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THE IMPACT OF VITAMIN D ON THE EPIDEMIOLOGY, PATHOGENESIS, AND TREATMENT OF ESOPHAGEAL ADENOCARCINOMA

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A THESIS

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ABSTRACT

Esophageal adenocarcinoma is now the most common form of esophageal cancer, and the prognosis for patients with this disease is poor. Understanding the mechanisms of disease development through premalignant Barrett’s esophagus, a consequence of GERD, and factors predicting a positive response to treatment is necessary to improve outcomes. Vitamin D is a hormone that acts as an immunomodulator and regulator of cellular proliferation with anticancer properties, and has been implicated in the prevention of some cancers. Its main source in light skinned individuals is the sun, and there is an epidemiological association between increased exposure to ultraviolet irradiation and decreased incidence of esophageal adenocarcinoma. Refluxed acid and bile create an inflammatory milieu that in part may be mediated by 1,25(OH)\(_2\)D by interacting with cyclooxygenase pathways, mitigating DNA damage, playing a role in bile acid metabolism, and influencing the immune cell population. 1,25-dihydroxyvitamin D may inhibit the Th1 response associated with reflux esophagitis while fostering the Th2 response associated with Barrett’s esophagus. Other immune cells including macrophages, dendritic cells, Th17 cells, and Tregs may be influential to the development or prevention of EAC, but their specific roles and interaction with 1,25(OH)\(_2\)D is undefined. Hedgehog and NF-κB signaling are increased in BE and EAC, and although some interaction with 1,25(OH)\(_2\)D may be present, it is difficult to credit this with a significant implication. The effects of 1,25(OH)\(_2\)D are mediated by VDR. Vitamin D receptor is expressed in normal gastric cardia, Barrett’s esophagus, and esophageal adenocarcinoma, but absent in normal esophageal squamous mucosa. Staining intensity of VDR assessed by immunofluorescence correlates inversely with
histologic grade, and staining is more intense in tissue that did not respond to neoadjuvant treatment, although no statistically significant correlations were found. Considering this, it is plausible that the extent of VDR expression is related to tumor progression and response to therapy, but it is equally as plausible that these data are merely random.
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1,25(OH)\(_2\)D 1,25-dihydroxyvitamin D, a.k.a calcitriol
25(OH)D 25-hydroxyvitamin D, a.k.a calcidiol
BE Barrett’s esophagus
CI confidence interval
COX-2 cyclooxygenase-2
CT computed tomography
CTL cytotoxic T lymphocyte
CYP27B1 1α-hydroxylase
EAC esophageal adenocarcinoma
EGD esophagogastroduodenoscopy
EGJAC esophagogastrectomy junction adenocarcinoma
ESCC esophageal squamous cell carcinoma
EUS endoscopic ultrasound
FFQ food frequency questionnaire
GC gastric cancer
GCA gastric cardia adenocarcinoma
GNCA gastric non-cardia adenocarcinoma
GERD gastroesophageal reflux disease
LCA lithocholic acid
NO nitric oxide
NSAIDs non-steroidal anti-inflammatory drugs
OR odds ratio
PET positron emission tomography
ROS reactive oxygen species
RR relative risk
RXR retinoid X receptor
SD standard deviation
Tregs regulatory T cells
UV ultraviolet
VDR vitamin D receptor
INTRODUCTION

Esophageal adenocarcinoma (EAC) is a cancer with a rapidly rising incidence and poor prognosis. It shares a significant association with gastroesophageal reflux disease (GERD) and the subsequent development of Barrett’s esophagus (BE), but how chronic reflux causes metaplastic and neoplastic change is unknown. Vitamin D has received considerable attention as an anticancer agent, primarily as consequence of epidemiologic evidence that suggests a protective effect of vitamin D on the development of breast and colon cancer, but its role in cancers of the upper gastrointestinal tract and in particular the esophagus is far less established. Presumably, the anticancer effects of vitamin D come from its role in immunomodulation and its impact on cellular proliferation, but specific mechanisms are elusive. Understanding the function of vitamin D in the setting of esophageal adenocarcinoma may provide important information pertaining to the prevention and treatment of this cancer.

The incidence of esophageal adenocarcinoma is rising [1, 2], from 3.6 per million in 1973 to 25.6 per million in 2006 [1-3], and accounting for 59% of all histologically confirmed carcinomas of the esophagus from 2004 to 2008 [4]. The vast majority of cases of esophageal adenocarcinoma arise from preceding Barrett’s esophagus [5], a condition defined by the American Gastroenterological Association “in which any extent of metaplastic columnar epithelium that predisposes to cancer development replaces the stratified squamous epithelium that normally lines the distal esophagus” [6]. A background of chronic inflammation concomitant with gastroesophageal reflux disease predisposes to Barrett’s esophagus [5], and promotes an environment rich with factors
that damage tissue, trigger genetic alterations, incite reactive changes in the immune cell population, and potentiate further inflammation. However, the typical symptoms of gastroesophageal reflux disease – epigastric burning, regurgitation, and belching [7] – may be absent in a large portion of patients with Barrett’s esophagus [8], complicating its identification.

The prognosis for patients with esophageal adenocarcinoma remains poor, with a 5-year relative survival less than 20% for cancers of all stages [4]. The prognosis for patients with resectable disease – stages I, II, and III – undergoing surgery is better, but marginally, with reports of 5-year overall survival between 25% and 39% depending on surgical modality and use of adjuvant therapies [9-12]. The use of neoadjuvant therapy – chemotherapy, radiation therapy, or chemoradiation therapy prior to surgery – may improve 5-year survival up to 59% [13], but the outcomes vary widely and the overall benefit when considering the adverse effects of treatment is controversial [14].

Factors portending poor post-operative survival include incomplete tumor resection [15] and positive lymph nodes [16]. In contrast, factors predicting a positive post-operative prognosis include a complete or major response to neoadjuvant treatment [16] and complete primary tumor resection (R0 resection) [17]. A univariate predictor of positive post-operative prognosis, T stage classification is inversely related to the response to neoadjuvant treatment [16] – patients with lower T stages are more likely to respond to neoadjuvant therapy and perform better post-operatively. Patients responsive to neoadjuvant treatment are also less likely to have metastatic lymph nodes during
pathologic staging [16], and are more likely to attain complete primary tumor resection, have improved 5-year survival, and fewer disease recurrences [18, 19], making the pathologic response to neoadjuvant therapy a key prognostic indicator in patients with adenocarcinoma of the esophagus.

Predicting a response to neoadjuvant treatment is difficult, however. Reports have demonstrated that 63-71% of patients with locally advanced esophageal adenocarcinoma undergoing neoadjuvant chemoradiation therapy show less than 50% histopathologic regression [20, 21]. Considering histopathologic tumor regression is the most significant independent prognostic indicator [22], identifying tumor characteristics and biomarkers that presage responsiveness to neoadjuvant treatment would certainly be a valuable clinical tool.

Vitamin D is a hormone the most well known function of which is to control bone mineralization and calcium homeostasis in higher organisms, but its actions as an immunomodulator and regulator of cellular proliferation with anticancer properties have also gained attention [23]. Approximately 80–90% of vitamin D is synthesized cutaneously as a result of exposure to ultraviolet (UV) rays, although this varies considerably with complexion [24]. Its circulating form, 25-hydroxyvitamin D (25(OH)D), is measured in the serum and is considered the most reliable way of estimating the vitamin D status of a patient.
Epidemiological observations insinuate a connection between vitamin D status and cancers of the breast and colon [25], and preclinical data support the hypotheses that vitamin D can limit proliferation of breast [26, 27], colon [28-33], and prostate [34] cancer cells, although there are limited clinical data supporting the preclinical findings. A potential role of vitamin D in cancer prevention and treatment is derived from its ability to regulate genes involved in the cell cycle, apoptosis, and angiogenesis, and to modulate immune function by influencing the transcription of enzymes involved in the synthesis of inflammatory mediators and cytokines of the T cell subset, macrophages, and dendritic cells [35].

The role of vitamin D status in cancer epidemiology is currently a heavily researched topic, and equally as heavily debated. The association between low serum 25(OH)D levels and risk of breast [36, 37] and colon [37, 38] cancers is well accepted, and a similar association with bladder cancer has also been purported [39]. The association of vitamin D status with cancer of the prostate [37] and skin [40] is less clear, but increasing serum 25(OH)D levels may be associated with an increased risk of prostate cancer [41, 42] and are associated with increased risk of melanoma and non-melanoma skin cancer [40, 43, 44]. However, with respect to melanoma this may be confounded by the carcinogenic affects of UV irradiation on the skin. In fact, in those with a diagnosis of malignant melanoma, higher serum 25(OH)D levels have been associated with less advanced tumor stage and decreased tumor depth [45]. In addition, some have suggested that there may be a role for vitamin D in controlling the progression of cutaneous malignancies [46], highlighting some of the equipoise that exists regarding the impact of vitamin D on
cancer. A suggested link between UV exposure and reduced risk of cancer has been proposed on the basis of ecologic evidence [47], but prospective analyses have yet to uncover a consistent relationship between vitamin D status and cancer mortality in general [48]. This is especially true for rarer cancers including esophageal and upper gastrointestinal cancers for which even retrospective studies have established no consistent associations [49], although no adequate summary of the epidemiologic evidence exists.

