 6-well plates (differentiated). The experiment included six different treatment groups namely-DMSO control (0.1%), 1,25 (OH)\textsubscript{2}D\textsubscript{3} (100nM), SFN (20 µM), TSA (1µM), 1,25 (OH)\textsubscript{2}D\textsubscript{3} + SFN and 1,25 (OH)\textsubscript{2}D\textsubscript{3} + TSA (APPENDIX G). After treatment, the cells were incubated for 24 hours before harvesting. Caco-2 cells were obtained from the American Type Culture Collection (HTB-37; American Type Culture Collection, Rockville, MD). 1, 25 (OH)\textsubscript{2}D\textsubscript{3} was purchased from Enzo Life Sciences, Plymouth Meeting, PA. Trichostatin A and SFN were purchased from Sigma-Aldrich, St. Louis, MO. Real-time PCR materials, including all primers and master mix were purchased from Applied Biosystems, Foster City, CA. Cell culture dishes were bought from Costar, Cambridge, MA. All other cell culture materials and materials for RNA isolation and cDNA synthesis were purchased from Life Technologies, Grand Island, NY.

6.3.2 RNA Isolation and Reverse Transcription

At the completion of the treatment period, cells were washed with saline, then treated with 1 mL Trizol per well of a 6-well plate, and cells were scraped to be harvested
for subsequent RNA extraction. Cells were frozen at -80°C until RNA was ready to be isolated in collection tubes. To isolate the RNA, 200 µL chloroform was added to each homogenate for phase separation. Isopropanol (500 µL) was then used to precipitate the RNA. Washing three times with 1 mL of 75% ethanol was followed by the addition of 40 µL of Diethyl pyrocarbonate (DEPC) water. The samples were then incubated for 10 minutes at 56°C to ensure maximum RNA solubility. RNA samples were then quantified using a Nanodrop machine (λ260/280). cDNA was prepared using the SuperScript III First Strand Synthesis System for RT-PCR (Life Technologies, Grand Island, NY). Details of the protocol are listed in APPENDIX H.

6.3.3 Real Time PCR

For all experiments, mRNA expressions of GAPDH, VDR, CYP24A1, CYP27B1, and TRPV6, were measured by ViiA™ 7 Real-Time PCR System (Applied Biosystems, Foster City, CA). Reactions consisted of 10 µL of SYBR GREEN Gene Expression Master Mix (Applied Biosystems, Foster City, CA) with forward and reverse primers (10 nM each) and 10 µL of DNAse/RNAse Free Water with 100 ng of cDNA. Duplicates were set up for each sample. The levels of VDR, CYP24A1, CYP27B1, and TRPV6 were measured by the comparative ΔΔCT method (ThermoFisher Scientific, 2016). For each sample, the relative abundance of GAPDH and the target genes were obtained. The difference between those two abundances (target gene- GAPDH) is the ΔCT for that sample which were the values used for statistical analysis. The mean ΔCT values were then normalized against the mean ΔCT values of the control sample, Dimethyl Sulfoxide (DMSO), resulting in a ΔΔCT value. 2 was raised to the – ΔΔCT to produce a relative quotient (RQ) for each sample,
with the RQ of the control being one. RQ values were used to create the mRNA expression curves for each gene. Primer sequences are available on APPENDIX A.

6.3.4 Histone Deacetylase (HDAC) Assay

We used the HDAC-Glo™ I/II Assay (luminescent) (Promega, 2017). The assay uses an acetylated, live-cell-permeant, luminogenic peptide substrate that can be deacetylated by HDAC activities. Deacetylation of the peptide substrate is measured using a coupled enzymatic system in which a protease in the Developer Reagent cleaves the peptide from aminoluciferin, which is quantified in a reaction using Ultra-Glo™ Recombinant Luciferase. The HDAC-mediated luminescent signal is persistent and proportional to deacetylase activity.

Cells were harvested 24 h after treatment with SFN (20 µM) and TSA exposure (1 µM), and cell lysates were analyzed for HDAC activity, as reported [10].

6.3.5 ATP Assay

We used the CellTiter-Glo® 2.0 Assay (Promega, 2017). This assay provides a homogeneous method to determine the number of viable cells in culture by quantitating the amount of ATP present. Luminescence readout is directly proportional to the number of viable cells in culture. Cells were harvested 24 h after co-treatment with SFN (20 µM) and TSA (1 µM), and cell lysates were analyzed for ATP activity as per protocol.
6.3.6 MT Assay

We used the RealTime-Glo™ MT Cell Viability Assay (Promega, 2017), and measured cell viability continually in the same sample well up to 48 hours. This gave us more information about the mode of action of our treatments about time dependence. The nonlytic nature and rapid response of the assay made it possible to monitor cell viability over time in the same well. The assay measures the reducing potential of viable cells, and is ATP-independent, providing an orthogonal method for viability or cytotoxicity determination.

Luminescence readout is proportional to the number of viable cells in culture. Cells were harvested 24 h after co-treatment with SFN (20 µM) and TSA (1 µM), and cell lysates were analyzed for ATP activity.

6.3.7 Caspase 3/7 Assay

We used the Caspase-Glo® 3/7 Assay (Promega, 2017) to measure apoptosis in proliferating Caco-2 cells. The assay provides a pro luminescent caspase-3/7 DEVD-aminoluciferin substrate and a proprietary thermostable luciferase in a reagent optimized for caspase-3/7 activity, luciferase activity and cell lysis. Adding the single Caspase-Glo® 3/7 Reagent in an "add-mix-measure" format results in cell lysis, followed by caspase cleavage of the substrate. This liberates free aminoluciferin, which is consumed by the luciferase, generating a "glow-type" luminescent signal that is proportional to caspase-3/7 activity.

Cells were harvested 24 h after co-treatment with SFN (20 µM) and TSA (1 µM), and cell lysates were analyzed for caspase 3/7 activity.
6.3.8 Migration Assay

A simple scratch test is used to determine the rates of closure following treatments to assess their efficacy. Caco-2 cells were seeded in 6-well plates at a density of 300,000 cells/well and allowed to attach overnight. Once the cells were 70% confluent, wounds were created using the etching at the bottom of the wells as guide, using a p-200 pipette tip. The wells were then washed with 1X sterile PBS and treated as designed. Photographs were taken on day 0 at four reference points (4 quadrants) and marked as the “0Hr”. Each wound was then followed over the course of 24 hours to determine the rate of closure in response to the treatments. Photographs were taken again and marked as “24Hr”. This entire experiment was repeated for an N=3. Percent closure was calculated as below:

\[
\text{% closure} = \frac{(0\text{Hr avg. wound size} - 24\text{Hr avg. wound size})}{0\text{Hr avg. wound size}}
\]

6.3.9 Statistical Analysis

Results from real-time PCR were analyzed using one-way ANOVA with a p-value of <0.05 used as the cut-off for statistical significance. The analysis was setup as a randomized block design with each experiment as a block. Dunnett’s Multiple Comparison Tests was also used to find which means of the treatment groups differed significantly from the control (DMSO) and used a P-value of <0.05 as a cut-off for statistical significance. All statistical analyses and figures were generated using GraphPad Prism (v 7.04). Tukey’s post-test was conducted to determine significant multiple comparison differences. For all assays, the results are expressed as Mean±SEM.
6.4 Results

6.4.1 Vitamin D-responsive gene expression

We hypothesized that the expression of vitamin D-inducible genes would be increased by concomitant treatment of cells with SFN and TSA. We measured, with and without the HDAC inhibitors, the mRNA expression of genes known to be involved in vitamin D action and cell metabolism: namely, VDR (vitamin D receptor), CYP24A1 (24 hydroxylase), CYP27B1 (1α hydroxylase) and TRPV6 (transient receptor potential, vanilloid family, member 6 calcium-specific channel protein) by real time quantitative polymerase chain reaction (RT-PCR); results are expressed as RQ values ($2^{\Delta\Delta CT}\pm$SEM).

(a) Undifferentiated Caco-2 Cells

In proliferating Caco-2 cells, VDR expression was overall significant (p=0.03) from one-way ANOVA and Browne-Forsythe test (p<0.0001). From Dunnett’s multiple comparison test, VDR was significantly increased by D+SFN (3.44±0.39, p=0.01) and D+TSA (3.10±0.85, p=0.01) when compared to control. Tukey’s multiple comparison maintained the significance when compared to vitamin D, D+SFN (p=0.02) and D+TSA (p=0.03).

CYP24A1 mRNA levels (N=4) were significantly affected by our experiment (p=0.01) from the one-way ANOVA and Browne-Forsythe test (p=0.02) analyses. There was about 372 fold increase by vitamin D alone (372.2±94.1, p=0.03). Vitamin D + SFN treatment did not increase CYP24A1 mRNA expression more than vitamin D alone; however, concomitant treatment of vitamin D + TSA resulted in a marked increase in CYP24A1 gene expression. ~3,500 fold (3555±695, p=0.003) in proliferating Caco-2
cells. Tukey’s multiple comparison test did not show any additional significant association among vitamin D and the combination groups D+SFN or D+TSA. CYP27B1 mRNA expression (N=6) was not affected by vitamin D or the combination treatment with epigenetic modifiers SFN and TSA.

TRPV6 mRNA expression (N=6) was increased by 44% by vitamin D alone (0.44±0.80, p=0.01). Combination treatment with vitamin D and TSA increased TRPV6 gene expression by 93% (0.93±1.01, p=0.0006), and this increase was significantly different than vitamin D alone (p=0.04) from Tukey’s posttest. Although SFN had no significant effect on TRPV6 both alone and in combination with vitamin D, TSA alone was significantly different from control (0.13±0.39, p=0.003). Relative mRNA expression, compared to the DMSO control treatment, for proliferating Caco-2 cells are presented in Fig 6.1.
Fig 6.1: mRNA Expressions of Vitamin D Responsive Genes in Proliferating Caco-2 Cells. (a) Vitamin D receptor (VDR) expression was increased more than D alone in D+TSA group; (b) TRPV6 showed the highest response in D+TSA group, (c) CYP24A1 showed similar response as TRPV6 in D+TSA group; and (d) there was no effect on CYP27B1 expression across the treatment groups. Results are from N>3 experiments for all genes and *p<0.05, and **p<0.01 represent significance.
(b) Differentiated Caco-2 Cells

In fully differentiated Caco-2 cells, CYP24A1 expression was increased ~650 fold by vitamin D treatment (643.2±122.2, p<0.0001) alone, but combination treatment with epigenetic modifiers and vitamin D blunted the increase. Albeit the decrease relative to D alone, D+SFN (142.2±29.53, p<0.0001) and D+TSA (191.3±29.38, p<0.0001) significantly increased CYP24A1 expression, ~140 fold and ~190 fold respectively, when compared to control. This association was intact when compared to D alone, i.e the combination treatments were significantly different from D alone, D+SFN (p=0.001) and D+TSA (p=0.009). The HDAC inhibitors SFN and TSA response on CYP24A1 were also significantly different from D alone, SFN (p<0.0001) and TSA (p<0.0001) from Tukey’s post test.

TRPV6 expression in fully differentiated Caco-2 cells was significantly increased in D+TSA group (2.8±0.78, p=0.01) only. SFN had no effect on TRPV6 expression both alone and in combination with vitamin D.
We found that neither vitamin D treatment nor vitamin D combination treatment with epigenetic modifiers influenced VDR and CYP27B1 mRNA expression (Figure 6.2).

Fig 6.2: mRNA expressions of vitamin D responsive genes in fully differentiated Caco-2 cells. (a) CYP24A1 showed the highest response in D only group and was significantly different from D+SFN and D+TSA groups. (b) TRPV6 was significantly increased in D+TSA group. (c) VDR and (d) CYP27B1 expressions were not affected by the treatments.
Results are from N>3 experiments for all genes and *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 represent significance.

(c) Animal Tissue Sample Analyses

We also analyzed small intestinal scrapings from APC 1638 mice (5 male+5 female). The animals were fed on a diet containing 450 mg of SFN/kg. We then analyzed the cDNA samples (prepared as per protocol in APPENDIX H) for VDR, CYP24A1, CYP27B1 and TRPV6. We found that TRPV6 expression was significantly increased (30±8.8, p<0.0001) in sulforaphane group when compared to no sulforaphane (control) group (Fig. 6.3).

![TRPV6 Bar Graph](image)

**Fig 6.3: TRPV6 Expression in Animal Tissue Samples.** Sulforaphane increased TRPV6 expression in small intestinal scrapings from TNFα- K/O mice. GAPDH was used as
internal loading control. Results are expressed as Mean±SEM of 10 animal samples (N=10).

6.4.2 Histone acetylation status

To test the relative HDAC inhibitor activity of SFN and TSA, we measured nuclear global HDAC I/II activity. Caco-2 cells were seeded at 10,000 cells/well in a 96-well, opaque walled plate. Cells were treated at 70% confluency with the desired treatments and incubated for 24 hours before the assay was conducted. The cells were in their proliferating stage while we carried out the HDAC assay. Our expectation was that the selected dosages of SFN (20 μM) and TSA (1 μM), would cause significant changes in histone deacetylation activity in Caco-2 cells.