The term “vitamin D” is often used generically to describe a number of forms and precursors of the active molecule. The plant-derived vitamin D$_2$ (ergocalciferol) and animal-derived vitamin D$_3$ (cholecalciferol) are the fat-soluble forms obtained in the diet [50]. However, unsupplemented dietary vitamin D is generally insufficient in meeting human needs [51]. Vitamin D$_3$ is also synthesized photochemically in the skin. Neither vitamin D$_2$ nor D$_3$ are physiologically active forms, and further hydroxylation in the kidney and liver is required.

Ultraviolet irradiation converts cutaneous 7-dehydrocholesterol to vitamin D$_3$, which then translocates to the blood along with dietary vitamin D$_2$ and D$_3$ from the lymphatics. In the blood, vitamin D travels bound to lipoproteins or vitamin D binding protein to the liver where it undergoes its first hydroxylation to form 25-hydroxyvitamin D (25(OH)D or “calcidiol”). Still not active, 25-hydroxyvitamin D undergoes one more hydroxylation in the kidney by the crucial enzyme 1α-hydroxylase (a.k.a. CYP27B1)
forming 1,25-dihydroxyvitamin D (1,25(OH)$_2$D or “calcitriol”), the physiologically active form.

1,25-dihydroxyvitamin D exerts its best-characterized effects by binding to the cytoplasmic vitamin D receptor (VDR) promoting heterodimerization with the retinoid X receptor (RXR) and nuclear localization, where it acts as a transcription factor [52]. A cell’s responsiveness to 1,25-dihydroxyvitamin D is purported to be related to the level of VDR in that cell, and 1,25-dihydroxyvitamin D upregulates VDR levels in a dose-dependent manner [53]. The vitamin D receptor is found in almost every tissue and cell in the body including parathyroid, pancreatic B cells, thyroid C cells, arterial smooth muscle cells, cardiac myocytes, osteoblasts, chondrocytes, striated muscle, esophagus, stomach, intestine, liver parenchymal cells, kidney nephron tubules, juxtaglomerular apparatus, podocytes, testis, ovary, uterus, T and B cells, bone marrow, thymus, lung alveolar cells, keratinocytes, hair follicles, and brain neurons [54, 55]. Furthermore, CYP27B1 is also found extrarenally in a number of tissues [56], in macrophages [57], dendritic cells [58], and T cells [59], and is regulated via different mechanisms than the renal enzyme [60]. This extrarenal analog of CYP27B1 may operate in a more substrate-dependent manner that the form found in the kidney [54].

In the mouse, VDR is expressed in some of its largest quantities throughout the digestive tract including the duodenum, jejunum, ileum, and colon [61, 62]. In the rat intestine, VDR is involved in the regulation of a large number of genes involved in calcium homeostasis and intestinal absorption, but also intra- and intercellular matrix
modeling, immune responses, inflammatory processes, angiogenesis, and genes for
proteases, enzymes, and their inhibitors [63]. In fact, endothelial cell tumors in VDR
knockout mice grow larger than in wild type mice, and wild type mice are amenable to
calcitriol-mediated tumor inhibition whereas VDR knockout mice are not [64]. In
epithelial cells, VDR and its ligand, 1,25-dihydroxyvitamin D, contribute to the
maintenance of the differentiated phenotype and promote pathways that defend cells
against risk for carcinogenic conversion due to their anti-proliferative, pro-differentiation,
pro-apoptotic, and anti-metastatic activities [65, 66].

The vitamin D receptor was isolated from the human intestine in 1987 [67]. The
possible anticancer role of 1,25-dihydroxyvitamin D and its signaling pathways [65] in the
gastrointestinal tract is evidenced by the association of malignancy in the human colon
with the loss of VDR activity [68], and a correlation between a single nucleotide
polymorphism in the VDR gene and the risk for colon cancer in human subjects [69].
However, currently the only evidence of VDR expression in the esophagus was
demonstrated by the detection of VDR mRNA using PCR [70]. 1,25-dihydroxyvitamin D
and VDR may regulate mechanisms that contribute to an environment associated with
metaplastic and neoplastic change in the esophagus, but its expression and physiological
role at this location has not been characterized.

Despite the rising rate of esophageal adenocarcinoma, evidence of a potential
epidemiological role of vitamin D status in other cancers (breast, colon, prostate, skin,
bladder), and the importance of a response to neoadjuvant treatment for prognosis of
patients with this disease, little is known about the relationship between vitamin D and the development and treatment of esophageal adenocarcinoma. Furthermore, the prevalence of vitamin D deficiency in U.S. adults has been estimated to be 41.6% [71]. If vitamin D deficiency is related to esophageal adenocarcinogenesis, the intervention to correct this would be a relatively simple and inexpensive way to reduce mortality from this disease. The goal of this thesis is to examine three original and interrelated subjects concerning the topic of esophageal adenocarcinoma and vitamin D, and the ancillary questions concomitant to them. These are, 1) the current evidence regarding the epidemiology of upper gastrointestinal cancers and vitamin D status, 2) the expression of VDR in the context of the histology of the lower esophagus and gastric cardia, including normal esophageal tissue and Barrett’s esophagus, and 3) the impact of VDR expression on the response to neoadjuvant chemoradiation therapy in patients undergoing surgery for esophageal adenocarcinoma. Prior to presenting these subjects, this thesis will explore the cellular and molecular implications of 1,25-dihydroxyvitamin D in the pathogenesis of Barrett’s esophagus and esophageal adenocarcinoma in order to provide a more robust foundation for discussing the goal of this thesis.

The overarching hypothesis is that vitamin D status influences the development of esophageal adenocarcinoma and its response to neoadjuvant treatment. Specifically, higher vitamin D levels decrease the incidence of esophageal adenocarcinoma through an antiproliferative action on the cells lining the distal esophagus, and through attenuation of the inflammatory immune response created by regurgitated stomach and duodenal contents; and, higher vitamin D levels inhibit the response to neoadjuvant therapies by
decreasing the amount of actively dividing cells and, therefore, those most susceptible to chemo and radiation therapy.

**Vitamin D and in the Pathogenesis of BE and EAC**

To explore the role of vitamin D in the pathogenesis of BE and EAC, a PubMed search was conducted using broad terms for publications related to vitamin D and esophageal disease. As expected, very little literature exists on this topic so the search was expanded to include any publications pertaining to vitamin D and cancer pathogenesis, immune function, inflammatory pathways, and other mechanisms of molecular signaling and transcription alteration that could pertain to vitamin D and esophageal disease. When information was lacking, which was frequent, parallels were drawn to other diseases and physiological processes to generate new research hypotheses and future directions. Relevant topics that are included in the subsequent discussion sections include: *Chronic Reflux, Inflammation, and Genetic Alterations, CD4*\(^+\) *Helper T Cells, Th1 Cells, Th2 Cells, Th17 Cells, Regulatory T Cells, Macrophages, Dendritic Cells, Wnt Signaling, Hedgehog Signaling, NF-κB, IL-6 and STAT Signaling.*

**Chronic Reflux, Inflammation, and Genetic Alterations**

The change from normal esophageal mucosa to intestinal metaplasia in Barrett’s esophagus requires alterations in the regulation of gene expression [72]. Refluxed acid and bile initiates an inflammatory process that has the potential to modify genetic phenotypes causing metaplasia or initiating carcinogenesis [73, 74]. Acid [75] and bile [76-78] can cause DNA damage directly or via generation of reactive oxygen species (ROS)
and nitric oxide (NO) (Figure 1) [79]. They may also influence epigenetic changes such as altered DNA methylation, contributing to changes in gene expression [80-82]. Some of the genes impacted by acid and bile reflux include those that influence morphology like the CDX [82-88] and SOX [89-91] families of transcription factors as well as those involved in the cell cycle [92].

Refluxed acid and bile can stimulate esophageal keratinocytes to release proinflammatory molecules including substance P, platelet-activating factor, and IL-8 [73, 93, 94], which recruit neutrophils and other blood leukocytes and stimulate these cells to produce H$_2$O$_2$, NO, ROS [73, 95] and HOCl, a ROS capable of inducing aberrant DNA methylation patterns (Figure 1) [80]. Bile acids are capable of inducing cyclooxygenase-2 (COX-2) [96], a key enzyme that is upregulated in the early stages of inflammation [97], and prostaglandin receptor expression in human esophageal adenocarcinoma cells [98]. A single nucleotide polymorphism in the promoter of COX-2 may increase the risk of developing esophageal adenocarcinoma [99], and there is in vitro and epidemiologic evidence that non-steroidal anti-inflammatory drugs (NSAIDs) are chemopreventive in Barrett’s esophagus and esophageal adenocarcinoma, though these effects may be mediated by mechanisms other than COX-2 inhibition [6].