All treatments significantly increased HDAC inhibitor activity (n=6) when compared to control. Vitamin D had 30 fold decrease in HDAC activity (-30.17±7.07, p=0.03) which was similar to D+SFN alone (-32.75±9.63) and D+TSA group (-30.42±4.2). SFN alone had a bigger effect on HDAC inhibition (-42.92±9.5) with the highest effect seen in TSA alone group as expected (-47.5±8.9).

The results of HDAC assay (normalized to control- DMSO) are presented in Fig 6.4.
Fig 6.4: HDAC Activity in Proliferating Caco-2 Cells. All treatments significantly decreased HDAC activity in the system. Each data point represents Mean±SEM (N=10). The data were analyzed by One-way ANOVA and *p <0.05, **p<0.01, and ***p<0.001 is significant. Tukey’s post test showed no significant difference among vitamin D and the combination treatments of D+SFN and D+TSA.
6.4.3 ATP-dependent Cell Viability Assay

To determine cell viability, we measured cellular ATP activity. Caco-2 cells were seeded at 10,000 cells/well in a 96-well, opaque walled plate. Cells were treated at 70% confluency with the desired treatments and incubated for 24 hours before the assay was conducted. Our expectation was that the selected dosages of SFN (20 μM) and TSA (1 μM) with vitamin D (100 nM) would be sufficient to decrease ATP-dependent viability in proliferating Caco-2 cells. There was no effect of vitamin D and the HDAC inhibitors on cell viability in an ATP-dependent manner, although Bartlett’s test (p<0.0001) and Browne-Forsythe Test (p=0.03) results came back as significant. Tukey’s posttest also showed no effect on ATP-dependent cell viability in vitamin D and the combination treatments. The results are presented as percent (%) control in Fig 6.5.

![Bar graph showing ATP-dependent cell viability in proliferating Caco-2 cells.](image)

**Fig 6.5: ATP-dependent Cell Viability in Proliferating Caco-2 Cells.** There was no significant difference among the treatment groups on cell viability. Values are
representative of three independent experiments (N=3). (Mean±SEM) represented as %
control. No significant difference was observed across the treatments.

6.4.4 ATP-independent Cell Viability Assay

To determine cell viability in an ATP-independent manner, we measured cellular
MT Cell Viability Substrate activity. Caco-2 cells were seeded at 10,000 cells/well in a 96-
well, opaque walled plate. Cells were treated at 70% confluency with the desired treatments
and incubated for 24 hours before the assay was conducted. Our expectation was that the
selected dosages of SFN (20 μM) and TSA (1 μM) with vitamin D (100 nM) would be
sufficient to decrease viability in proliferating Caco-2 cells. Two- way ANOVA test was
performed to test for time X treatment interaction.

Compared to control, all treatments were significant different at all time points
(p<0.0001) with an overall significant interaction(p<0.0001). Tukey’s post test showed
significant difference in D+SFN (p<0.0001) and D+TSA (p<0.0001) at 12 hours, D+TSA
only (p=0.02) at 24 hours, again D+SFN (p<0.0001) and D+TSA (p<0.0001) at 48 hours
with observed increase in cytotoxicity by SFN and TSA. The results are presented as
relative luminescence in Fig 6.6.
Fig 6.6: ATP-independent Cell Viability in Proliferating Caco-2 Cells. All treatments significantly changed cell viability in a time-dependent manner. Maximum decrease in cell viability was observed at 24H. Each data point represents 4 replicates and results are expressed as (Mean±SEM).

6.4.5 Apoptosis in Proliferating Caco-2 Cells

We used Caspase-Glo® 3/7 lytic assay and measured the degree of caspase cleavage of the substrate. The cleavage liberates free aminoluciferin, which is consumed by the luciferase enzyme, generating the luminescent signal that is proportional to caspase-3/7 activity (used as a proxy measure of apoptosis). In other words, the higher the activity, the higher the luminescent signal. Caco-2 cells were seeded at 10,000 cells/well in a 96-well, opaque walled plate. Cells were treated at 70% confluency with the desired treatments and incubated for 24 hours before the assay was conducted. Our expectation was that the
selected dosages of SFN (20 μM) and TSA (1 μM) with vitamin D (100 nM) would be sufficient to induce apoptosis in proliferating Caco-2 cells. The results are presented as relative luminescence in Fig 6.7.

![Graph showing relative luminescence for different treatments.](image)

**Fig 6.7: Apoptosis in Proliferating Caco-2 Cells.** *All treatments had significant increase in caspase 3/7 activity. Values are representative of four independent experiments (N=4) and represented as Mean±SEM. Tukey’s post test showed no significant difference between D+SFN and D+TSA when compared to D alone.*

**6.4.6 Migration Assay**

Caco-2 cells were plated and treated as per protocol described previously. The representative pictures per treatment (one of four wounds created) are shown in **Fig 6.8**. We then calculated the % closure (*Fig. 6.9*) for each treatment compared to the control per the formula shown in the methods section. Compared to control (N=3), D alone group
(1.18±0.15) showed protective effect in Caco-2 cell migration. The combination groups D+SFN (0.70±0.06) and D+TSA (0.87±0.02), as well as the individual HDAC inhibitors, SFN (0.79±0.08) and TSA (0.88±0.10) alone showed no significant difference in migration.

Fig 6.8: Migration assay in Proliferating Caco-2 Cells. The combination treatments had no change in migration, D alone showed a tendency to prevent migration while the rest of the treatment groups were unable to reflect this change.
Fig 6.9: Percent (%) Closure in Proliferating Caco-2 Cells. No significant difference was observed when compared to control in migration but was overall significant \( (p=0.03) \). D+SFN reduced migration significantly when compared to D \( (p=0.02) \) from Tukey’s posttest.

6.5 Discussion

Colorectal cancer (CRC) is a major cause of cancer morbidity and mortality in the US. Factors involved in the pathogenesis of colorectal cancer appear to be complex and heterogeneous and include dietary and lifestyle factors as well as inherited and somatic mutations [9]. It is believed that although the underlying form of genomic or epigenomic instability determines the types of mutations that occur in colon cancer, the selective pressures that lead to the clonal evolution of the tumors are largely the same across all
colorectal cancers. This can explain why certain genes are more frequently mutated in colon cancers than in other cancers (e.g. breast cancer, prostate cancer) and vice versa.

Epigenetic mechanisms that modify chromatin structure are divided into the following: DNA methylation, histone modifications, nucleosome remodeling and non-coding RNAs. The complex interaction of these mechanisms regulate the way genes are expressed in different cell types, developmental stages and disease states, including cancer. [10]

Histone modification is controlled by a balance in activity between histone acetyltransferase (HAT) and histone deacetylase (HDAC) activities. The former adds an acetyl group to histones resulting in uncoiling or “opening” of the chromatin structure, facilitating gene transcription. The HDACs remove acetyl groups from the histones, leading to increased binding of DNA to histone proteins, and the coiling or “closing” of chromatin, which inhibits transcription [11]. The HDACs are known to be critical in the regulation of expression of genes important for cell survival, proliferation, differentiation, and apoptosis [12]. HDACs also act as members of a protein complex responsible for recruitment of transcription factors to the promoter region of genes, including those of tumor suppressors, and regulation of acetylation status of specific cell cycle regulatory proteins [13]. High HDAC expression and histone hypoacetylation have been observed in cancer with associated transcriptional repression of genes, providing a rationale for the investigation of HDAC inhibitors in cancer therapeutics [14].

Sulforaphane is an isothiocyanate derived from cruciferous vegetables and is particularly enriched in broccolis and broccoli sprouts. Sulforaphane has been received great attention due to its promising chemo preventive and therapeutic effects established in a
variety of human cancer cell lines and in preclinical cancer models, including colon cancer. [15] Although more cited for its antioxidative and anti-inflammatory defense mechanisms, the induction of proliferation arrest and apoptotic death primarily contribute to the anticancer mechanisms against post-initiated/transformed cells. [15, 16, 17] However, the precise molecular mechanisms of sulforaphane-elicited anticancer effect are not thoroughly elucidated. [18]

Vitamin D receptor (VDR) is a transcription factor that binds to specific vitamin D response elements (VDREs) within the promoter regions of its primary target genes [19]. Most VDR target genes contain multiple VDREs. In the absence of a ligand, VDR acts via co-repressor proteins i.e. histone deacetylases (HDACs) [20]. HDACs can also inactivate non-histone proteins, e.g. p53 by deacetylation. Therefore, HDACs have multiple influences in cellular processes. [21] Studies in animals (xenografted tumors, chemically-induced carcinogenesis, genetic models for CRC) and cultured cells show that 1,25(OH)2D3 exerts a variety of antitumor effects on colon carcinoma cells, including inhibition of proliferation, increase in differentiation, sensitization to apoptosis, and inhibition of angiogenesis and metastasis [22] Moreover, two studies have shown that APCmin mice lacking the VDR gene develop bigger intestinal tumors [23] Zheng et al., 2012). In addition, 1,25(OH)2D3 inhibits the pro-tumoral properties of CRC-associated fibroblasts and, accordingly, high VDR expression in these fibroblasts is associated with a favorable clinical outcome for metastatic CRC patients [208]. Mechanistically, these effects are based on regulation by 1,25(OH)2D3 of crucial genes controlling the cell cycle CDKN1A/ p21CIP1, CDKN1B/p27KIP1, c-MYC, and others), apoptosis (BAX, and
others), invasion (metalloproteases, and others), the epithelial-to-mesenchymal transition (CDH1/E-cadherin, and others) and angiogenesis [24].

A series of studies have shown that the expression of VDR and CY27B1 in colon epithelial cells increases at early steps of tumorigenesis (adenomas and well differentiated carcinomas) as compared to that in normal cells but it strongly declines in advanced, poorly differentiated tumors [25,26]. Thus, although rarely mutated, VDR and Cyp27b1 are downregulated in a relatively high proportion of advanced colorectal tumors. [27].

24-Hydroxylase, encoded by the CYP24A1 gene, is the key enzyme that catabolizes 1,25(OH)2D3 to the less active calcitroic acid. It has been reported that CYP24A1 is overexpressed in several human tumor types and that changes in CYP24A1 expression are associated with cancer development and progression. A recent study showed that CYP24A1 expression is closely associated with CRC progression, and might be a novel prognostic biomarker for colorectal cancer. [28]

TRPV6 mRNA was first shown to be regulated by 1,25(OH)2D3 [29] in Caco-2 cells. The dependence of TRPV6 mRNA expression on the VDR-mediated genomic actions of 1,25(OH)2D3is supported by increased intestinal TRPV6 mRNA expression after injection of mice with 1,25(OH)2D3[30,31]. It regulates TRPV6 expression by a process that requires new mRNA and protein synthesis and the point of regulation lies most likely at the transcriptional level [32].

It is clear from the results that TSA enhanced vitamin D activity in more than one genes related to Vitamin D. The responses differed within the same gene based on the condition of Caco-2 cells (differentiated vs. proliferating) as they were gene-specific. SFN, really had the opposite effect of TSA in fully differentiated cells. However, in proliferating
cells, it showed a greater impact in combination with vitamin D in two of the four genes. Overall, we saw a mixed association between vitamin D and SFN from what we hypothesized and therefore merits further exploration.

It has been proposed that gene mutations and epigenetic alterations contribute to colon cancer formation through the activation of oncogenic pathways and the inactivation of tumor suppressor genes, which regulate hallmark behaviors of cancers (Fearon et al., 2011). Insights into the biology of colorectal cancers have been gained from the identification of genes commonly mutated in these cells signaling pathways in colon cancer and have led to paradigms that have informed the study of epigenetic alterations in cancer. These insights are also currently being used to develop new diagnostic and prognostic assays and potential therapies for colorectal cancer.

In summary, we state that the components of the vitamin D pathway can potentially be used both as treatment and preventive strategies for colorectal cancer. In colorectal cancers with low VDR expression, it may be possible that future treatments could target the genomic and epigenomic level alterations present to increase VDR expression by modulating expression of transcription factors or utilizing HDAC inhibitors, respectively.

6.6 Bibliography


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CHAPTER 7
DISCUSSION

Epigenetics refers primarily to alterations in gene expression at the promoter regions of genes. Unlike genetic mutations, epigenetic alterations do not change the actual genetic sequence and, therefore, are part of a new avenue in cancer research [1].

7.1 Epigenetic Alterations in Cancer

The epigenetic landscape is largely a reflection of factors that determine the state of the chromatin, which determines whether the DNA is accessible to proteins that control gene transcription or not. A relaxed or “open” chromatin state allows for gene transcription, whereas a condensed or “closed” chromatin state prevents gene transcription [2]. The epigenetic mechanisms currently believed to play a role in cancer include:

1) DNA methylation of cytosine bases in CG rich sequences, called CpG Islands;
2) Post-translational modifications of histones, which regulate the packaging structure of the DNA (called chromatin);
3) microRNAs and noncoding RNAs; and
4) nucleosome positioning

7.1.1 DNA Methylation

Gene silencing may occur due to methylation of DNA in the promoter region of genes [3]. DNA methyltransferase enzymes (DNMTs) add a methyl group (-CH3) to the DNA structure causing hypermethylation and possibly a reduced expression of tumor suppressor genes. DNA methylation refers to the enzymatic addition of a methyl group to
the 5-position of cytosine by DNA methyltransferases (DNMT) to produce 5-methylcytosine, a normal base in DNA [4]. Most CpGs (a dinucleotide sequence) are methylated in normal mammalian cells with unmethylated CpGs being typically present only in regions of DNA called CpG Islands. CpG islands are regions in the genome where the percentage of the CpG dinucleotides is higher than expected - greater than 200–500 bases in length with greater than 50% GC content [5]. CpG islands, although protected from methylation under normal circumstances, can become aberrantly methylated in cancer. Methylation of CpG islands within the promoter region is correlated with transcriptional silencing although it appears that decreased gene expression is only characteristic of a subset of methylated genes in CRC [6, 7].