1,25-dihydroxyvitamin D may alter the progression of metaplasia and neoplasia by mitigating the disease-promoting effects of COX-2 and the genotoxicity of ROS. In prostate cancer cells, 1,25-dihydroxyvitamin D is capable of regulating many enzymes involved in prostaglandin synthesis and signaling. Moreno and colleagues demonstrated
the ability of 1,25-dihydroxyvitamin D to downregulate expression of COX-2 and the prostaglandin receptor, EP2, and to upregulate 15-prostaglandin dehydrogenase, the enzyme responsible for inactivation of prostaglandins [100] (Figure 1). It is possible that these mechanisms are similarly present in the esophagus.

Figure 1. Bile and acid reflux induces keratinocytes to produce chemokines and reactive oxygen species and to upregulate pro-survival genes like COX-2. Chemokines recruit PBLs like neutrophils, monocytes and T cells, which produce additional cytotoxic factors. There is evidence that HOCl produced by neutrophils can induce hypomethylation of various genes. Additionally, hypomethylation of the CDX1 promoter has been shown to upregulate its expression. It is possible that epigenetic changes like this could impact CDX2 and SOX9 expression as well, but the evidence is not overwhelming. 1,25-(OH)2D may limit inflammation by inhibiting COX-2 expression and stimulating prostaglandin degradation; it may also be capable of attenuating ROS-mediated DNA damage. Effects on peripheral blood leukocytes are varied. 1,25-(OH)2D; 1,25-dihydroxyvitamin D; 15-PGDH: 15-prostaglandin dehydrogenase; PAF: Platelet-activating factor; PBL: Peripheral blood leukocyte; PGE2: Prostaglandin E2; ROS: Reactive oxygen species; SP: Substance P.
1,25-dihydroxyvitamin D is also capable of reducing DNA photoproducts in epidermal keratinocytes, even when added after UV irradiation, suggesting a mechanism that is different from the absorption of DNA-damaging UV rays [101]. If this is the case, it is possible that 1,25-dihydroxyvitamin D could mitigate DNA damage as a consequence of gastroesophageal reflux; however, there is no evidence currently supporting this conjecture. Additionally, 1,25-hydroxyvitamin D can reduce the production of NO [101], another possible agent contributing to DNA damage of the esophageal epithelium.

Evidence in mice suggests that 1,25-dihydroxyvitamin D may play a role in the direct metabolism of bile acids. Lithocholic acid (LCA), a toxic secondary bile acid, is partially catabolized by CYP3A [102]. CYP3A transcription can be activated by an LCA-activated transcription factor [96, 102], and also by the VDR-RXR heterodimer [103] (Figure 2). Lithocholic acid is also capable of binding VDR as a ligand and inducing transcription of CYP3A [102]. Even though LCA has not been specifically implicated in the development of Barrett’s esophagus and esophageal adenocarcinoma, in general there is substantial evidence regarding the pathogenic role of bile acids [96]. In fact, 86% of patients with GERD have at least traces of bile acids in their refluxate compared with 58% of control subjects, and the concentration of bile acids in GERD patients is considerably higher than controls [104]. Furthermore, CYP3A is involved in the detoxification of other bile acids and toxic substances [105]. Investigation in VDR knockout mice demonstrated a 30–200% increase in bile acid pools, suggesting
significant dysregulation of bile acid metabolism in the absence of a properly functioning vitamin D signaling pathway [106]. Importantly, this effect may be caused by decreased repression of the bile acid synthesizing enzyme, CYP7A1 [106, 107]. Regulation of bile acid metabolism by VDR was corroborated elsewhere, but attributed to increased urinary excretion and the impact of bile acid transporters [108]. The putative role of 1,25-dihydroxyvitamin D in the regulation of bile acid metabolism insinuates the potential for disease modification, but this is currently speculative and warrants further attention.

Figure 2. Both lithocholic acid and 1,25-dihydroxyvitamin D interact with vitamin D receptor to upregulate transcription of CYP3A, an enzyme involved in bile acid detoxification. Activation of VDR is also implicated in repression of CYP7A1 activity, a rate-limiting enzyme in bile acid synthesis. 1,25-(OH)₂D₃: 1,25-dihydroxyvitamin D; LCA: Lithocholic acid; LCA-TF: Lithocholic acid transcription factor; RXR: Retinoid X receptor; VDR: Vitamin D receptor.
**CD4⁺ Helper T Cells**

The leukocyte population of the esophagus may play a critical role in the progression of disease. The types of immune cells involved and their effector functions depend on the proinflammatory mediators produced in the chronically inflamed tissue. T-helper lymphocytes are major governors of the immune response type, traditionally considered either a type 1 or type 2 response depending on whether it is mediated by Th1 or Th2 cells, respectively. In vivo, however, the response type exists on a spectrum governed by four subsets of CD4⁺ cells: Th1, Th2, regulatory T cells (Tregs), and IL-17-producing Th17 cells. Interestingly, naive (antigen-inexperienced) T cells express very low levels of VDR and have impaired classical T-cell receptor (TCR) signaling. Signaling by an alternative TCR pathway upregulates VDR expression, which subsequently activates genes encoding molecules involved in the classical pathway. The alternative TCR signaling requires initial activation of MAPK p38 resulting in successive induction of VDR together with the activation of PLC-γ1, which are both required for subsequent classical TCR signaling and T-cell activation.

**Th1 Cells**

Th1 cells promote cytolytic activity to effect clearance of intracellular pathogens and tumor cells, and they are capable of causing significant tissue damage. A preponderance of CD8⁺ cytotoxic T lymphocytes (CTLs) and macrophages, effector cells of a Th1 response, has been observed in the reflux esophagitis that precedes and is associated with Barrett’s esophagus (Figure 3).
1,25-dihydroxyvitamin D can inhibit activation of a Th1 immune response by limiting the secretion of type 1 cytokines such as IL-12 from macrophages and IFN-γ from Th1 cells (Table 1 & Figure 4). These two cytokines take part in a positive feedback loop that potentiates a Th1 response, and in which the beneficial effect of 1,25-dihydroxyvitamin D is involved [115] (Figure 4). In a murine model of induced colitis, 1,25-dihydroxyvitamin D is capable of significantly decreasing expression of the Th1 lineage transcription factor, T-bet, a decrease that was concomitant with the attenuation in severity of colitis observed [116] (Table 2 & Figure 4). However, VDR knockout mice also have reduced levels of Th1 cells [117] and there is no change in Th1 cytokine production in the supernatants of T cell cultures obtained from mice treated with 1,25-dihydroxyvitamin D [118]. Therefore, it is unclear if the protective effect of 1,25-dihydroxyvitamin D in colitis is via reduction in Th1 cells and their activity. It would be reasonable to perform similar studies in mouse models of gastroesophageal reflux to determine if 1,25-dihydroxyvitamin D is capable of reducing Th1 counts in reflux esophagitis.

**Th2 Cells**

Th2 cells promote a humoral response against extracellular pathogens, opsonization, and mucosal immunity. In Barrett’s esophagus, the total number of leukocytes is increased compared with reflux esophagitis, with the majority consisting of plasma cells [114] (Figure 3). Recently, Lind and colleagues found comparable
proportions of CD3⁺CD4⁺ and CD4⁺CD103⁺ Th lymphocytes in the tissue of Barrett’s esophagus and duodenum from Barrett’s esophagus patients and control subjects [119].

Figure 3. Effect of 1,25-dihydroxyvitamin D on the function of immune cells involved at various stages in the pathogenesis of esophageal adenocarcinoma.

Different immune cell populations have been observed in the progression from healthy to reflux esophagitis, Barrett’s esophagus and esophageal adenocarcinoma, but it is still unclear whether these immune cells play a causal role or are just associated with the metaplasia–dysplasia–adenocarcinoma sequence in the esophagus. 1,25-(OH)₂D₃ may regulate the differentiation and activity of these cells. In this figure, the relationship between 1,25-(OH)₂D₃ and the immune cells involved in esophageal disease is tenuous. 1,25-(OH)₂D₃: 1,25-dihydroxyvitamin D; DC: Dendritic cell; M1: Classically-activated macrophage; M2: Alternatively-activated macrophage.

Since the specialized intestinal epithelium in Barrett’s esophagus is largely similar to duodenal epithelium, these findings suggest that the T lymphocytes in Barrett’s esophagus are not due to active inflammation as suggested earlier [114], but could be due to metaplastic intestinal type changes in Barrett’s esophagus. These data suggest that reflux esophagitis is associated with a Th1-mediated environment, whereas a Th2-mediated environment characterizes Barrett’s esophagus. However, it would be misleading to characterize the Barrett’s esophagus response as simply Th2 because Th1 effector cells, CTLs and macrophages, exist in large numbers in Barrett’s esophagus despite being present in significantly decreased proportions compared with reflux esophagitis [114]. In fact, although the absolute number of Th1 effector cells does
attenuate slightly in Barrett’s esophagus compared with reflux esophagitis, the real contrast is the increased number of plasma cells that appear in Barrett’s esophagus [114].