Unmethylated CpG islands within the promoter region of genes are correlated with an open chromatin structure (euchromatin) whereas methylated CpG islands are correlated with a condensed, closed chromatin structure (heterochromatin) and transcriptional silencing [8]. For example, normal colonic epithelium generally has unmethylated CpG islands in the promoter regions of genes, whereas aberrant hypermethylation of promoter associated CpG islands is a hallmark of neoplasms.

The concept of “CpG island shores” [9] (areas of less dense CpG dinucleotides) have been linked to hypermethylation in cancer [10]. The methylation of these CpG island shores are generally tissue specific and has recently been shown to be altered in colorectal cancer [10, 11]. This driving role of aberrant methylation of CpG island shores in the development of cancer is still controversial and remains to be supported with additional studies.
It is important to recognize that DNA methylation is a normal mechanism in the mammalian genome by which cells regulate gene expression. Most of the CpG sites in the human genome, which are located outside of promoter regions, are heavily methylated. The methylation state of a gene is regulated by a family of DNA methyltransferases (DNMTs). When the DNMTs are not functioning properly, either due to structural abnormalities caused by epigenetic changes or functional inabilities, formation of malignant cells and/or progression leads to the development of a specific cancer e.g. colorectal, breast, prostate cancer etc.

7.1.1.1 DNMT Inhibitors

DNMT inhibitors, also referred to as demethylating agents, have been under preclinical and clinical investigation for over 30 years [12]. After formation of an irreversible complex with DNMT, the enzymes degrade [13] and prevent methylation of daughter DNA in CpG islands during DNA replication. At low concentrations (30-300nM), these inhibitors (e.g. 5-Aza-2’-deoxycytidine or AZA) exhibit potent DNA hypomethylation properties, whereas high concentrations (∼3–10 µM) are cytotoxic [14]. Other DNMT inhibitors in an earlier phase of development include 5-aza-2’-deoxycytidine (DAC) analogues such as SGI-110 and zebularine.

Although DNA methylation is one of the two key epigenetic mechanisms in cancer, we focused on histone modification for our research, which will be discussed now.
7.1.2 Histone Modification

DNA is packaged into chromatin and coils around structural histone proteins in cells. The histones are responsible for maintaining the shape and structure of chromatin. Many post-translational alterations can occur at specific amino acids (e.g. lysine) in the exposed tail of histone proteins, which result in a conformational change in the chromatin structure and in the transcription of important genes necessary to suppress carcinogenesis, such as tumor suppressor genes. These chromatin alterations include acetylation, methylation or phosphorylation [15].

Histone acetylation is controlled by a balance in activity between histone acetyltransferase (HAT)-adds the “acetyl” group (open state) and histone deacetylase (HDAC)-removes the “acetyl” group (closed state). The HDACs are known to be critical in the regulation of expression of genes important for cell survival, proliferation, differentiation, and apoptosis [3]. HDACs also act as members of a protein complex that recruit transcription factors to the promoter region of genes, e.g. tumor suppressor genes, and regulation of acetylation status of specific cell cycle regulatory proteins [16].

7.1.2.1 HDAC Inhibitors

High HDAC expression and histone hypoacetylation have been observed in cancer with associated transcriptional repression of genes, providing a rationale for the investigation of HDAC inhibitors in cancer therapeutics [17].

Aberrant HDAC activity has been documented in a variety of tumor types and led to the development of HDAC inhibitors as anticancer therapeutics [18]. Modest clinical benefits were previously reported with relatively weak HDAC inhibitors such as valproic
acid and phenylbutyrate in advanced solid tumors or hematologic malignancies [19]. More powerful HDAC inhibitors include both class-specific (I and II) inhibitors (entinostat and romidepsin) and pan HDAC inhibitors (vorinostat, belinostat and panobinostat). Clinical studies in solid tumors are ongoing with HDAC inhibitors, alone or in combination with other agents.

7.2 Epigenetics and CRC

Epigenetic alterations, like gene mutations, contribute to the pathogenesis and molecular heterogeneity of various cancers. This is highlighted by the identification of a CpG island methylator subtype of colorectal cancer (CRC) that has a distinct epigenome with a high frequency of methylated genes [20]. Our understanding of epigenomic alterations in colorectal cancer, however, is less advanced than that of our understanding of gene mutations, but substantial progress has recently been made in this area [2]. In the following sub-sections, we will discuss the role of epigenetics in CRC, including the contribution of epigenetics to the molecular heterogeneity of colorectal cancer, and the clinical applications of these epigenetic alterations as biomarkers for early detection, diagnosis, and management of patients with CRC.

7.2.1 Epigenetic pathophysiology of CRC

The classic view of cancer is that it arises because of the accumulation of mutations in key tumor-suppressor genes or oncogenes, which deregulate homeostatic functions and cause the transformation of normal cells into cancer cells. Sequencing of colon cancer genomes has revealed that there is only a small number of functionally important gene
mutations out of the hundreds of mutations found in the average colon cancer genome. A recent analysis of approximately 13,000 genes revealed mutations in the coding sequence of approximately 67 genes in the average colon cancer genome, of which a subset of 12 genes were proposed to be the genes most likely to be involved with cancer formation in individual cancers [21]. The current cancer genome sequencing efforts demonstrate that most cancer genomes carry hundreds of mutations, providing a major challenge to determine which of these mutations play a pathogenic role in the formation of the cancer (“driver mutations”) and which are merely a consequence of this process (“passenger mutations”).

Furthermore, CRC can be sub-grouped based on the type of genomic instability that they display. Different patterns of mutant genes demonstrate the underlying genomic instability in cancer and influences the susceptibility to and selection for specific mutations.

It is believed that although the underlying form of genomic or epigenomic instability determines the types of mutations that occur in CRC, the selective pressures that lead to the clonal evolution of the tumors are largely the same across all CRCs. This concept can explain why certain genes are more frequently mutated in colon cancers than in other cancer types (e.g. breast cancer, prostate cancer) and vice versa.

It has been proposed that gene mutations and epigenetic alterations contribute to colon cancer formation through the activation of oncogenic pathways and the inactivation of tumor suppressor genes, which regulate hallmark behaviors of cancers [22].

7.3 Epigenetics and BC

Breast cancer is generally induced by the accumulation of altered gene regulations which cause abnormal cell growth and expansion. In addition to genetic mutations,
epigenetics also play an important role in breast cancer tumorigenesis. Studies have focused on initiating molecular mechanisms in cancer development; identification of new biomarkers to predict breast cancer aggressiveness and the potential of epigenetic therapy [23].

### 7.3.1 Epigenetic pathophysiology of BC

#### 7.3.1.1 DNA Methylation

Aberrant methylation plays an important role in BC development. DNA hypomethylation has been generally demonstrated on a genome-wide scale in cancer studies while hypermethylated DNA regions of certain genes, especially normally unmethylated CpG islands, were frequently reported. A growing number of studies have focused on hypermethylated genes in breast cancer which have crucial roles in cell-cycle regulation, apoptosis, tissue invasion and metastasis, angiogenesis and hormone signaling [24, 25]. Aberrant methylation profiles of these genes are associated with BC stage and prognosis, therefore methylation status of key cell cycle regulating genes have been proposed as reliable markers for BC [26].

Global DNA hypomethylation is prevalent in breast tumors. Up to 50% of the cases represent reduced 5-methylcytosine content when compared with matched-control normal tissues [23]. Whole genome distribution of aberrant DNA methylation in eight breast cancer cell lines and normal human mammary epithelial cells was analyzed by [27]. It was shown that hypomethylation was distributed throughout the whole genome and simultaneous hypermethylation occurred at CpG-rich regions. In addition, hypomethylation was three- to five-times more frequent than hypermethylation. Studies
have shown that promoter hypermethylation of tumor suppressor genes has mostly resulted in downregulation of gene expression.

### 7.3.1.2 Histone Modification

In breast cancer, investigations carried out on histone modifications are relatively newer than DNA methylation studies. Post-translational histone modifications have a critical role in breast tumorigenesis and aggressiveness of prognosis. In addition, they are potential therapeutic targets. Since different breast cancer subtypes represent distinct gene expression profiles, it is important to clarify the effect of histone marks on gene expression levels in breast tumors. However, there are limited studies about histone modification patterns in breast cancer tumors, though the numbers of investigations are increasing in this field.

In a study of global histone modifications in breast cancer tumors, histone modification levels were significantly higher in steroid receptor [27]. The study concluded that reduced detection levels of these histone marks would be correlated with poor prognostic characteristics. Another study investigated the arginine and lysine methylation of BRCA1 in breast cancer cell lines and breast tumor tissue samples [28] and found that BRCA1 is methylated at arginine and lysine residues both in cell lines and tumor samples. However, lysine methylation was only detected in triple negative MDA-MB-231 cells, while arginine methylation was detected in both MDA-MB-231 and MCF-7 cells. MCF-7 cells are estrogen, progesterone receptor positive, HER2 receptor negative and show wild-type p53. Per these results, they proposed that methylation may affect the ability of BRCA1 binding to specific promoters or affects protein-protein interactions that alter the recruitment of BRCA1 to these promoters.
Another study demonstrated lysine histone modification patterns in breast cancer cell line MCF-7 and normal mammary epithelial cell line MCF-10A and found no significant difference [29]. In another study, histone modification profiles in MCF-7 cells demonstrated that the promoters were the most abundant regulatory elements [30]. Modified histones have gained importance as biomarkers of breast cancer prognosis. The investigations carried out on mechanisms of histone modifications are also promising for the development of efficient HDAC inhibitor therapies.

7.4 Epigenetics of Vitamin D

Insufficient intake or metabolism of vitamin D appears to play a key role in the development of a multitude of diseases affecting the central nervous system, the skeleton and various organs where metabolic disturbances may contribute to the generation of malignancies [31]. In the following section, we will discuss the effects of different epigenetic components of vitamin D metabolism.

7.4.1 Regulation of Vitamin D Receptor (VDR)

Although several rapid and non-genomic actions of active vitamin D have been described [32], most of the effects of vitamin D are mediated by the vitamin D receptor (VDR). VDR is the only protein that binds 1,25(OH)2D3 effectively at sub-nanomolar concentration [33]. This finding somewhat simplifies the understanding of vitamin D signaling, since the physiological effects of the hormone largely overlap with the actions of the transcription factor VDR, a ligand-activated transcription factor.
Unbound 1,25(OH)2D3 freely enter a cell and bind to a VDR present in the cytoplasm or the nucleus [34]. VDR is classified as a class II nuclear receptor, which forms a heterodimer with retinoid X receptor (RXR). This heterodimer binds to the vitamin D response elements (VDREs), which are in the promoter region of key genes [35]. Many vitamin D target genes, e.g. p21, p27 and e-cadherin, have been found to regulate cell cycle arrest and cell differentiation [36, 37]. Therefore, it has been proposed that 1,25(OH)2D3 or its pharmaceutical analogs can possibly be used as a therapeutic for cancer by mediating VDR activity.

VDR is one of approximately 1900 transcription factors, which are encoded by the human genome [38]. In addition, VDR is a member of the superfamily of nuclear receptors, most of which are specifically activated by lipophilic molecules [39]. The transcription factor VDR is the only high-affinity target for 1,25(OH)2D3 within the cell nucleus [33]. Its lipophilic properties allow 1,25(OH)2D3 to pass through all biological membranes, i.e., gene regulation by vitamin D does not involve additional plasma membrane-associated signal transduction steps. Moreover, VDR is rather ubiquitously expressed, i.e., most human tissues and cell types are responsive to 1,25(OH)2D3 [40].

Proliferation of the non-malignant breast epithelial cell line, MCF-12A, was sensitively and completely inhibited by 1,25(OH)2D3 (70 nM) [41] while MDA-MB-231 cells demonstrated a resistance towards 1,25(OH)2D3 (> 100 nM) which correlated significantly with reduced Vitamin D receptor (VDR).

7.4.1.1 Mechanism of Action

Vitamin D exerts its activity in association with its receptor (VDR) and another nuclear receptor, the retinoid X receptor (RXR), in combination with histone
acetyltransferase (HAT). The chromatin of a previously inactive gene can become relaxed upon binding of transcription factors to DNA recognition sequences. This process is counteracted by recruitment of factors having HDAC activity. Accessory transcription factors (ATFs) may be localized proximal or distal of VDR, with distances of up to several hundreds of base pairs, which can be overcome by loop domains of the transcriptional complex [31].

7.4.2 Regulation of CYP27B1

CYP27B1 possesses 1α-hydroxylase activity, which is critical for the last step in vitamin D activation. This enzyme is found in the inner mitochondrial membrane and converts 25(OH)D3 to its active form 1,25(OH)2 D3. It is mainly expressed in the proximal tubule of the kidneys, but it is also expressed in many other vitamin D target tissues, albeit at lower activity levels [42]. The CYP27B1 gene has been reported to harbor a CpG island in its promoter [43]. However, recent DNA sequence updates have shifted the CpG island from the CYP27B1 promoter region into the gene coding sequence.

7.4.2.1 Mechanism of Action

The promoter region of CYP27B1 contains a negative vitamin D response element or nVDRE [44]. This region is responsible for 1,25(OH)2D3-dependent trans repression. This repression seems to be achieved through recruitment of both HDACs and DNMTs by VDR/RXR to the promoter region of CYP27B1 [45]. In cancer, expression of CYP27B1 is often downregulated. This may be explained by increased methylation of the CpG island located within CYP27B1. Methylation of CYP27B1 in various diseases cause reduced
local activation of 1,25(OH)2D3 thus reducing local levels of active vitamin D and restricting its functions [46].

In the breast cancer cells MDA-MB231, CYP27B1 hypermethylation led to gene silencing, while in prostate cancer cell lines, combination of the DNMT1 inhibitor and the HDAC inhibitor TSA resulted in increased activity of CYP27B1 [47].

7.4.3 Regulation of CYP24A1

The 1,25(OH)2D3 24-hydroxylase is an inner mitochondrial membrane enzyme, which catalyzes and inactivates both 25(OH)D3 and 1,25(OH)2D3 [48-50]. Its primary site of expression are the kidneys, playing a crucial role in regulating systemic vitamin D metabolite levels. However, CYP24A1 is also found in many other vitamin D target tissues i.e. skin, gut etc. The promoter of CYP24A1 is spanned by a CpG island which can be regulated by DNA methylation [46] and has several response elements including two VDREs.

7.4.3.1 Mechanism of Action

In healthy kidney, skeletal muscle, whole blood, brain, skin fibroblasts, and sperm the CYP24A1 promoter is not methylated [51], although the expression levels are highly variable. Although, methylation of CYP24A1 was low (5%) peripheral blood lymphocytes [52], it was highly methylated in full term human placenta (56%). In colon cancer cell lines, administration of anti-tumor drug, 5-aza-2-deoxycytidine (DAC), induced CYP24A1 expression in a cell line-specific manner, independent of the methylation level of the promoter. In these cells, induction of CYP24A1 expression by DAC seemed to be
independent of CYP24A1 promoter methylation [53]. Moreover, the methylation level of the CYP24A1 promoter was comparably low both in colon adenocarcinomas and the adjacent mucosa, although the expression of CYP24A1 was significantly higher in the tumors [54]. Taken together, the regulation of CYP24A1 by DNA methylation appears to be tissue-dependent, both in health and disease.

One study investigated the mRNA expression levels of the CYP24A1 gene in malignant and normal breast tissues [55] and found that the mRNA expression of CYP24A1 was significantly upregulated in the tumor tissues (P<0.01). This major difference revealed that the normal breast tissues transcriptionally expressed CYP24A1 slightly. These results are suggestive of dysregulation of the vitamin D signaling and metabolic pathways during tumorigenesis in breast cancer.

7.4.4 Regulation of TRPV6

TRPV6 is a member of the vanilloid subfamily of transient receptor potential (TRP) proteins and functions as an epithelial calcium channel in organs, such as the intestine, kidney, and placenta [56]. TRPV6 mRNA expression is strongly regulated by 1,25(OH)2D3 in the intestine and in Caco-2 (human colon) cells. 1,25(OH)2D3 regulates TRPV6 expression by a process that requires new mRNA and protein synthesis and the point of regulation lies likely at the transcriptional level. In Caco-2 cells, 1,25(OH)2D3 is the primary hormonal regulator of intestinal calcium absorption by a process involving an increase in transcellular calcium transport [57]. However, the molecular mechanism has not been fully described. TRPV6 mRNA was first shown to be regulated by 1,25(OH)2D3 [58] in Caco-2 cells. The dependence of TRPV6 mRNA expression on the VDR-mediated
genomic actions of 1,25(OH)2D3 is supported by increased intestinal TRPV6 mRNA expression after injection of mice with 1,25(OH)2D3 and that TRPV6 expression was lower in vitamin D receptor knockout mice as compared to wild type mice [56]. Few studies have investigated TRP genes in human breast cancer and have demonstrated that TRPC6 and TRPV6 expression increased in both breast cancer tissues and cell lines, with both TRPC6 and TRPV6 regulating cell proliferation [59]. In a study examining the effect of tamoxifen on TRPV6 function and intracellular calcium homeostasis in MCF-7 breast cancer cells, tamoxifen decreased the transport rates of calcium [60].

7.4.4.1 Mechanism of Action

The TRPV6 gene codes for an epithelial calcium channel protein known to play an important role in intestinal calcium absorption. Upregulation of TRPV6 mRNA expression in the colon may be an important mechanism of vitamin D-mediated calcium absorption. TRPV6 gene expression in Caco-2 cells was enhanced in presence of Vitamin D Receptor Elements (VDREs) in the promoter region [56].

Migration and invasion are important features of malignant cells and their therapeutic inhibition might be critical to avoid metastasis of solid tumors. One study attempted to characterize the abilities of TRPV6 expressing cells, by studying their migration potential and the effect of channel [61]. They found that, TRPV6 was strongly expressed in breast adenocarcinoma tissue and TRPV6 mRNA expression was up-regulated between 2fold and 15-fold compared with the average in normal breast tissue. Whereas TRPV6 is expressed in the cancer tissue, its role as a calcium channel in breast carcinogenesis is poorly understood. An in vitro model showed that TRPV6 can be regulated by estrogen, progesterone, tamoxifen, and 1,25(OH)2D3 and has a large
influence on breast cancer cell proliferation [59]. TRPV6 may be a novel target for the development of calcium channel inhibitors to treat breast adenocarcinoma expressing TRPV6.

7.5 Epigenetics of Sulforaphane

Cruciferous vegetables (crucifers), include species predominantly from Brassicaceae family and the more common members are cultivars of Brassica oleracea genus including broccoli, cabbage, cauliflower, Brussel sprouts, and kale; as well as Raphanus genus, i.e. various types of radish. The health benefits of these vegetables are partly due to the nature of the phytochemicals they contain and the glucosinolates [62], the enzymatic hydrolysis products that can modify genes [63]. Nutraceuticals can target survival, proliferation, invasion, angiogenesis, and metastasis, thereby influencing various steps of tumor cell development [64].

Sulforaphane is a bioactive food component or nutraceutical, with potential health benefits that are mainly exerted through epigenetic modification of genes. The principal role of sulforaphane in these epigenetic modifications is believed to be primarily due to its histone deacetylase (HDAC) inhibitor activity.

7.5.1 Phase II Detoxification and SFN

Potentially harmful, foreign compounds (e.g. carcinogens) [65, 66] often involve Phase I and II components, which eventually permits their excretion from the cells. A compound which activates Phase I and Phase II enzymes is called a bifunctional inducer; and, if it activates only Phase II enzymes, it is a monofunctional inducer [67]. Upon
treatment in the cells with oxidants including H2O2, oxidative stress and conformational changes occur. Therefore, for an optimal cellular detoxification environment, Phase II reactions should be at a rate that prevents intermediate products of Phase I from accumulating. Aliphatic sulforaphane acts as a monofunctional inducer, whereas the indole isothiocyanates (ITCs) from mature broccoli are bifunctional inducers derived from another glucosinolate, glucobrassicin [68].

7.5.2 Histone Deacetylase Inhibition

In a recent study to test whether SFN inhibits HDAC activity in vivo, an average daily dose of 7.5 μmol per animal for 21 days, suppressed the growth of human PC-3 prostate cancer cells by 40% in male nude mice [69]. There was a significant decrease in HDAC activity in the xenografts, as well as in the prostates and mononuclear blood cells (MBC), of mice treated with SFN, compared to controls. In human subjects, a single dose of 68 g BroccoSprouts inhibited HDAC activity significantly in peripheral blood mononuclear cells (PBMC) 3 and 6 hrs. following consumption from the same study.

In human embryonic kidney 293 cells, SFN dose-dependently increased the activity of TOPflash reporter and inhibited HDAC activity, resulting in an increase in acetylated histones [70]. Bioactive dietary supplements such as green tea polyphenols (GTPs) and sulforaphane (SFN) inhibit DNA methyltransferases (DNMTs) and histone deacetylases (HDACs), respectively, which are of central importance to cancer prevention. In a recent study of estrogen receptor ER-negative MDA-MB-231 cells, GTPs and SFN alone or in combination lead to the reactivation of ER expression which was consistently correlated with promoter hypomethylation and hyperacetylation [71]. General hypothesis regarding
SFN action was that the ability of SFN to target aberrant acetylation patterns, in addition to effects on phase II enzymes, might make it an effective chemoprevention agent.

### 7.6 Epigenetics of vitamin D in CRC

The components of the vitamin D pathway can potentially be used both as treatment and preventive strategies for CRC. The active metabolite 1,25 (OH)2 D3 targets the Wnt/β-catenin signaling pathway by up regulating key tumor suppressor genes, which promotes an epithelial phenotype, but is only useful when the VDR is present [72]. In colorectal cancers with low VDR expression, it may be possible that future treatments could target the genomic and epigenomic level alterations present to increase VDR expression by modulating expression of transcription factors or utilizing HDAC inhibitors, respectively.

Colorectal cancer can be treated by targeting transcription factors that down-regulate the vitamin D receptor, which is essential for vitamin D to mediate its effects in promoting an epithelial phenotype. Furthermore, potential preventative measures to protect individuals from colon cancer include- increasing serum levels of 25(OH)D3 by either sun exposure, diet or supplements.

### 7.6.1 Mechanistic links between vitamin D and CRC

High levels of serum 25(OH)D3 are related to lower incidence rates in many cancers, particularly colon cancer [73-75]. 25(OH)D3 is a hepatic metabolite in the vitamin D pathway that is converted by the 1α-hydroxylase enzyme in the kidneys to form 1,25(OH)2D3. The vitamin D pathway includes both endogenous (cutaneous synthesis)
and exogenous sources (diet, supplements) of vitamin D, so both can cause a significant increase in serum 25(OH)D3.

Unbound 1,25(OH)2D3 enters a cell and bind to a VDR present in the cytoplasm or the nucleus [34]. VDRs are expressed in normal colonic cells, but it has been shown that VDR expression levels decrease in the later stages of colon cancer; the mechanism behind this phenomenon is not fully understood [76]. Therefore, treatment with 1,25(OH)2D3 may not be as effective in the later stages of colon carcinogenesis due to the development of cellular resistance to 1,25(OH)2D3. It has been suggested that both genomic and epigenetic modifications might be involved in the reduction of VDR expression [77-82]. For instance, Malinen and colleagues demonstrated that the down regulation of 25(OH)D3 due to reduced expression of CYP27A1 via HDACs can be overcome with the use of HDAC inhibitors [83]. Furthermore, it is proposed that an individual could reduce their risk of colorectal cancer approximately 30–50% by either increasing vitamin D intake to 2000 IU/day or increasing their sun exposure to raise blood levels of 25 (OH)D3 to greater than 30 ng/ml [84].

7.7 Epigenetics of vitamin D in BC

A large proportion of breast cancer cells contain the VDR protein, however the level of expression is variable within individual cells [85] and, therefore, the biological response to vitamin D also varies between patients. The primary effect of exposure of breast cancer cells to 1,25 (OH)2D3 is cell cycle arrest due to changes in the functional status of the proteins that regulate cell cycle [86]. An effect of 1,25(OH)2D3 on cell proliferation can also be mediated in an indirect way, via interference to the function of estrogen receptors (ER)[87]. 1,25 (OH)2D3 and its analogues decrease the expression of
ERα, which in turn reduces the level of mitogenic signals to breast cancer cells from estrogens [88]. Another mechanism of the anticancer action of 1,25 (OH)2D3 against breast cancer cells is that it down-regulates the expression of aromatase, which catalyzes a step-in estrogen synthesis [89].

One of the key factors regarding the activity of 1,25 (OH)2D3 is its availability in the BC environment. This is maintained by the balance between synthesis and catabolism. CYP27B1 is present in some breast cancer cells, to control the autocrine synthesis of 1,25 (OH)2D3, but this enzyme is also active in breast cancer microenvironment. It has been shown that breast adipocytes produce CYP27B1, bio-activate 25(OH)D3 to 1,25 (OH)2D3 and in a paracrine fashion deliver 1,25 (OH)2D3 to the breast epithelium [90]. On the other hand, the availability of 1,25 (OH)2D3 is maintained by its degradation, maintained by the 24-hydroxylase enzyme (CYP24A1). In normal tissues, this enzyme is expressed in response to 1,25 (OH)2D3 exposure, providing a negative feedback mechanism that maintains a steady concentration of 1,25 (OH)2D3 in the plasma [91]. Genome hybridization studies have revealed that in certain human breast cancers the CYP24A1 gene is amplified and this may cause a reduction in the level of 1,25 (OH)2D3 and cause cells to proliferate unduly [87].