A mouse model of reflux esophagitis demonstrated significantly increased expression of Th2 cytokines IL-4, IL-10, and IL-13 in mice with Barrett’s esophagus compared to non-Barrett’s epithelium [120]. This study implicated IL-4 as a cytokine with a crucial role in the expression of CDX2 (discussed under results section titled, “Molecular Signaling Mechanisms and Transcription Alteration”) and the early development of Barrett’s esophagus [120]. Furthermore, it appears that active inflammation does not seem to predominate in Barrett’s esophagus compared with reflux esophagitis, and Barrett’s esophagus tissue most often shows no histological signs of inflammation in concordance with significantly decreased proinflammatory cytokines – IL-1, IL-8 and IFN-γ – compared with reflux esophagitis [121].

The impact of 1,25-dihydroxyvitamin D on the Th2 population is equivocal. There is no change in Th2 cytokine production in the supernatants of T-cell cultures obtained from mice treated with 1,25-dihydroxyvitamin D [118]. This is supported by an

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Levels in reflux esophagitis</th>
<th>Levels in Barrett’s Esophagus</th>
<th>Levels in esophageal adenocarcinoma</th>
<th>Impact of 1,25-dihydroxyvitamin D</th>
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<tr>
<td>IFN-γ</td>
<td>+++</td>
<td>+</td>
<td>?</td>
<td>↓</td>
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<tr>
<td>IL-6</td>
<td>++</td>
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<td>IL-4</td>
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<td>TGF-β</td>
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↑: 1,25-dihydroxyvitamin D increases cytokine levels; ↓: 1,25-dihydroxyvitamin D decreases cytokine levels; +: Mild increase; ++: Moderate increase; +++: Significant increase.
earlier report that 1,25-dihydroxyvitamin D has little impact on Th2 cytokines [115] but it is capable of attenuating IL-4 production if T cells are exposed to 1,25-dihydroxyvitamin D when still in a naive state [122]. This perceived effect of 1,25-dihydroxyvitamin D on IL-4 production may be mischaracterized considering reports of little VDR expression in naive T cells [113].

In contrast, Daniel and colleagues, using human peripheral blood mononuclear cells, demonstrated the ability of 1,25-dihydroxyvitamin D to increase production of Th2 cytokines, IL-4 and IL-10 (Table 2 & Figure 4), while also inhibiting production of TNF-α, IFN-γ and IL-1β, though it was not established whether this was a direct transcriptional effect downstream after activation of VDR or a consequence of Th1 inhibition [123]. In a murine model of induced colitis, 1,25-dihydroxyvitamin D is capable of increasing the Th2 lineage factor, GATA-3 [116] (Table 2 & Figure 4). Further support from another study reported that 1,25-dihydroxyvitamin D promoted differentiation of naive T lymphocytes into a Th2 phenotype by increasing the GATA-3 transcription factor and promoting IL-4 production [124]. The variability in these results insinuates the intricacy of 1,25-dihydroxyvitamin D action in vivo. Additionally, because of the many discrepancies between Barrett’s pathogenesis and a chemically induced colitis model, conclusions are tenuous. However, since Barrett’s pathogenesis can also be viewed as a chemically induced inflammatory process, perhaps it is a useful analog.
**Th17 Cells**

Th17 cells are potent promoters of inflammation and are implicated in autoimmunity. To date, the presence of Th17 cells in Barrett’s esophagus or esophageal adenocarcinoma has not been investigated, but their potential role is interesting because of their potent proinflammatory properties and relationship to Treg cells. Th17 cells have been associated with other chronic inflammatory conditions of the digestive tract, such as ulcerative colitis and Crohn’s disease [125], and the presence of Th17 cells predicts poor prognosis in colorectal cancer patients [126]. One proposed mechanism through which Th17 cells promote neoplasia is through the secretion of IL-17, a cytokine that promotes angiogenesis, upregulation of survival genes [127], and activation of NF-κB signaling [110].

The best evidence for the action of 1,25-dihydroxyvitamin D on Th17 cells has been obtained from the study of Th1/Th17-mediated disease. Daniel and colleagues demonstrated the ability of 1,25-dihydroxyvitamin D to reduce dendritic cell expression of IL-23 by almost half and reduce the level of IL-6 protein detected by more than half [116] (Table 2 & Figure 4). Both IL-23 and IL-6 are promoters of differentiation to the Th17 phenotype. In addition, IL-23 is chemotactic to macrophages and is an inhibitor of CTL-mediated tumor cell destruction [128]. If Th17 cells contribute to Barrett’s pathogenesis, the effects of 1,25-dihydroxyvitamin D on dendritic cells and their secreted cytokines may impact the disease process.
Regulatory T Cells (Tregs)

Tregs include natural Tregs that modulate the immune response and inducible Tregs that are nonspecific immunosuppressors [127]. Tregs have dual implications in the promotion of carcinogenesis, suppressing the chronic inflammation that promotes aberrant tissue growth but also the antitumor functions that inhibit neoplastic proliferation [128]. In patients with esophageal squamous cell carcinoma, Tregs positively correlate with survival rate and do not suppress antitumor immunity [129]. In contrast, both progression towards anaplasia and increasing tumor grade positively correlate with the presence of Tregs in oral squamous cell carcinoma [130], and the invasiveness of hepatocellular carcinoma positively correlates with intratumoral Treg density [131].

Prognosis of esophageal squamous cell carcinoma is significantly better in patients with a subjectively evaluated abundance of both CD4\(^+\) and CD8\(^+\) cells, and a higher Treg count actually correlated with greater numbers of CD8\(^+\) and CD4\(^+\) cells. Since Tregs are immunosuppressive, increases in these cells could be due to the host defense mechanism. Notably, in patients with an abundance of CD4\(^+\) and CD8\(^+\) cells, Treg count did not influence prognosis [129], suggesting that high Treg counts are not causative in inducing high CD4\(^+\) and CD8\(^+\) counts but a concomitance of increased T cell counts in general.

Proteomic analysis has demonstrated an increase in the frequency of Tregs in Barrett’s esophagus and esophageal adenocarcinoma compared with normal controls.
In patients with esophageal adenocarcinoma, higher Treg counts in the center of the tumor were associated with lower stage of disease and were positively correlated with the density of CD8\(^+\) CTLs [133]. However, increased Treg counts were not correlated with improved prognosis. The findings suggest that the increased levels of Tregs in Barrett’s esophagus and esophageal cancer are an indicator of an increased host defense mechanism in general, and that the number of CD4\(^+\) and CD8\(^+\) T cells plays a bigger role in disease progression and prognosis.

### Table 2. Impact of 1,25-dihydroxyvitamin D on immune cells involved in reflux-related esophageal disease.

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Effect of 1,25-dihydroxyvitamin D</th>
</tr>
</thead>
<tbody>
<tr>
<td>Th1</td>
<td>Decreases Th1 cytokines (IL-12, IFN-γ); repression of T-bet</td>
</tr>
<tr>
<td>Th2</td>
<td>Increases Th2 cytokines (IL-4, IL-10); increases GATA-3; no effect has also been reported</td>
</tr>
<tr>
<td>Th17</td>
<td>Decreases cytokines that promote Th17 differentiation (IL-6, IL-23)</td>
</tr>
<tr>
<td>Treg</td>
<td>Increases Treg capacity to inhibit proliferation of CD4+CD25 cells; increases Treg cytokines (IL-10, TGF-β); increases FoxP3</td>
</tr>
<tr>
<td>Macrophage</td>
<td>Decreases macrophage cytokines (TNF-α, IL-12)</td>
</tr>
<tr>
<td>Dendritic cells</td>
<td>Decreases dendritic cell cytokines (IL-6, IL-23); arrests dendritic cells in immature development state; spontaneous apoptosis has been reported</td>
</tr>
</tbody>
</table>

Tregs are responsive to 1,25-dihydroxyvitamin D. Gorman and colleagues observed the capability of CD4\(^+\)CD25\(^+\) cells (Tregs) from mice treated with 1,25-dihydroxyvitamin D or UVB irradiation to inhibit proliferation of CD4\(^+\)CD25\(^-\) cells (non-Treg T cells) when cultured with antigen and APCs [118]. These cells, when adoptively transferred in naive mice, were also capable of suppressing immune responses. 1,25-dihydroxyvitamin D is capable of upregulating Treg production of IL-10 and TGF-β in
mice [116], and along with the steroid dexamethasone, it increases expression of the Treg lineage factor, Foxp3, significantly more than dexamethasone alone (Figure 4).

**Macrophages**

Macrophages are present in both reflux esophagitis and Barrett’s esophagus [114] (Figure 3), although they may be of different types. The macrophages associated with the Th1 response – M1 macrophages – are activated by Th1 cytokines like IFN-γ, are proinflammatory and capable of tissue damage [134]. In fact, IFN-γ is increased in esophagitis compared with normal and Barrett’s tissue [121] (Table 1). The macrophages associated with the Th2 response – M2 phenotype – are activated by Th2 cytokines like IL-4 and act to limit inflammation and promote proliferation, tissue remodeling, and angiogenesis, and have the potential to secrete TNF [134, 135]. Perhaps not coincidentally, TNF-α and IL-4 are both increased in Barrett’s esophagus compared with esophagitis [121] (Table 1). Based on the general classification of M1 and M2 phenotypes of macrophages, the presence of both TNF-α and IL-4 might suggest the presence of both M1 and M2 phenotype of macrophages in Barrett’s esophagus. However, it is the M1/M2 polarization and the ratio of these functionally active cells that would determine the predominant macrophage response in Barrett’s esophagus and esophagitis and, thus, should be compared.

Taking into account the type of inflammation and inflammatory cytokines present, it is possible that macrophages take on an M1 phenotype in reflux esophagitis and an M2 phenotype in Barrett’s esophagus, but the subtype of macrophages has not yet
been characterized [114]. Understanding how Th1- and Th2-mediated responses contribute to the metaplasia–adenocarcinoma sequence is more complex. One could hypothesize a role for both types of inflammation in the development of intestinal metaplasia in the esophagus. For example, evidence suggests that tissue damage associated with M1 macrophages and the Th1-like inflammatory response is responsible for the genetic alterations necessary for metaplastic change (see discussions on Th1 cells and 1,25-dihydroxyvitamin D and esophageal disease). And yet, the M2 macrophages and the Th2 environment seem ideal for cultivation of the extensive tissue repair and remodeling that takes place in esophageal metaplasia.

Research has demonstrated the interplay between 1,25-dihydroxyvitamin D and production of cathelicidin, an antimicrobial peptide, in macrophages. Activation of Toll-like receptors in macrophages causes increased expression of VDR and 1-α-hydroxylase, which subsequently simulates production of cathelicidin [57]. Additionally, 1,25-dihydroxyvitamin D can modulate the function of macrophages by limiting the release of TNF-α and IL-12, proinflammatory cytokines released by M1 cells that promote pathologic inflammation, Th1 differentiation, and release of IFN-γ (Figure 4). 1,25-dihydroxyvitamin D can also inhibit production of TNF-α mRNA and protein in peritoneal macrophages [136] and IL-12 production in primed and stimulated monocytes in vitro, and has similar effects on dendritic cells [137]. This inhibition occurs by transcriptional repression via the VDR-RXR heterodimer, partially by interfering with NF-κB-mediated upregulation of transcription.
**Dendritic Cells**

Increased numbers of dendritic cells are observed in Barrett’s esophagus and esophageal carcinoma [138], drawing into question their role in the disease process. It is uncertain whether their presence is a consequence of or a stimulus for esophageal disease, and what their prognostic significance is. For example, in the past, dendritic cells in the context of squamous cell carcinoma of the esophagus have been correlated with a positive prognosis [139]. Theoretically, dendritic cells can recognize tumor antigens and induce the innate and adaptive immune system to eradicate malignant cells [140]. More recently, the presence of dendritic cells in gastric cancer was an inauspicious indicator [141]. Mature dendritic cells are immunogenic, but immature and semi-mature dendritic cells are immunosuppressive, tolerogenic, or regulatory due to a lack of costimulatory molecules [142]. In esophageal adenocarcinoma, dendritic cells have been identified as CD83⁺, a marker for mature dendritic cells [138, 143].

A novel theory for dendritic cells indicating a pathologic change was proposed by Bobryshev and colleagues, who observed a dendritic cell-enriched infiltrate in the cardiac mucosa of the stomach [143]. Traditionally, the presence of cardiac mucosa and its mucous-secreting tubular cardiac glands in the proximal stomach is considered normal, as is the presence of cardiac glands in the distal esophagus. Although there is no systematic study to examine the development of cardiac mucosa with age, the theory for the presence and length of the cardiac mucosa increasing with age has been challenged and not confirmed. Most of the findings suggest that the presence of cardiac mucosa is not
Figure 4. The possible impact of 1,25-dihydroxyvitamin D on differentiation of immune cells and secreted cytokines. These mechanisms are tenuous. 1,25-(OH)2D3; 1,25-dihydroxyvitamin D; CTL: Cytotoxic T lymphocyte; CYP: Cytochrome P450; DC: Dendritic cell; M1: Macrophage of the M1 phenotype; M2: Macrophage of the M2 phenotype; VDR: Vitamin D receptor.

age-related and might be present from early life. Based on research in a population aged 20 years and younger showing the absence of cardiac mucosa between the squamous mucosa of the distal esophagus and the oxyntic (fundaic) mucosa of the proximal stomach, Bobryshev and colleagues suggested that cardiac mucosa is technically a metaplastic phenomenon, and a possible first step in the pathogenesis of Barrett’s esophagus [143]. Thus, the extent of cardiac mucosa could be associated with duration of reflux symptoms.
and the presence of erosive esophagitis in the squamous mucosa, or a predisposing factor to Barrett’s esophagus and esophageal adenocarcinoma. This suggests a relationship between cardiac mucosa and metaplasia in reflux esophagitis at the gastroesophageal junction. However, the underlying mechanism for such a relationship has not yet been examined. Investigators observed clustering of dendritic cells, which they deemed reminiscent of autoimmune diseases, and proposed an autoimmune mechanism as a potential player in Barrett’s pathogenesis [143]. This is purely speculative, however. The mechanistic link between dendritic cells and pathologic change in the region of the gastroesophageal junction is currently unexplained.

Dendritic cells express 1-α-hydroxylase and thus generate 1,25-dihydroxyvitamin D, which acts in an intracrine, paracrine, and autocrine fashion [144]. 1,25-dihydroxyvitamin D is capable of arresting dendritic cells in an immature, CD83 state of differentiation [58, 145]. The immature dendritic cells foster the development of Treg cells and suppress alloreactivity and autoimmunity thereby promoting immune tolerance [146].

The reciprocal expression of VDR and 1-α-hydroxylase in dendritic cells, as characterized by Hewison and colleagues, may further highlight the interplay between 1,25-dihydroxyvitamin D and dendritic cells [58]. Monocyte dendritic cell precursors possess upregulated VDR expression and low 1-α-hydroxylase expression. As they mature, however, VDR is downregulated and 1-α-hydroxylase is upregulated. This suggests a mechanism by which dendritic cells regulate their own population. When the
population is relatively immature, it expresses VDR but synthesizes little 1,25-
dihydroxyvitamin, so inhibition is minimal. As the population matures, it upregulates 1-
α-hydroxylase thereby increasing synthesis of 1,25-dihydroxyvitamin D, which acts in a
paracrine fashion to inhibit dendritic cell precursor monocytes and immature dendritic
cells from reaching full maturation (Figure 4).

It is possible that regulation of 1,25-dihydroxyvitamin D production by dendritic
cells in the diseased esophagus is dependent on the state of disease progression – reflux
esophagitis, metaplasia, or neoplasia. While evaluating the presence of the FcεRI IgE
receptor – a receptor on Langerhans cells, eosinophils, mast cells, and basophils – in
eosinophilic esophagitis, reflux esophagitis, and controls, Yen and colleagues observed
an increased number of FcεRI cells in reflux esophagitis biopsies compared with controls,
and also characterized a positive correlation between the number of FcεRI-positive cells
and Langerhans cells, mature dendritic cells of the esophagus [147]. This suggests that
dendritic cells are increased in reflux esophagitis. It is possible that 1,25-
dihydroxyvitamin D could contribute to the attenuation of pathologic inflammation
associated with reflux esophagitis by limiting the development of dendritic cells [144] to
their immunogenic mature form [142]. However, the investigation by Yen and colleagues
was in children with an average age under 10 years, vastly limiting the generalization to
the population with Barrett’s-associated esophageal disease [147]. Also, the magnitude of
the contribution of mature dendritic cells in reflux esophagitis is not substantiated.
Mature dendritic cells are increased in Barrett’s esophagus [143]. The extent of their role in fostering the Th2 response characteristic of Barrett’s esophagus [114] is uncertain, but if they are implicated, they could contribute to a response that fosters tissue remodeling and growth, processes that may support metaplastic change. Promotion of immunosuppressive immature dendritic cells by 1,25-dihydroxyvitamin D could help limit a Th2 response that may contribute to metaplasia.

Mature dendritic cells are present in adenocarcinoma of the esophagus as well, and in greater numbers than Barrett’s esophagus [114] (Figure 3). Indeed, a robust dendritic cell response may be crucial for eradicating neoplastic cells [148, 149]. If dendritic cells are prognostically favorable, then the ability for 1,25-dihydroxyvitamin D to attenuate dendritic cell maturation would militate against the immune response. However, the exact implication of the number, type, and location of dendritic cells in the neoplastic tissue and the correlation with disease prognosis has not been elucidated [140, 148].

There is epidemiological evidence that serum 25-hydroxyvitamin D insufficiency is associated with a number of autoimmune or immune-related diseases [150]. Bobryshev and colleagues have proposed a novel autoimmune-type mechanism for metaplasia of the esophagus based on the observation of leukocyte clusters that were similar to those of other autoimmune conditions [138]. Promotion of Treg cells could help limit autoreactive immune cells that may be involved in this disease process [151]. 1,25-dihydroxyvitamin D could contribute by directly enhancing Treg function [151] or by arresting dendritic
cells in an immature state [145]. Furthermore, 1,25-dihydroxyvitamin D may possess the potential to induce spontaneous apoptosis of dendritic cells [145], the putative cells of the autoimmune-like process observed by Bobryshev and colleagues in Barrett’s esophagus [138].