7.7.1 Mechanistic links between vitamin D and BC

The mechanism behind reduction in BC risk related to vitamin D intake is mainly due to cell cycle arrest and apoptosis by 1,25(OH)2D3 [86, 92]. 1,25(OH)2D3 can also induce morphological changes associated with apoptosis in breast cancer cells [93]. 1,25(OH)2D3 can also inhibit metastasis of cancer cells and has potential anti-angiogenic
properties that can inhibit tumor cell invasion [94]. Vitamin D can act as an anti-inflammatory agent such that 1,25(OH)2D3 can down-regulate the expression of cyclooxygenase-2, which plays a role in prostaglandin synthesis in human breast cancer [89]. Many epidemiological studies have reported positive associations between gene polymorphisms and risk of BC; these include vitamin D related genes such as VDR, vitamin D-binding protein (VDBP), as well as the CYP24A1 gene [95].

Several studies have examined the effects of 1,25(OH)2D3 on mammary carcinogenesis in cells and animal models and found a protective role of 1,25(OH)2D3 in BC development. The relationship of vitamin D with risk of breast cancer may be subtype-specific, with evidence of stronger effects of vitamin D for more aggressive breast cancer, especially in African women.

7.8 Epigenetic effects of SFN in CRC

Colorectal cancer (CRC) has high morbidity and mortality rates worldwide and is a global health problem. The conventional radiotherapy and chemotherapy regimen for CRC not only has a low cure rate but also causes side effects [96]. Many studies have shown that adequate intake of fruits and vegetables in the diet may have a protective effect on CRC occurrence, possibly due to the special biological protective effect of the phytochemicals in these foods. SFN is one such compound present in cruciferous vegetables. In colon cancer cells, sulforaphane is associated with decreased cell proliferation, cell cycle arrest and apoptosis [97].

A few mechanisms that are known to be affected by SFN in CRC are as below:
7.8.1 Inhibiting phase I metabolic enzymes

Cytochrome P450 is a major component of phase I metabolic enzymes and can convert xenobiotics into more electrophilic, reactive, mutagenic or even carcinogenic bioactive compounds. 1,25(OH)2D3 in Caco2 cells, can induce some cytochrome enzymes which can mediate the metabolism of carcinogens, making them less carcinogenic. Previous studies have shown that SFN competitively inhibited or covalently modified some cytochrome enzyme isoforms and reduced their expression, thereby inhibiting DNA-adduct and chemical carcinogenesis [98, 99]. In another study, SFN was found to significantly reduce the expression of the enzyme mRNA in Caco-2 cells [100].

7.8.2 Inducing phase II metabolic enzymes

Phase II metabolism is composed mainly of binding reactions, wherein carcinogens and their metabolite reactive oxygen species (ROS) bind to endogenous ligands such as glutathione and glucuronic acid. Currently, many studies have confirmed that SFN induces the expression of several phase II metabolic enzymes in CRC cells, including glutathione reductase (GR), glutathione S-transferase (GST), aldehyde reductase (AR), NAD[P]H: quinone oxidoreductase (NQO) and uridine 5’-diphospho (UDP)-glucuronosyltransferase (UGT) [96]. Glutathione reductase converts the active and oxidized glutathione to stable and reduced glutathione (GSH). Glutathione transferase catalyzes the binding of electrophilic substrate with GSH to induce its excretion. AR and NQO catalyze the conversion of metabolically active aldehydes and quinones to relatively stable alcohols and hydroquinones respectively, reducing their activities and damaging effects. UGT catalyzes
the transferring of β-glucuronic acid from UDP-glucuronic acid to active substrates, thereby increasing their solubility and facilitating their excretion.

Treatment of Caco-2 cells with 25 μM SFN for 24 h significantly increased mRNA and protein expression levels of UGT1A1, 1A8 and 1A10 as well as their enzymatic activity [100]. It has also been shown that SFN and SFN-GSH significantly improved mRNA and protein expression levels of UGT1A1, mRNA expression level of GSTA1 and activity of bilirubin in HepG2 and HT-29 cells [101].

7.8.3 Inhibiting HDAC activity

Nrf2 pathway is likely to play a role in the inhibition of HDAC by SFN. A study has found that carcinogen 1,2-dimethylhydrazine more likely induced CRC in wild-type Nrf2+/+ mice than in mutant Nrf2-/- mice and that HDAC level in wild-type mice was significantly increased as compared to that in Nrf2-/- mice upon treatment with 400 ppm SFN in diet (Rajendran et al., 2015). SFN treatment had a more profound effect on reducing tumors in wild-type mice than in Nrf2-/- mice. Other studies have shown that SFN inhibited the activity of HDAC by promoting its degradation in the cytoplasm (Yin et al., 2016).

7.8.4 Cell cycle arrest

Previous studies have shown that SFN arrested cell cycle in many types of CRC cells. For example, SFN arrested Caco-2 cells in the G1/G2 [100] and G2/M phases [102], and arrested HCT116 cells in the G2/M phase [103]. SFN also augmented the expression of p21, which exerted inhibitory activity on cyclin-dependent kinase (CDK) and regulated
the levels of many cell cycle proteins. While arresting HT-29 cells in the G0/G1 phase, SFN significantly induced the expression of p21 and reduced the expression level of some regulatory proteins, which is vital for G1 phase, such as cyclin A, cyclin D1 and c-Myc, at high doses (>25 μM) [104].

7.8.5 Inhibiting tumor angiogenesis

Kim et al., 2015, have found that SFN significantly reduced the expression level of vascular endothelial growth factor (VEGF) and hypoxia inducible factor-1α (HIF-1α), both of which are associated with angiogenesis and tumor metastasis, and inhibited tumor metastasis in a dose-dependent manner in hypoxia-treated HCT116 cells [105]. SFN might inhibit CRC progression and tumor angiogenesis in a similar manner.

7.8.6 Enhancing the efficacy of chemotherapy drugs

Studies have shown that both SFN and antineoplastic agent oxaliplatin could inhibit the proliferation of Caco-2 cells in a dose-dependent manner. Pretreatment of HT-29 cells with 2.5 μM SFN activated the cancer drug PR-104A and reduced its EC50 by 3.6-fold. Similar results were observed with SW620 cells. However, SFN had little effect on normal colon cells [106]. Therefore, SFN combined with traditional chemotherapy drugs might reduce drug resistance and enhance drug efficacy. The potential chemopreventive effect of SFN on CRC has also been confirmed by in vivo animal studies. Animal experiments have shown that 2-6 h after oral gavage of 5-20 μmol/L SFN, the highest level of SFN was detected in small intestine, prostate, kidney and lung (in a descending order) [107]. Therefore, SFN might provide a targeted chemopreventive effect in the prevention of CRC.
7.9 Epigenetics of SFN in BC

Phytochemicals have been studied extensively for the treatment of various diseases and disorders [96]. They exhibit a wide range of safety and target multiple pathways and targets in breast cancer cells [108]. Current evidence suggests that naturally occurring phytochemicals can effectively target breast cancer stem cells, [109, 110] and are proposed to be useful in the treatment of BC.

Consumption of cruciferous vegetables such as broccoli, bok choy, Brussel sprouts etc. correlates with decreased risk of cancer induction and this protective effect has been shown to be in part due to the presence of an isothiocyanate (ITC) glucoraphanin [62, 111]. The four important ITCs formed from glucosinolates by the activity of myrosinase are benzyl-ITC, allyl-ITC, phenylethyl-ITC (PEITC) and methylsulphinylbutyl-ITC (sulforaphane). PEITC [112] and sulforaphane [113].

Sulforaphane was found to inhibit proliferation, angiogenesis, and metastasis as well as induce cell cycle arrest and apoptosis in breast cancer cells. Sulforaphane reactivated expression and reduced proliferation in MDA-MB-231 cells, probably via histone modifications and DNA demethylation-facilitated activation of ER-α [71]. Sulforaphane treatment caused cell cycle arrest at S- and G2/M-phase with enhanced levels of p21WAF1 and p27KIP1 and decreased cyclin A, cyclin B1 and CDC2 expression in breast cancer cells [114]. It also decreased the production of IL-1β, IL-6, TNF-α, interferon-γ, IL-4, platelet-derived growth factor and VEGF in breast cancer cells [115].

In addition, sulforaphane has a marked effect on cell cycle checkpoint controls and cell survival and/or apoptosis in various other cancer cell lines, though the molecular mechanisms that remain poorly understood [116-120].
The effect of SFN on various human cell lines, animal models as well as human subjects have been listed below:

Table 7.1: Summary of SFN effects in cancer

<table>
<thead>
<tr>
<th>Subjects</th>
<th>SFN Dosage</th>
<th>Duration</th>
<th>Outcome Measure</th>
<th>Effect</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human</td>
<td>200 µmol</td>
<td>1 hour</td>
<td>SFN metabolites in breast tissue</td>
<td>(left)1.45±1.1 2 and (right) 2.00±1.95 pmol/mg tissue</td>
<td>[121]</td>
</tr>
<tr>
<td>Human</td>
<td>102 µmol</td>
<td>1 hour</td>
<td>NQO1 mRNA expression</td>
<td>Increase mRNA expression by 200%</td>
<td>[122]</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>SFN Dosage</th>
<th>Duration</th>
<th>Outcome Measure</th>
<th>Effect</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>HEK293 (human embryonic Kidney cells)</td>
<td>5µM SFN + 100ng/L TSA</td>
<td>48 hours</td>
<td>HDAC activity</td>
<td>Reduced HDAC activity SFN+TSA &lt;60% SFN&lt;30% TSA &lt;25%</td>
<td>[69]</td>
</tr>
<tr>
<td>SW620 (human colon cancer cell)</td>
<td>100 µM</td>
<td>72 hours</td>
<td>Cell Proliferation</td>
<td>&lt;20% than control</td>
<td>[123]</td>
</tr>
<tr>
<td>Caco2, HT-29 and SW480 (human colorectal cancer cells)</td>
<td>1, 5, 10 and 20 µmol</td>
<td>24 and 48 hours</td>
<td>β-defensin-2 (HBD-2) gene expression</td>
<td>At 24 hours: 1.6X increase (by 20 µmol) in HBD-2 At 48 hours: 2X increase (by 20 µmol) in HBD-2</td>
<td>[124]</td>
</tr>
<tr>
<td>HepG2 cells (human hepatic cancer cells)</td>
<td>1.0, 2.5, 5.0 and 10.0 µmol</td>
<td>6 hours</td>
<td>CYP1A1 mRNA expression</td>
<td>Increased mRNA expression: 1.0 µmol= 2.2X 2.5 µmol= 2.8X 5.0 µmol= 3.8X 10.0 µmol= 3.6X</td>
<td>[125]</td>
</tr>
<tr>
<td>Animals</td>
<td>SFN dosage</td>
<td>Exposure time</td>
<td>Outcome measure</td>
<td>Effect</td>
<td>References</td>
</tr>
<tr>
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</tr>
<tr>
<td>LNCap C4-2 (human prostate cancer cells)</td>
<td>10 µmol 20 µmol 40 µmol</td>
<td>24 hours</td>
<td>Androgen Receptor (AR) And Prostate Specific Antigen (PSA) protein levels</td>
<td>Decrease in AR and PSA protein expression by 25% compared to control when treated with 40 µmol SFN</td>
<td>[126]</td>
</tr>
<tr>
<td>Mice triple negative breast cancer (TNBC) cells</td>
<td>Various Concentrations of SFN</td>
<td>72 h</td>
<td>Effects of SFN treatments on Cell proliferation, cell cycle arrest and apoptosis</td>
<td>SFE exhibited antiproliferative effects against multiple kinds of TNBC cells ex and in vivo</td>
<td>[127]</td>
</tr>
<tr>
<td>Animals</td>
<td>SFN dosage</td>
<td>Exposure time</td>
<td>Outcome measure</td>
<td>Effect</td>
<td>References</td>
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</tr>
<tr>
<td>F344 rats</td>
<td>5 µM</td>
<td>2 weeks</td>
<td>colonic aberrant crypt foci (ACF) and multicrypt foci</td>
<td>Reduction in the total ACF from 153 to 109–115 (P &lt; 0.01) and multicrypt foci from 52 to 35 (more than four crypts/focus; P &lt; 0.05).</td>
<td>[128]</td>
</tr>
<tr>
<td>Sprague-Dawley Rats (female)</td>
<td>glucosinolates (25 and 100 µmol) and isothiocyanates (25, 50, and 100 µmol)</td>
<td>5 days</td>
<td>mammary tumor formation</td>
<td>Reduced incidence at all doses of glucosinolate and isothiocyanate treatment (P = 0.0197 and 0.0190, respectively)</td>
<td>[68]</td>
</tr>
<tr>
<td>Sprague-Dawley Rats (female)</td>
<td>SFN 75 and 150 µmol</td>
<td>5 days</td>
<td>Multiplicity of tumors</td>
<td>Reduction in tumor multiplicity by 75 and 150 µmol, 0.45 and 0.26 times respectively ((P = 0.01)</td>
<td>[99]</td>
</tr>
</tbody>
</table>
It is important to understand the metabolism of drugs or pharmaceutical substances (also known as xenobiotic metabolism) or bioactive food components like SFN, to fully elucidate its mechanism of action. The goal of xenobiotic metabolism is to transform various non-polar metabolites generated by multiple chemical reactions, to a more polar form that can then be more easily eliminated from the body. There are three phases of xenobiotic metabolism: (i) Phase I reactions introduce or expose functional groups on the drug to increase the polarity of the compound and activate them. The primary and first pass site of this metabolism occurs during hepatic circulation. Additional Phase I metabolism occurs in gastrointestinal epithelial, renal, skin, and lung tissues. Phase I reactions are broadly grouped into three categories, oxidation, reduction, and hydrolysis (ii) In Phase II reactions, these activated xenobiotic metabolites are conjugated with charged species such as glutathione (GSH), sulfate, glycine, or glucuronic acid. The addition of large anionic groups (such as GSH) detoxifies reactive electrophiles and produces more polar metabolites that cannot diffuse across membranes, and may, therefore, be actively transported. (iii) Conjugates and their metabolites are excreted from cells in Phase III of their metabolism.

Sulforaphane (SFN) has shown to be an effective cancer chemopreventive agent in several animal carcinogenic models as presented in the summary table above (Table 7.1) and is thought to induce Phase II detoxification enzymes with promoter antioxidant response elements [129-131]. Recent work has implicated multiple mechanisms of sulforaphane action, with the majority of studies focusing on SFN as a potent Phase II enzyme inducer and additional evidence for cell cycle arrest and apoptosis. Early research
focused on Phase II enzyme induction by sulforaphane as well as on the inhibition of enzymes involved in carcinogen activation, but there has been growing interest in other mechanisms of chemoprotection by sulforaphane. Upregulation of Phase II metabolism is likely a critical mechanism leading to cancer prevention by sulforaphane in the “initiation” phase, helping to more rapidly eliminate genotoxins from the body.

To date, very few human clinical trials have evaluated the effects of sulforaphane on cancer outcome; however, several pilots and Phase I human sulforaphane trials have

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CHAPTER 8
SUMMARY AND CONCLUSIONS

How the complex interactions of human exposures, metabolism, and genetics are related to the development of colorectal and breast cancers being influenced by vitamin D and sulforaphane is not yet known.

A more complete understanding of vitamin D and sulforaphane in human carcinogenesis from organ site–specific biologic mechanisms, prospective serologic analyses, genetic variation, and short-term clinical trials including metabolomic profiling holds the key to elucidating the effect of vitamin D in colorectal and breast cancers in the future.

Insights into the biology of these cancers have been gained from the identification of genes commonly mutated in the cell signaling pathways and have led to paradigms that enriched the study of epigenetic alterations in cancer. These insights are also currently being used to develop new diagnostic and prognostic assays and potential therapies for colorectal cancer. Possible mechanisms include, effects of vitamin D on cell proliferation and differentiation, and apoptosis.

It is evident from the existing literature that clinical trials of vitamin D supplementation at both the blood and tissue levels are needed to pinpoint the specific mechanisms in both colorectal and breast cancer. Finally, examination of vitamin D in relation to cancer survival is a relatively uncharted area that deserves much greater attention.
Large-scale clinical cohort studies are critical and required to confirm the chemopreventive effects of SFN on CRC and BC.

Future studies should determine whether sulforaphane in food might play a chemopreventive role through the molecular mechanisms discussed previously, as well as determine its effective concentration, bioavailability and interactions with other dietary components. Understanding the distribution, metabolism and excretion of SFN in vivo could become a significant avenue of research.
### APPENDIX A

#### TABLE: GENE PRIMER SEQUENCES

<table>
<thead>
<tr>
<th>Genes</th>
<th>Forward Seq.</th>
<th>Reverse Seq.</th>
</tr>
</thead>
<tbody>
<tr>
<td>VDR</td>
<td>5’-GAG GGA ACA GAC AGG AGA AAT G-3’</td>
<td>5’-TAT CGT GAG TAG GCA GGA GAG-3’</td>
</tr>
<tr>
<td>TRPV6</td>
<td>5’-GAC CTG CGT GGG ATA ATC AA-3’</td>
<td>5’-CAG GAA GCG AAG TGA GAA CA-3’</td>
</tr>
<tr>
<td>Cyp24a1</td>
<td>5’-TGT GTG TGT GTC CGT GTA TG-3’</td>
<td>5’-CCT GCA CCA CAG ATC CTA AAT-3’</td>
</tr>
<tr>
<td>Cyp27b1</td>
<td>5’-CCA TGT GGC AGA AGG GAT AA-3’</td>
<td>5’-AAA CCG TAA ACC AGG CTA GG-3’</td>
</tr>
<tr>
<td>GAPDH</td>
<td>5’-CAT GGG TGT GAA CCA TGA GA-3’</td>
<td>5’-GGG TGC TAA GCA GTT GGT-3’</td>
</tr>
</tbody>
</table>
**APPENDIX B**

**TABLE: SUMMARY OF STUDIES SELECTED IN OUR META-ANALYSIS OF VITAMIN D AND BC, PUBMED 2000-2017**

| STUDY # | Country | Study name; Study Design; Sample Size | Menopausal status; Baseline age; Follow-up time | Exposur e; Outcome assessment | 25(OH)D Mean±SD % deficient | Dietary vitamin D Mean±SD | Supp. use, % | Adjustme nt | Findings | Conclusion |
|---------|---------|---------------------------------------|------------------------------------------------|-------------------------------|-----------------------------|-----------------------------|-------------------------|---------------|-----------|-----------|------------|
| 1 [129] | USA     | Nurses’ Health Study; Nested case-control; Cases (N=701) and controls (N=724) | Total 30-55y Mean: ~57.1 y* | 25(OH)D: IA; Self-reported diagnosis of BC during follow-up. | Mean: 32.3 ng/mL* | n/a | n/a | BMI, menopausal status, menopausal hormone use, height, age at menarche, parity and age at first birth, weight at age 18, age | Women in the highest quintile of 25(OH)D had RR=0.73; (95% CI: 0.49-1.07; P-trend = 0.06) | High levels of 25(OH)D may be modestly associated with reduced risk of BC. |

QS=0+1+1+2=4 (Range:0-8)
<table>
<thead>
<tr>
<th></th>
<th>USA</th>
<th>Cancer Preventio n Study II Nutrition Cohort; Prospective cohort; N=68,567</th>
<th>Post 50-74y Mean: ~62 y* Up to 9y of follow-up</th>
<th>Dietary/s uppleme ntal calcium and vitamin D, 68-item food frequen cy questionaire (FFQ); BC incidence ascertain</th>
<th>n/a</th>
<th>Dietary vitamin D: Mean: 167 IU/d**</th>
<th>Total vitamin D: Mean: 320 IU/d</th>
<th>Age, energy, history of breast cyst, family history BC, height, weight gain since age 18, alcohol use, race, age at menopause, age at first birth and number of Using suppleme ntal vitamin D intake was not associate d with BC risk, overall. The associati on suggeste d a protectiv e effect among</th>
<th>Our results support the hypothe sis that dietary calcium and/or some other compon ents in dairy products may modestl y reduce</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>[185]</td>
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</tbody>
</table>

QS=2+2+2+2=8 (Range:0-8)
ed with NDI.

live births, education, mammography, history, and HRT use.

women with estrogen receptor-positive tumors comparing highest to lowest intake of dietary vitamin D (RR=0.74; 95% CI, 0.59-0.93; P-trend = 0.006)

Reduced BC risk was associated with use of vitamin D or multivitamin and other variables included in the fully adjusted models. Age, education, and ethnicity were not included in the fully adjusted models. Vitamin D could help prevent BC.

QS=0+2+2+2=6 (Range:0-8)
<table>
<thead>
<tr>
<th>4</th>
<th>USA</th>
<th>Iowa Women’s Health Study (IWHS)</th>
<th>Post 55-69 y ~61.5 y*</th>
<th>127-item FFQ Dietary supplement questionnaire; Incident cases of BC were n/a</th>
<th>Mean total vitamin D intake: ~520 IU/d* n/a</th>
<th>Baseline age (y), smoking status, age at menarche, age at reported menopause, first degree relative</th>
<th>The adjusted RR of BC for women consuming &gt;800 IU/day versus &lt;400 IU/day total</th>
<th>Vitamin D intake of &gt;800 IU/day appears to be associated with a small decrease in risk of BC</th>
</tr>
</thead>
<tbody>
<tr>
<td>(N=1,135)</td>
<td>pathology report indicating invasive BC, age-matched population-based controls. Controls: 22% menarche (&lt;12, 12, 13, 14+), first degree family history of BC (yes/no), ever breast-fed (yes/no), and age at first birth (&lt;20, 20-24, 25-29, 30+, nulliparous).</td>
<td>OR=0.62; 95% CI:0.49-0.79.</td>
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<tr>
<td>QS=2+2+2+2=8(Range:0-8)</td>
<td>Iowa Women’s Health Study (IWHS) Prospective cohort N=34,321</td>
<td>Follow-up up to 18y</td>
<td>127-item FFQ Dietary supplement questionnaire; Incident cases of BC were n/a</td>
<td>Mean total vitamin D intake: ~520 IU/d* n/a</td>
<td>Baseline age (y), smoking status, age at menarche, age at reported menopause, first degree relative</td>
<td>The adjusted RR of BC for women consuming &gt;800 IU/day versus &lt;400 IU/day total</td>
<td>Vitamin D intake of &gt;800 IU/day appears to be associated with a small decrease in risk of BC</td>
<td></td>
</tr>
</tbody>
</table>
identified between 1986 and 2004 through linkage to the State Health Registry of Iowa, part of the National Cancer Institute’s Surveillance, Epidemiology and End Results program (SEER).

with BC, estrogen use, age at first live birth, number of live births, education category, BMI category, activity level, live on a farm, mammogram history, daily energy, fat and alcohol intakes. vitamin D was 0.89 (95% CI: 0.77-1.03). RRs were stronger among women who were ER+ or PR+ status. The association of high vitamin D intake with BC was strongest in the first 5 years after baseline dietary among postmenopausal women.
A nested case-control study within a randomized trial compared lowest intake group, and diminished over time.

<table>
<thead>
<tr>
<th>#</th>
<th>USA</th>
<th>Women’s Health Initiative</th>
<th>Post 50-79 y (Mean: ~63 y*)</th>
<th>25(OH)D ( \leq 10 \text{ nmol/L} ) *</th>
<th>Mean total vitamin D intake: 350 IU/d*</th>
<th>Supplementation use: Vitamin D/multivitamin: Cases: 47%; Controls: 48%</th>
<th>Baseline age (y), weight, and baseline percentage of energy from total fat</th>
<th>Baseline 25(OH)D levels were not associated with subsequent BC risk</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>Women’s Health Initiative</td>
<td>Post 50-79 y (Mean: ~63 y*)</td>
<td>25(OH)D ( \leq 10 \text{ nmol/L} ) *</td>
<td>Mean total vitamin D intake: 350 IU/d*</td>
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<td>Baseline age (y), weight, and baseline percentage of energy from total fat</td>
<td>Baseline 25(OH)D levels were not associated with subsequent BC risk</td>
<td></td>
</tr>
</tbody>
</table>

**QS=0+2+1+2=5 (Range:0-8)**
(N=1,067)

BCs were confirmed by both local and central medical record and pathology report review by trained adjudicators who were blinded to randomized allocation, with such records available = .20)
<table>
<thead>
<tr>
<th>Study</th>
<th>Country</th>
<th>Study Design</th>
<th>Case-Control Study Details</th>
<th>Case Number</th>
<th>Control Number</th>
<th>Follow-Up Time</th>
<th>25(OH)D</th>
<th>RR of BC</th>
<th>Risk Factors Assessed</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>USA</td>
<td>Prostate, Lung, Colorectal, and Ovarian (PLCO) Cancer Screening Trial</td>
<td>Nested case-control study within a screening trial</td>
<td>N=1,005</td>
<td>N=1,005</td>
<td>3.9 y</td>
<td>26.7 ng/mL</td>
<td>1.04</td>
<td>BMI at age 18 to 20, age at menarche, age at menopause, HRT use, history of benign breast disease, family history of BC, combined parity, age at first birth, smoking status, alcohol intake, and total calcium intake.</td>
<td>No inverse association between circulating 25(OH)D and BC risk</td>
</tr>
</tbody>
</table>

Note: QS = 0+2+2+2 = 6 (Range: 0-8)
A total of 92% of the ascertained BC cases were confirmed through review of medical records. QS=0+1+1+2=4 (Range: 0-8)