**Molecular Signaling Mechanisms and Transcription Alteration**

Altered cell signaling might contribute to Barrett’s esophagus pathogenesis by impacting expression of gene families implicated in esophageal metaplasia, such as CDX and SOX. CDX2 is absent in normal squamous epithelium of the esophagus but is expressed in Barrett’s esophagus and in squamous cells proximal to esophageal Barrett’s metaplasia [120]. Combining data from two separate studies of human endoscopic esophageal biopsies, CDX1 and CDX2 were detected in all 25 specimens of Barrett’s esophagus and in none of the 12 specimens of normal squamous epithelium nor the four specimens of gastric body mucosa [82, 84]. The SOX9 gene of the SOX family is not expressed in the adult murine esophagus [89], but it is expressed in normal human intestinal epithelium [152], and combining data from two separate studies, SOX9 expression was discovered in all 56 Barrett’s esophagus specimens and 71 of 84 esophageal adenocarcinoma specimens [89, 90].

The signaling pathways involving Wnt, Hedgehog (HH), and NF-κB are observed in Barrett’s esophagus or esophageal adenocarcinoma and regulate these genes. In addition, there is a role for the immune cell population and cytokine milieu, including IL-6, IL-12, and IL-17, as mediators of NF-κB and JAK-STAT signaling pathways with
involvement in the pathogenesis of Barrett’s esophagus and esophageal adenocarcinoma. There is support for the regulation of many of these signaling pathways by 1,25-dihydroxyvitamin D and its unhydroxylated forms.

**Wnt Signaling Pathway**

Wnt proteins are a family of ligands involved in signaling pathways that mediate cell proliferation, stem cell activity, differentiation, and tissue polarity [153]. In the canonical Wnt pathway, Wnt ligands bind to their receptor, Frizzled, and stimulate the translocation of β-catenin to the nucleus where it activates the TCF/LEF transcription factor to influence gene expression [154] (Figure 5a). Multiple Wnt proteins exist and are expressed differentially amongst the cell layers in the normal human esophagus [155]. The abnormal expression and regulation of Wnt and the Wnt signaling pathways have been implicated in the Barrett’s esophagus–adenocarcinoma disease process [156].

From observations of human biopsy specimens, it appears that the activity of Wnt signaling increases in Barrett’s esophagus and esophageal adenocarcinoma. It may do so steadily as the disease progresses from normal squamous epithelium of the esophagus to metaplasia, dysplasia, and adenocarcinoma – Wnt antagonists, familial adenomatous polyposis coli-associated protein and SFRP, steadily decrease [156]. Alternatively, Wnt signaling may be increased in esophageal adenocarcinoma but absent in nondysplastic Barrett’s esophagus. Combining data from three separate studies, 48 of 90 human esophageal adenocarcinoma specimens were positively stained for β-catenin in the
nucleus [90, 156, 157], an indicator that Wnt signaling was activated, but was reported absent in nondysplastic Barrett’s esophagus [157].

Considerable evidence exists for involvement of the Wnt signaling pathway in the expression of multiple genes with implications in Barrett’s esophagus or associated adenocarcinoma. However, the conditions under which Wnt signaling goes awry and whether it is a cause or effect of disease is unclear. For example, the Cdx1 promoter possesses a TCF/LEF response element, and culture of murine embryos with Wnt induces Cdx1 expression [158]. Yet, Wnt signaling is present in healthy esophagus tissue [155], so for significant alterations in the pathogenesis of Barrett’s esophagus – for example, by inducing abnormal CDX gene expression – it is critical to demonstrate that normal levels of Wnt signaling in the esophagus do not induce CDX expression. Alternatively, an initiating epigenetic change could be required for Wnt signaling to become aberrant. For example, altered methylation of a TCF response element in the promoter of a CDX gene could allow Wnt signaling to promote transcription. This is analogous to the findings by Wong and colleagues where TNF-α induces expression of CDX1 genes that lack a fully methylated promoter [82].

Members of the DICKKOPF (DKK) family of proteins are Wnt signaling antagonists, interfering with the normal assembly of the Wnt receptor complex and its ability to release β-catenin from degradation [154]. In human colon cancer, Aguilera and colleagues demonstrated 1,25-dihydroxyvitamin D as a slow, indirect inducer of DKK-1 transcription [33], whereas in another study, 1,25-dihydroxyvitamin D represses DKK-4
transcription [32]. In the esophagus, however, there was a statistically significant increase in DKK-1/4 expression in esophagitis compared with normal and Barrett’s esophagus tissue, which appeared to express DKK-1/4 similarly [155]. Thus, despite a role for 1,25-dihydroxyvitamin D in Wnt signaling regulation, it is difficult to conclude that DKK is dysregulated in Barrett’s esophagus and related disease and that it is an important target for disease treatment or prevention (Figure 5a).

**Hedgehog (HH) Signaling Pathway**

In humans, three proteins of the HH family exist. These include SHH, IHH and DHH, all of which are ligands for the membrane receptor, PTCH. Hedgehog ligand binding releases the transmembrane protein, SMO, from inhibition, which leads to the activation of the GLI transcription factors to a transcription-inducing form (Figure 5b). Hedgehog signaling regulates numerous processes embryologically including cell differentiation and tissue patterning. In the adult, it takes part in stem cell maintenance and tissue repair [159]. Hedgehog signaling activation can grow columnar-type tissue that expresses SOX9 and the glandular markers cytokeratin 8 and 18, using a denuded rat trachea as a scaffold [89].

In the human adult gastrointestinal tract, expression of SHH is absent in the esophagus, restricted to fundic glands of the stomach and bases of intestinal crypts [160]. In human esophageal squamous cell cultures, increased expression of HH ligand is observed when incubated at pH <4 [89], the pH used clinically to define an episode of
Figure 5. Sites of intervention by 1,25-dihydroxyvitamin D in the signaling pathways involved in the pathogenesis of Barrett’s esophagus and esophageal adenocarcinoma. (A) WNT and (B) HH signaling in Barrett’s esophagus and esophageal adenocarcinoma and implications of 1,25-dihydroxyvitamin D. (C) TNF-α, IL-17 and IL-1 act on different receptors that transduce through NF-κB signaling (shown acting on one generic receptor here). 1,25-(OH)₂D₃ suppresses levels of TNF-α (Table 2), and in APCs, it inhibits the action of NF-κB transcription factor RelB. This effect was not observed in the setting of Barrett’s esophagus and esophageal adenocarcinoma. (D) IL-6-mediated JAK-3–STAT-3 signaling has been observed in esophageal adenocarcinoma, but the inhibition of IL-12-mediated STAT-3 signaling has not. 1,25-(OH)₂D₃; 1.25-dihydroxyvitamin D; DKK: Dickkopf; HH: Hedgehog; RXR: Retinoid X receptor; SMO: Smoothened; VDR: Vitamin D receptor.
reflux [161]. A mouse model of esophagojejunostomy provided evidence that bile acid can also increase HH signaling [89].

Combining data from two separate studies, SHH expression was reported in 118 of 119 Barrett’s esophagus and 83 of 89 esophageal adenocarcinoma biopsy specimens taken from human subjects [89, 162]. Also, HH signaling is associated with the epithelial pit patterning [162] of Barrett’s esophagus: small round, characterized by gastric metaplasia (no goblet cells); long oval, characterized by mixed gastric-intestinal metaplasia (some goblet cells) and tubular, characterized by complete intestinal metaplasia (significant goblet cell presence) [163]. In this study, significantly lower SHH and PTCH1 was observed in goblet versus non-goblet columnar epithelium and SHH expression was positively correlated with MUC5AC expression, a marker of goblet cell metaplasia [162]. This suggests that HH signaling is lower in Barrett’s esophagus with tubular patterning, which is considered a more complete form of metaplasia, than small round patterning. These results are certainly confusing considering one would expect expression of SHH to display a similar relationship to goblet cell metaplasia as it does a marker of goblet cell metaplasia.

It has been proposed that PTCH inhibits SMO by secreting a cholesterol molecule, and it has been subsequently shown that unhydroxylated vitamin D is a potent effector of inhibition via this mechanism [164] (Figure 5b). Vitamin D limits proliferation of basal cell carcinoma in mice and blocks HH signaling to an extent similar to cyclopamine, a known HH signaling inhibitor, and does this more effectively than its
precursor, 7-dehydrocholesterol and its derivatives, 25-hydroxyvitamin D and 1,25-
dihydroxyvitamin D [165]. Cyclopa
tamine can reduce the growth of esophageal
adenocarcinoma cell lines by 75–95% [166]. These findings, together with the prevalence
of aberrant HH signaling in Barrett’s esophagus and esophageal adenocarcinoma, as
discussed earlier, further highlight the potential role for vitamin D and its hydroxylated
forms in Barrett’s esophagus and associated esophageal disease.