<p>| 7 | Germany [178] | Population-based case-control study from southern Germany (Freiburg and Rhein-Neckar-Odenwald) | Pre 30-50 y Mean: ~42.6 y* | 25(OH)D: IA; Cases were identified through frequent monitoring of hospital admissions, surgery | 51.3 nmol/L | n/a | n/a | Stratified by age and adjusted for time of blood collection, number of births, first-degree family history, age at menarche, duration of Compare with the lowest category (&lt;30 nmol/L), the ORs (95% CI) for the upper categories (30–45, 45–60, ≥60) | There is a protective effect of vitamin D for premenopausal BC |</p>
<table>
<thead>
<tr>
<th>Cases (N=289) and controls (N=595)</th>
<th>schedule and pathology reports in 38 hospitals.</th>
<th>breast-feeding, BMI, alcohol consumption.</th>
<th>n/a</th>
<th>n/a</th>
<th>Reproductive risk factors, history of benign breast disease, family history, education, alcohol use, postmenopausal hormone</th>
<th>No association between 25(OH)D and BC (OR = 1.09, 95% CI 0.70-1.68, P = 0.60) for the top vs</th>
<th>Results do not support an association between adulthood serum 25(OH)D and postmenopausal BC.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>8</strong></td>
<td>USA</td>
<td>[125]</td>
<td>Cancer Prevention Study-II (CPS-II) Nutrition Cohort Nested case-control within Prospective cohort study</td>
<td>Post 47-85 y Mean: ~69.5 y**</td>
<td>25(OH)D: IA; Eligible cases included women who reported a new diagnosis of BC on a biennial Mean: 49.1-59.5 nmol/L depending on season.</td>
<td>n/a</td>
<td>n/a</td>
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</table>

 QS=0+1+1+2=4 (Range: 0-8)
| Cases (N=516) and controls (N=516) | CPS-II Nutrition Cohort Survey between the date of their blood draw and 30 June, 2005 (n = 514) or who did not report an incident BC but for whom fatal BC was identified through linkage with the National Death Index (n = 2). | use, diet, recreationa l physical activity and zip code (for latitude). | bottom quintile. |

QS=0+0+2+1=3(Range:0-8)
<table>
<thead>
<tr>
<th>9</th>
<th>Denmark</th>
<th>Case-control study</th>
<th>Total 29-87 y Mean: ~58 y**</th>
<th>25(OH)D: LC; Mammo graphic, followed by pathologic examination. Information on studied subjects from The Danish National Hospital Discharge Register and the Danish Cancer Register were also retrieved.</th>
<th>Cases: 69 ± 23 nmol/L Controls: 76 ± 28 nmol/L</th>
<th>n/a</th>
<th>n/a</th>
<th>Controls matched with cases on menopausal state, and time of year of blood sampling (± 2 mo). Compared with the lowest tertile of 25(OH)D levels, risk of BC was significantly reduced among women in the highest tertile (RR=0.52; 95% CI: 0.32-0.85)</th>
<th>Risk of BC was inversely associated with 25(OH)D levels</th>
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<td></td>
<td>QS=0+1+0+1=2(Range:0-8)</td>
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</table>
| No | Country | Study Design | Cases (N=311) | Controls (N=311) | Vitamin D level was not related to BC risk

Vitamin D may not be related to BC risk.

Serum 25(OH)D level was not associated with an increased risk neither at the 1st nor at the 2nd pregnancy samples (OR = 1.4, 95% CI 0.6–3.4; OR 1.4, 95% CI 0.7–2.8, respectively), but was associated with an increased risk of PABC (OR =

| Pre 30-34 y Mean: ~33 y** | Missing information on diagnosis of BC. | n/a | n/a | Controls matched to cases by parity, age, year, and season. | Mean: 43 nmol/L * | n/a | n/a |

25(OH)D: IA;
<table>
<thead>
<tr>
<th></th>
<th>France E3N Cohort [121]</th>
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<tr>
<td>Cases (N = 636)</td>
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<tr>
<td>Controls (N= 1,272)</td>
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</tbody>
</table>

Total Cases (56.9± 6.4) and controls (56.9± 6.4)

25(OH)D:

IA:

Every 2-3 y, questionnaire was sent out. In each questionnaire, participants were asked whether a cancer had been diagnosed, and if so, pathology reports were

Cases: 24.4±10.9 ng/mL

Control: 25.1 ± 11.0 ng/mL

n/a

n/a

BMI, HRT use, history of mammography history and of breast benign disease, family history of BC, parity, smoking status, use of oral contraceptives, age at menarche, and physical activity, alcohol consumption, total energy, calcium/vitamin D

2.7; 95%CI 1.04–6.7)

There is a decrease risk of BC with increasing 25(OH)D3 serum concentrations (odds ratio, 0.73; 95% confidence interval, 0.55-0.96; P-trend = 0.02) among women in the highest tertile)
requested from the attending physician.

dietary intakes, vitamin D/calcium supplement use, serum calcium, PTH, estradiol, progesterone, estradiol (pmol/L, continuous) and progesterone (nmol/L, continuous).

<table>
<thead>
<tr>
<th>Study</th>
<th>Country</th>
<th>Study Design</th>
<th>Sample Details</th>
<th>25(OH)D Cases</th>
<th>25(OH)D Controls</th>
<th>BMI at age 18 y and at the time of blood collection, ages at menarche and first birth, parity.</th>
<th>Circulating 25(OH)D levels and BC risk</th>
</tr>
</thead>
<tbody>
<tr>
<td>12</td>
<td>USA</td>
<td>Nurses’ Health Study II</td>
<td>Both all women (50.9±12.6), pre-menopausal (39.7±7.83), BC cases were identified on biennial question</td>
<td>25.4±9.5 ng/mL</td>
<td>25.0±9.6 ng/mL</td>
<td>No significant association was observed between plasma 25(OH)D levels and BC risk.</td>
<td>Circulating 25(OH)D levels were not significantly associated with BC risk.</td>
</tr>
<tr>
<td>Study ID</td>
<td>Country</td>
<td>Design</td>
<td>Cases (N = 194)</td>
<td>Controls (N= 194)</td>
<td>Total (40-70y) Mean: ~58.3 y*</td>
<td>25(OH)D: IA; Histologically confirmed primary, incident, BC, with</td>
<td>Cases: 32.7±14.4 ng/mL</td>
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</tr>
<tr>
<td>13 [175]</td>
<td>USA</td>
<td>Case-control study</td>
<td>Cases (N = 194)</td>
<td>Controls (N= 194)</td>
<td>25(OH)D: IA; Histologically confirmed primary, incident, BC, with</td>
<td>Cases: 32.7±14.4 ng/mL</td>
<td>Controls: 37.4±15.9 ng/mL</td>
</tr>
</tbody>
</table>

Cases (N = 613) Controls (N= 1,218) post-menopausal (58.68 ±7.46) naires; the National Death Index was searched for non-responders. All BC cases occurred after blood collection but before 1 June 2007.

family history of BC, and history of benign breast disease.

and BC risk (top vs. bottom quartile multivariate RR = 1.20, 95% CI (0.88 to 1.63), P-value, test for trend = 0.32)
no prior cancer history except nonmelanoma skin cancer.

ng/mL vs. CF: 37.4 ng/mL; *P = .02*

like) and worse prognostic indicators had lower mean 25(OH)D levels

<table>
<thead>
<tr>
<th></th>
<th>USA, Sweden</th>
<th>Nested Case-control From two cohorts: New York University Women’s Health Study and the Northern Sweden Mammary Screening Cohort</th>
<th>Total 34-65 y Mean: ~52.6 y*</th>
<th>25(OH)D: IA; For the NYUWH S, incident cases of invasive BC were identified by mailed questionnaires or follow-up</th>
<th>Cases: 53.0±14.9 nmol/L</th>
<th>n/a</th>
<th>n/a</th>
<th>Age at menarche, age at first birth/parity, family history of BC, BMI, past HRT use, and alcohol consumption.</th>
<th>No association was observed between circulating levels of 25(OH)D and overall BC risk (multivariate-adjusted model OR = 0.94; 95% CI: 0.76-1.16)</th>
<th>Circulating 25(OH)D levels were not associated with BC risk overall</th>
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<tr>
<td>14</td>
<td>[182]</td>
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</table>

QS=0+2+2+2=6 (Range:0-8)
Cases (N = 1,585) Controls (N = 2,940) telephone interviews every 2 to 4 years after 1991, supplemented by linkages to state cancer registries in New York, New Jersey, Florida and the US National Death Index. Medical records were reviewed for the highest vs. lowest quintile. *P* trend = 0.30
confirm self-reported cases. Using a capture-recapture analysis, we estimated that combining active and cancer registry-based follow-up resulted in a BC ascertainment rate of 95%. For the NSMSC, annual linkages to the Swedish National
Cancer Registry were used to identify incident cases of BC in the cohort.

<table>
<thead>
<tr>
<th>QS=0+0+1+2=3(Range:0-8)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>15</strong> Saudi Arabia Case-control study</td>
</tr>
</tbody>
</table>
medical record review to be free of cancer.

<table>
<thead>
<tr>
<th>N</th>
<th>Iran</th>
<th>Population based case-control study</th>
<th>Cases Pre 34-36 y Mean: ~35y**</th>
<th>25(OH)D: 1A Daily intake of calcium and</th>
<th>Cases: 15.2±8.2 ng/mL Control: n/a</th>
<th>Vitamin D supplement, % yes: Cases: 0.0%</th>
<th>Daily sunlight exposure, covering body against sunlight, calcium</th>
<th>The lack of vitamin D and calcium supplementation increased</th>
<th>Vitamin D may have a role in BC incidence but it needs</th>
</tr>
</thead>
<tbody>
<tr>
<td>16</td>
<td></td>
<td>[179]</td>
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QS=0+0+1+2=3 (Range: 0-8)

6.1 (2.4, 15.1) for women with a serum 25(OH)D concentration, 10 ng/mL and 4.0 (1.6, 10.4) for women with a serum concentration of 10 - 20 ng/mL (P-trend = 0.0001)
| (N=60) and controls (N=116) | vitamin D and all dietary resources of mentioned factors were collected. We selected cases from patients who underwent surgery from 2010 to 2012 in Emdad Shahid Beheshti University hospital. Cases were | 15.5±7.5 ng/mL Overall: 15.4 ng/mL* | Controls: 9.7% supplement s, vitamin D supplement s, fish and egg intakes and weekly profile of egg consumption. slightly the risk of premenopausal BC (p=0.009 , OR=1.115, CI 95%=1.049-1.187) | further proof |
identified from both self-reports registration and confirmed by pathological reports. The pathological feature of cases was collected from pathological reports in the pathology archive of the mentioned hospital.

\[ QS=0+1+2+2=5 \text{(Range:0-8)} \]
<table>
<thead>
<tr>
<th>17</th>
<th>USA</th>
<th>Nested case-control study within the Multiethnic Cohort Study</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Cases: N=707</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Controls: N=707</td>
</tr>
<tr>
<td>25(OH)D</td>
<td>Post Mean: ~67.8 y**</td>
<td></td>
</tr>
<tr>
<td>LC</td>
<td>Incident invasive BC cases were identified by linkage to the Surveillance, Epidemiology, and End Results Program registries in the states of Hawaii and California through October, 2010, including 729</td>
<td></td>
</tr>
<tr>
<td>Overall:</td>
<td>Mean 25(OH)D: 31.4 ng/mL*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Vitamin D deficiency (&lt;16 ng/mL): 7.2%*</td>
<td></td>
</tr>
</tbody>
</table>

**Vitamin D deficiency (<16 ng/mL): 7.2%**

Overall: Mean 25(OH)D: 31.4 ng/mL*

Post Mean: ~67.8 y**

Body mass index, parity, family history of BC, use of multivitamin and calcium supplement s, season, sunburn history and engagement in strenuous sports.

20 ng/mL increases of plasma 25(OH)D3 [OR=0.28; 95% CI: 0.14-0.56] and 25(OH)D [OR=0.43; 95% CI: 0.23-0.80] were inversely associated with BC risk among white women, but not among women in other racial/ethnic groups.

Circulating 25(OH)D3 and 25(OH)D were associated with a reduced risk of postmenopausal BC among whites, but not in other ethnic groups, who reside in low latitude regions.
<table>
<thead>
<tr>
<th></th>
<th>USA</th>
<th>Nurses’ Health Study II</th>
<th>Pre 45-46 y</th>
<th>25(OH)D</th>
<th>Median 25(OH)D</th>
<th>n/a</th>
<th>n/a</th>
<th>Body mass index (BMI) at age 18 and at blood collection, age at menarche, parity and age at first birth, history of benign breast disease, family history of BC, and alcohol consumption.</th>
<th>No association between plasma calculated free 25(OH)D and risk of BC overall (highest vs. lowest quartile RR=1.21; 95% CI: 0.83–1.77), P-trend = 0.50</th>
<th>There is no association between circulating free 25(OH)D or circulating VDBP levels with BC risk among mostly pre-menopausal women.</th>
</tr>
</thead>
<tbody>
<tr>
<td>18</td>
<td>[183]</td>
<td>Nested case-control within a cohort.</td>
<td>Mean: ~45.1±4.4 y**</td>
<td>BC cases were identified through the biennial questionnaires: Cases had no previousely reported cancer diagnosis before blood</td>
<td>Cases: 26.2 nmol/L</td>
<td>Control: 61.4 nmol/L</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cases (N = 584) Controls (N= 584)</td>
<td></td>
<td>25(OH)D :</td>
<td>Cases:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

QS=0+1+1+2=4(Range:0-8)
Collectio
n and were diagnose
d after blood collectio
n but before June 1, 2007.

**Abbreviations:** 95%CI=95% confidence interval; 25(OH)D=25-hydroxyvitamin D; BC=BC; BMI=Body Mass Index; HR=Hazard Ratio; HRT=Hormone Replacement Therapy; IA=Immmoassay; LC=Liquid Chromatography; n/a=Not applicable; NDI=National Death Index; OR=Odds Ratio; P-trend=P-value for the trend test; QS=Quality Score; RR=Risk Ratio;

*Estimated based on available categorical data and ranges within each category, with sample sizes for each category.
**Estimated based on available data on mean and SD for cases and controls and sample sizes of cases and controls.
APPENDIX C
SEARCH TERMS

APPENDIX D
SUPPLEMENTAL METHODS

Quality score

The quality score is composed of 5 items, and each item was allocated 0, 1 or 2 points. This allowed a total score between 0 and 8 points, 8 representing the highest quality. The following items are included in the score:

1. Study design
   0 for case-control studies
   1 for cross-sectional studies
   2 for longitudinal studies (retrospective or prospective) or non-randomized intervention studies

2. Population
   Observational studies
   0 if n <500
   1 if n 500 to 2000
   2 if n >2000

3. Outcome (see table below)

<table>
<thead>
<tr>
<th></th>
<th>0</th>
<th>1</th>
<th>2</th>
</tr>
</thead>
<tbody>
<tr>
<td>BC</td>
<td>If not reported or if diagnosis was based on a proxy for disease (i.e. use of medication that is not specific to the disease or symptoms)</td>
<td>If diagnosis was self-reported or retrieved from reports (e.g. medical records) not collected specifically for study or was based on the use of disease-specific medication (e.g. antidiabetic medication)</td>
<td>If diagnosis was made during study using adequate test or official registries were checked by a researcher</td>
</tr>
</tbody>
</table>

4. Adjustments
   0 if findings are not controlled** for at least age and gender
   1 if findings are controlled for age and gender
   2 if an intervention study is adequately randomized or if findings are additionally controlled for other key covariates (e.g. age at menarche, parity, family history of BC etc.)
**‘Controlled for’ here refers to: adjusted for in the statistical analyses (e.g. with multiple regression); stratified for in the analyses (e.g. pre- and post-menopausal separately); or narrow selection criteria of study participants on this covariate (e.g. Pre-menopausal women only).**
<table>
<thead>
<tr>
<th>Meta-analyzed study</th>
<th>Author/year</th>
<th>Assessment</th>
<th>Study design</th>
<th>Population</th>
<th>Outcome</th>
<th>Adjustments</th>
<th>QS</th>
</tr>
</thead>
<tbody>
<tr>
<td>[129]</td>
<td>Bertone-Johnson/2005</td>
<td>A</td>
<td>B</td>
<td>C</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>[185]</td>
<td>McCullough/2005</td>
<td>A</td>
<td>B</td>
<td>C</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>[177]</td>
<td>Knight/2007</td>
<td>A</td>
<td>B</td>
<td>C</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>[184]</td>
<td>Robien/2007</td>
<td>A</td>
<td>B</td>
<td>C</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>[127]</td>
<td>Chlebowski/2008</td>
<td>A</td>
<td>B</td>
<td>C</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>[128]</td>
<td>Freedman/2008</td>
<td>A</td>
<td>B</td>
<td>C</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>[178]</td>
<td>Abbas/2009</td>
<td>A</td>
<td>B</td>
<td>C</td>
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<tr>
<td>Reference</td>
<td>Author/Year</td>
<td>A</td>
<td>B</td>
<td>C</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>[180]</td>
<td>Agborsangaya/2010</td>
<td>A</td>
<td>B</td>
<td>C</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>[121]</td>
<td>Engel/2010</td>
<td>A</td>
<td>B</td>
<td>C</td>
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<td></td>
<td></td>
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<tr>
<td>[181]</td>
<td>Eliassen/2011</td>
<td>A</td>
<td>B</td>
<td>C</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>[175]</td>
<td>Peppone/2012</td>
<td>A</td>
<td>B</td>
<td>C</td>
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<td></td>
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<tr>
<td>[182]</td>
<td>Scarmo/2013</td>
<td>A</td>
<td>B</td>
<td>C</td>
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<td></td>
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<tr>
<td>[176]</td>
<td>Yousefi/2013</td>
<td>A</td>
<td>B</td>
<td>C</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>[179]</td>
<td>Bidgoli/2014</td>
<td>A</td>
<td>B</td>
<td>C</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>[118]</td>
<td>Kim/2014</td>
<td>A</td>
<td>B</td>
<td>C</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
APPENDIX E
BEGG’S FUNNEL PLOT

Begg’s Test
adj. Kendall’s Score (P-Q) = 35
Std. Dev. of Score = 35.46
Number of Studies = 22
z = 0.99
Pr > z = 0.324
z = 0.96 (continuity corrected)
Pr > z = 0.338 (continuity corrected)

Egger’s test

<table>
<thead>
<tr>
<th>Std_Eff</th>
<th>Coef.</th>
<th>Std. Err.</th>
<th>t</th>
<th>P&gt;t</th>
<th>[95% Conf. Interval]</th>
</tr>
</thead>
<tbody>
<tr>
<td>slope</td>
<td>-.0223186</td>
<td>.0158607</td>
<td>-1.41</td>
<td>0.175</td>
<td>-.0554034 .0107662</td>
</tr>
<tr>
<td>bias</td>
<td>.6823854</td>
<td>.8081164</td>
<td>0.84</td>
<td>0.408</td>
<td>-1.003316 2.368087</td>
</tr>
</tbody>
</table>

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APPENDIX F
QUALITATIVE FINDINGS

FIGURE: GRAPHS BY EXPOSURE AND FINDINGS
## APPENDIX G
### CALCULATION OF TREATMENTS

<table>
<thead>
<tr>
<th></th>
<th>Vitamin D (Enzo: BML-DM200-0050)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Stock Solution</strong></td>
<td>50 µg in 50 µL EtOH</td>
</tr>
<tr>
<td></td>
<td>= 2.4 mM (2.4X10^-3 M)</td>
</tr>
<tr>
<td><strong>Working Solution</strong></td>
<td>Dilute to 1:1000</td>
</tr>
<tr>
<td></td>
<td>2µL stock solution in 2000 µL (2mL) EtOH</td>
</tr>
<tr>
<td></td>
<td>= 2.4 µM (2.4X10^-6 M)</td>
</tr>
<tr>
<td><strong>Treatment Solution</strong></td>
<td>Dilute to 42 µL working solution in 13mL media</td>
</tr>
<tr>
<td></td>
<td>= 100 nM</td>
</tr>
</tbody>
</table>

|                          | Trichostatin A (TSA) (Sigma: T1952)                                                                                   |
| **Stock Solution**       | 10 mM Concentration                                                                                                   |
| **Working Solution**     | Dilute to 1:1000                                                                                                       |
|                          | 2 µL Stock Solution in 2mL DMSO                                                                                        |
|                          | = 10 µM                                                                                                                |
| **Treatment Solution**   | 1 µM = 200 µL working solution in 2mL media                                                                             |

|                          | L- Sulforaphane (SFN) (Sigma: S6317)                                                                                   |
| **Stock Solution**       | 20 mM in DMSO                                                                                                          |
| **Working Solution**     | 17.5 µL Stock Solution in 3.5 mL DMSO                                                                                  |
|                          | = 100 µM concentration                                                                                                 |
| **Treatment Solution**   | 20µM= 400µL working solution in 2mL media                                                                               |
APPENDIX H
LABORATORY PROTOCOL

RNA Isolation

1. Cell Harvest
   a. Remove the media
   b. Use 1mL Tri-reagent (Trizol) per well (6-well plates)
   c. Scratch the cells from the well with a cell scraper
   d. Pipet suspension till thick and stringy, transfer to 1.5 mL Eppendorf tubes
   e. Samples can be stored at -80°C if necessary
   f. If frozen, samples need to be kept at room temperature for 5 minutes before starting the phase separation

2. Phase Separation (samples need to be kept on ice)
   a. Add 150 µL chloroform reagent to the homogenate
   b. Invert vigorously for 15 seconds
   c. Allow the mixture to sit at room temperature for 5 minutes
   d. Centrifuge at 12,000g for 15 minutes at 4°C

3. RNA Precipitation
   a. Transfer the aqueous phase into a fresh 1.5 mL Eppendorf tubes
   b. Add 0.5 mL of 100% isopropanol and mix gently by inversion
   c. Keep on ice for 15 minutes
   d. Centrifuge at 12,000g for 20 min at 4°C

4. RNA Washing and Solubilization
   a. Remove the supernatant (be careful not to disturb the pellet)
   b. Add 1 mL of 75% Ethanol (EtOH)
   c. Kick samples at the bottom to dislodge the pellet
   d. Centrifuge at 7,500g for 5 minutes at
   e. Remove EtOH (careful not to lose the pellet at the bottom)
   f. Briefly spin samples down, remove the remaining liquid
   g. Air dry (keep samples open for several minutes)
   h. Add 40 µL DEPC water to the samples, invert and spin down
   i. Incubate 10 minutes at 56°C to homogenize the RNA and then keep on ice for several minutes

B. RNA Quantification
   a. Turn on the NanoDrop machine
   b. Blank the machine with 1 µL of DEPC water
   c. Take 1 µL of sample
   d. Press OK to get readings at 260/280 nm
   e. Dilute samples as necessary (we need at least 2µg RNA for PCR)
C. cDNA Synthesis
   a. The following protocol is designed to convert 5µg of total RNA into cDNA.
   b. Mix and briefly centrifuge each component before use.
   f. Combine the following in 0.5 mL DNA/RNAse free tubes (final volume 10 µL):
      - 1 µL Primer: 50 mM oligo (DT)
      - 1 µL 10 mM dNTP mix
      - 8 µL of 5 µg RNA and DEPC water
      - Incubate for 5 minutes at 65°C then place on ice for at least 1 minute (Protocol Library: Wood Lab)
   g. Prepare the following mix (for one reaction):
      - 2 µL 10X RT buffer
      - 4 µL 25 mM MgCl₂
      - 2 µL 0.1 M DTT
      - 1 µL RNaseOUT (40 U/µL)
      - 1 µL SuperScript III- RT (200 U/µL)
   h. Add 10 µL of cDNA Synthesis mix to each RNA/primer mixture (mix gently and collect by brief centrifugation)
   i. Incubate as follows:
      - 10 minutes at 25°C followed by
      - 50 minutes at 50°C
      - Terminate the reaction at 85°C for 5 minutes, then chill on ice
   j. cDNA synthesis reactions can be stored at -30°C to -10°C or used for PCR immediately

D. Real Time qPCR

1. cDNA Quantification
   a. Take 1 µL of each sample and measure the concentrations by using the NanoDrop machine.
   b. Total Volume = 1 µL cDNA and DEPC water to make each sample have 50 µg/mL cDNA.

2. Preparation of MasterMix (each tube will have 20 µL of final volume)
   a. 10 µL TaqMan Gene Expression Master Mix (Applied Biosystems #4369016)
   b. 1 µL TaqMan Gene Expression Assay
      - Housekeeping gene (GAPDH) with VIC dye- Applied Biosystems Hs 99999905_m1
   c. 1 µL TaqMan Gene Expression Assay
**Target gene (VDR) with FAM dye - Applied Biosystems Hs 99999905_m1**

**Target gene (TRPV6) with FAM dye - Applied Biosystems Hs 01114089_g1**

**Target gene (DKK1) with FAM dye - Applied Biosystems Hs _m1**

**Target gene (CYP24a1) with FAM dye - Applied Biosystems Hs 00167999_m1**

**Target gene (CYP27b1) with FAM dye - Applied Biosystems Hs 00168017_m1**

d. 4 µL of DEPC water
e. 4 µL cDNA (200ng/ mL total cDNA)

**Formula for the amount pipetted into each tube:**

\((\# \text{ of reactions}) \times (\text{Volume of reagent per reaction}) + (\# \text{ of reactions}) \times (\text{Volume of reagent per reaction}) \times (.10) = \text{Amount in the MasterMix}\)

[Note: 10% more is added because the mixture is sticky and pipetting can be difficult]

**3. Preparation of qPCR plate**

a. Pipette 16 µL of prepared MasterMix into each micro centrifuge tube (Applied Biosystems # 4346906)
b. Pipette 4 µL of cDNA samples into each reaction tube (run in duplicates)
c. Homogenize samples by spinning the tubes down

**4. ViiA-7 Real-Time PCR System**

a. Turn on the machine
b. Place the plate holding all reaction tubes into the plate reader
c. Click on ViiA-7 software on Desktop
   
   - Give the new experiment a name
   - Set up the reaction plate: 96 well
   - Method of comparison: standard curve
   - Protocol: TaqMan Standard MasterMix
d. Setting up the experiment
   
   - Define target genes (choose different colors for each gene)
   - Select VIC dye for GAPDH and FAM for all the target genes
   - Mark concentrations for each treatment
   - Assign Target and samples: mark the boxes with appropriate labels and concentrations
   - Select ROX dye for passive reference
e. Select Start and then click on the experiment number to start the experiment (Note: clicking on Start alone will not initiate the experiment)
f. The experiment will be completed in 1.45 hours for the data to be exported to an excel file for analysis.


67. Pradhan AD, Manson JE: Update on the vitamin D and omega-3 trial (VITAL)(). The Journal of steroid biochemistry and molecular biology 2016, 155:252-256.


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