**NF-κB**

NF-κB and its signaling pathway (Figure 5c) have been implicated in neoplasia
and its associated inflammation in multiple tissues [167]. NF-κB expression increases at
each step in the progression from reflux esophagitis to Barrett’s-associated esophageal
adenocarcinoma [168]. NF-κB proteins are a family of five subunits of transcription
factors that exist as homo- or hetero-dimers. These subunits include RelA (aka p65),
RelB, RelC, p50 and p52 [167]. The dimers remain in the cytoplasm until their
translocation to the nucleus is triggered by the dissociation of the inhibitory subunit, I-
κB. In addition to many agents, this signaling can be stimulated by TNF-α and IL-1 [167].
The ability of neoplastic cells to evade apoptosis and secrete proinflammatory, cancer-
promoting cytokines is attributed to NF-κB [135].

In vivo, NF-κB signaling is increased after administration of bile acid to the
esophageal lumen [78]. In addition, increased NF-κB signaling could help cells evade
apoptotic cell death induced by a harsh reflux environment [169]. In support of this
hypothesis, human Barrett’s cell lines, but not normal esophageal squamous cell lines, are
able to resist apoptosis induced by DNA damage, but not after inhibition of NF-κB signaling [169]. Notably, UVB irradiation was used to induce DNA damage in this study, so the exact role of NF-κB with respect to Barrett’s esophagus in the presence of reflux still remains unclear.

Bile acids increase expression of the NF-κB RelA, one of the subunits of the active heterodimer along with p50, in human esophageal adenocarcinoma cell lines [170]. Conversely, inhibition of NF-κB transcription decreases levels of both p65 – NF-κB signaling upregulates its own transcription factor – and MUC2, a mucin protein secreted in large amounts in the intestine and colon and an indicator of metaplastic change in the esophagus [170]. Transfection of the human colorectal cancer cell lines with p65 induces CDX1 expression [82], and NF-κB-mediated CDX2 expression increases in the presence of bile acids, albeit in monkey kidney cells [171]. These results suggest that NF-κB signaling may regulate insult by bile acid and transcriptional change in metaplasia of the esophagus.

The NF-κB family proteins are crucial transcription factors in the regulation of inflammation and immune function [167], and aberrant signaling may be integral to the promotion of neoplasia [135]. The NF-κB transcription factor RelB has a regulatory binding region for VDR/RXR on its promoter that recruits an inhibitory complex of factors when it is bound. Consequently, 1,25-dihydroxyvitamin D analogs can inhibit NF-κB signaling, albeit this action is purportedly restricted to APCs (Figure 5c). The
ability of 1,25-dihydroxyvitamin D to inhibit NF-κB signaling in esophageal keratinocytes or glandular epithelial cells has not been explored.

**IL-6 and STAT Signaling**

IL-6 (gp130), a cytokine released from both macrophages and lymphocytes, is increasingly expressed in the progression from normal esophagus to esophagitis and Barrett’s esophagus [172] (Table 1). Secretion of the cytokine is increased by esophageal adenocarcinoma cells when exposed to bile acid in a medium with pH <4.0 [173]. IL-6 signal transduction takes place via the JAK–STAT pathway [110], and regulates the cell cycle genes C-MYC and cyclin-D1 among other roles [174]. IL-6 binding to its receptor activates JAK leading to phosphorylation of STAT-3 (p-STAT) [110] (Figure 5d).

Biopsies from patients with esophageal adenocarcinoma and high-grade dysplastic Barrett’s esophagus display increased p-STAT in comparison to low-grade dysplastic Barrett’s esophagus, non-dysplastic Barrett’s esophagus, normal squamous esophageal epithelium and duodenal epithelium [173]. STAT-3 is present in the nucleus of cells exposed to bile acid at pH <4 [173], and when cultured with deoxycholic acid, cells with inhibited STAT signaling display increased cellular apoptosis [173, 175]. This suggests that STAT signaling is an important facilitator of resistance to the reflux environment, and may contribute to aberrant cellular proliferation.

1,25-dihydroxyvitamin D is capable of limiting STAT-3 phosphorylation in T cells stimulated by IL-12 in a mouse model of multiple sclerosis [176] (Figure 5d). IL-12
and IL-6 effect STAT signaling through separate receptors [110], so the efficacy of 1,25-dihydroxyvitamin D to inhibit IL-6-induced STAT-3 phosphorylation is uncertain. However, in this model, the beneficial effect of inhibited STAT signaling is through limitation of its promotion of Th1 differentiation [176], a T-cell response implicated in Barrett’s pathogenesis (see discussion on Th1 lymphocytes) and a therapeutic modality that could also be effective in Barrett’s esophagus and associated disease.

With this background established, this thesis will now explore the epidemiology of EAC and vitamin D, the expression of VDR at the gastroesophageal junction, and the correlation between VDR staining and response to neoadjuvant therapy in patients with EAC.

MATERIALS AND METHODS

Epidemiology of EAC and Vitamin D Literature Review

A PubMed search was conducted for publications listed under the MeSH terms "Vitamin D" and "Esophagus" and "Adenocarcinoma," which yielded no results. Search parameters were then expanded to include literature addressing vitamin D and sun exposure and any esophageal malignancies or gastric carcinomas published in the past decade. Abstracts of search results were surveyed for studies that examined the epidemiology of serum 25-hydroxyvitamin D levels or surrogates thereof and any of the above-mentioned cancers. These publications were then examined in more detail for their methods, results, and conclusions.
VDR Expression at the Gastroesophageal Junction and in EAC Biopsy Specimens from Patients who Received Neoadjuvant Therapy

Biopsy and post-neoadjuvant therapy resection specimens from 15 patients who had been treated between 2004 and 2009 for esophageal adenocarcinoma at the Creighton University Medical Center, Omaha, NE, were procured retrospectively. The Creighton University Institutional Review Board approved the use of these tissues for this study. All patients received neoadjuvant therapy and subsequent surgical resection. Neoadjuvant chemotherapy regimens were variable but most patients received 5-fluorouracil and cisplatin (Table 5). Most patients also received neoadjuvant radiation therapy (Table 5). The biopsies and resection specimens were examined and evaluated independently by two investigators (PS, WJH). Tumor staging prior to treatment was assessed by computed tomography (CT), positron emission tomography (PET), endoscopic ultrasound (EUS), and esophagogastroduodenoscopy (EGD). Thirteen of 15 biopsy specimens were from T3 disease; the remaining were T2.

Assessment of tumor differentiation on initial biopsy was performed using a three-grade classification system. Well-differentiated tumors were defined by the presence of well-formed glands containing malignant columnar cells displaying small regular nuclei. The complete absence of gland formation, or the presence of bizarrely shaped glands, identified poorly differentiated tumors. Moderately differentiated tumors possessed well-formed glands, but the cells were less columnar or frankly cuboidal, with reduced cell polarity and more dysplastic nuclei than those observed in well-differentiated tumors.
Histopathologic examination of all resected specimens consisted of thorough evaluations of tumor stage, residual tumor (R) category, grading, and number of examined and involved lymph nodes. Specimens were grouped as complete responders (C) if there was no residual tumor remaining assessed by pathologic examination of the resected specimen. Subjects were grouped as non-responders (N) if there was evidence of tumor in the resection specimen and if the T staging did not change (increase or decrease) at postoperative assessment. From each resection specimen, areas of the normal esophagus, Barrett's esophagus, and normal gastric tissue were identified.

**Immunofluorescence**

Formalin-fixed paraffin-embedded biopsy tissues were used for immunofluorescence labeling. After deparaffinization and rehydration, antigen retrieval was performed prior to immunostaining. Sections were incubated for 2h in block/permeabilizing solutions containing PBS, 0.25% Triton X-100, and 5% (v/v) goat serum at room temperature. The slides were subsequently incubated with a primary antibody solution including mouse anti-VDR (Santa Cruz Bio-tech, Santa Cruz, CA; sc-13133) (1:200 in PBS) at 4 °C overnight. After washing with PBS four times for 5 min each, a secondary antibody (1:200 dilution of affinity purified goat anti-mouse cyanine 3 (cy3) antibody, PBS, 0.1% Triton X-100, 1% goat serum) (Jackson Immuno-Research, Westgrove, PA) was applied to the sections for 2 hours in the dark. Negative controls were run in parallel with complete omission of primary antibody. Sections were washed with PBS four times for 5 min. Nuclei were counterstained with 4′, 6-diamidino-2-
phenylindole (DAPI). A single layer of nail polish was placed around the edge of slide to prevent escape of mounting media from the coverslip.

**Analysis**

Slides were visualized with a BX51 microscope, photographed with an Olympus DP71 camera using the same exposure time for each slide, and analyzed using Image J software (National Institutes of Health). Each image was opened in Image J and converted to an RGB stack. The threshold for each image was set to minimize the background fluorescence that would be incorporated into the software's measurement of intensity. The image was then analyzed by the software, generating a value that represented the mean staining intensity of that sample.

Because the normality of residuals assumption was violated, Mann-Whitney was used for evaluation of differences in VDR intensity with respect to NA treatment response. Again, because the normality of residuals assumption was violated, Kruskal-Wallis was employed for evaluation of differences in staining intensity with respect to tumor grade. For evaluation of VDR intensity as a predictor of overall and disease free survival, simple linear regression was use. Data were transformed to rank order coefficients in order to meet the assumptions required to utilize simple linear regression. Once transformed, no violation of independence, homoscedasticity, or normality of residuals was indicated. Further, no violation of linearity was indicated via scatterplot.
RESULTS

Epidemiology of Esophageal Adenocarcinoma and Vitamin D: Literature Review

Overall, 9 observational studies examining the relationship between 25-hydroxyvitamin D levels (or a surrogate for 25-hydroxyvitamin D levels) and upper gastrointestinal cancer were reviewed [177-187]. The results are summarized in Tables 1 and 2. One of these studies examined esophageal squamous cell dysplasia and was included because of the disease’s relationship to esophageal cancer. No consistent relationship was reported between serum 25-hydroxyvitamin D levels or a surrogate and upper gastrointestinal cancers; 4 studies reported negative correlations between vitamin D status and upper gastrointestinal cancer, three reported positive correlations, one reported no correlation, and one reported both positive and negative correlations. The results did not seem to trend systematically with the year of publication. All 3 studies examining esophageal cancer and UV exposure reported negative correlations.

The 4 studies that reported lower incidence of upper gastrointestinal cancer with higher levels of 25-hydroxyvitamin D or surrogates thereof were Tran and colleagues [178], Lipworth and colleagues [185], Giovannucci and colleagues [183], and Boscoe and Schymura [177]. Tran and colleagues assessed cumulative ambient UVB radiation exposure and its relationship to esophageal cancer [178]. Investigators reported an 18% decrease in risk for esophageal adenocarcinoma, a 17% decrease for esophagogastric junction adenocarcinoma, and a nonstatistically significant decrease in esophageal squamous cell carcinoma (ESCC) for each standard deviation (SD) increase in UVB irradiance, which was 107 J/m2. Lipworth and colleagues, in a case–control study in
### Table 3. Characteristics and outcomes of the nine studies surveyed.

<table>
<thead>
<tr>
<th>Author</th>
<th>Study Design; # Cancer Cases; Skin Type</th>
<th>Study Location; Study Period</th>
<th>Vitamin D Status Assessment</th>
<th>Confounding Variables Included in Analysis</th>
<th>Correlation Reported, HR/OR/RR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tran et al 2012</td>
<td>Case-control; 995; 95%+ white</td>
<td>Australia; 2002-2005</td>
<td>UVB irradiance (J/m²)</td>
<td>age, sex, BMI, state of residence at recruitment, heartburn, reflux symptoms, education, smoking, alcohol, h. pylori serostatus</td>
<td>EAC: OR 0.82\textsuperscript{a} (0.72-0.93); OR 0.59\textsuperscript{b} (0.35-0.99)</td>
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<td></td>
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<td></td>
<td>EGJAC: OR 0.83\textsuperscript{c} (0.73-0.94); OR 0.55\textsuperscript{d} (0.34-0.90)</td>
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<td></td>
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<td></td>
<td>ESCC: OR 0.94\textsuperscript{e} (0.82-1.09); OR 0.91\textsuperscript{f} (0.51-1.64)</td>
</tr>
<tr>
<td>Mulholland 2011</td>
<td>Case-control; 218; not reported</td>
<td>Ireland; 2002-2005</td>
<td>FFQ</td>
<td>age, sex energy intake, smoking, education, BMI, occupation, alcohol, NSAID use, h. pylori serostatus, glycemic index intake, saturated fat intake, location</td>
<td>EAC: OR 1.99 (1.03-3.86)</td>
</tr>
<tr>
<td>Abnet et al 2010</td>
<td>Case-control; 1065; 61% white, 33% Asian, 3% black</td>
<td>China, Finland, U.S. including Hawaii; 1974-2006</td>
<td>25OHD</td>
<td>smoking, alcohol, education, BMI, history of gastric surgery</td>
<td>EAC: P for trend 0.70</td>
</tr>
<tr>
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<td>ESCh: P for trend 0.77</td>
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<td>GCA: P for trend 0.88</td>
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<td>GNCA: P for trend 0.83</td>
</tr>
<tr>
<td>Chen et al 2010\textsuperscript{g}</td>
<td>Ecological; --; not reported</td>
<td>China; 1988-1992</td>
<td>UVB irradiance (mW/m²)</td>
<td>only sex, rural v urban county, ultraviolet irradiance, and cancer incidence/mortality were examined</td>
<td>ESCh: Incidence ratio: 0.73 (0.68-0.78)</td>
</tr>
<tr>
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<td>EC: mortality ratio: 0.92 (0.90-0.94)</td>
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<tr>
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<td>GC: Incidence ratio: 0.87 (0.83-0.91)</td>
</tr>
<tr>
<td></td>
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<td></td>
<td>GC: mortality ratio: 0.97 (0.95-0.99)</td>
</tr>
<tr>
<td>Lipworth et al 2009</td>
<td>Case-control; 304; not reported</td>
<td>Italy; 1992-1997</td>
<td>FFQ</td>
<td>age, sex, study center, education, smoking, alcohol, energy intake</td>
<td>ESCh: OR 0.84\textsuperscript{h} (0.71-0.99) OR 0.58\textsuperscript{i} (0.40-0.85)</td>
</tr>
<tr>
<td>Chen et al 2007</td>
<td>Nested case-control from prospective cohort; 979; not reported</td>
<td>China; 1986-1991</td>
<td>25OHD</td>
<td>age, sex, BMI, smoking, alcohol, serum selenium, cholesterol and retinol, cholesterol and a-tocopherol</td>
<td>ESCC: HR 1.06(1.01-1.13)</td>
</tr>
<tr>
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<td>GCA: 1.03(0.96-1.10)</td>
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<td></td>
<td>GNCA: 0.98(0.86-1.12)</td>
</tr>
<tr>
<td>Abnet et al 2007</td>
<td>Cross-sectional; 230; not reported</td>
<td>China; 1986-1991</td>
<td>25OHD</td>
<td>age, sex, height, weight, tooth loss</td>
<td>ESCD: RR 1.86\textsuperscript{j} (1.35-2.62)</td>
</tr>
<tr>
<td>Giovannucci et al 2006</td>
<td>Prosp ective cohort; 93; mainly white cohort</td>
<td>U.S.; 1986-2000</td>
<td>Model predicting 25OHD; model included skin color</td>
<td>age, height, smoking, calorie intake, alcohol, red meat, calcium, retinol, total fruits and vegetables</td>
<td>EC: RR 0.37 (0.17-0.80)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>GC: RR 0.58 (0.26-1.33)</td>
</tr>
<tr>
<td>Boscoe and Schymura 2006\textsuperscript{h}</td>
<td>Ecological; --; blacks and whites were analyzed separately</td>
<td>North America; 1993-2002</td>
<td>UVB irradiance (kJ/m²/year)</td>
<td>age, poverty, income, smoking, exercise, alcohol, outdoor occupation, urban/rural, air quality</td>
<td>EC: Incidence ratio 1.27\textsuperscript{k} (1.21-1.34)</td>
</tr>
<tr>
<td></td>
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<td></td>
<td></td>
<td>Mortality ratio 1.36\textsuperscript{k} (1.31-1.41)</td>
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<td>EC (blacks): RR 1.3-1.5\textsuperscript{l}</td>
</tr>
</tbody>
</table>

**NOTE:** Statistically significant values are bolded. 25OHD = 25-Hydroxyvitamin D; EAC = Esophageal Adenocarcinoma; EC = Esophageal Cancer; EGJAC = Esophagogastric Junction Adenocarcinoma ESCC = Esophageal Squamous Cell Carcinoma; ESCD = Esophageal Squamous Cell Dysplasia; FFQ = Food Frequency Questionnaire; GC = Gastric Cancer; GCA = Gastric Cardia Adenocarcinoma; GNCA = Gastric Non-cardia Adenocarcinoma UVB = Ultraviolet B. \textsuperscript{a}This OR is for each increase in 107 J/m² of cumulative ambient UVB exposure. See text for further explanation. \textsuperscript{b}This OR is for highest v. lowest tertile. See text for further explanation. \textsuperscript{c}A total of 424.088 cancer cases were used including nasopharynx, esophagus, stomach, colon, rectal, liver, lung, breast, cervix, bladder, leukemia. Exact figures for esophagus and gastric cancers were not published. \textsuperscript{d}These are statistics reported for overall incidence and mortality ratios, but these ratios varied depending on urban or rural counties. See text and Table 4 for further detail. \textsuperscript{e}This OR is for vitamin D status reported as a continuous variable. \textsuperscript{f}Highest quartile v lowest quartile. \textsuperscript{g}This study examined over 3 million cancer cases of all types but did not specify numbers of individual types of cancer. \textsuperscript{h}Ratio was reported for receiving annual average 650 kJ/m² versus 1540 kJ/m². See text for further explanation. \textsuperscript{i}Authors did not report specifics on this risk ratio. See text for details.
Italy, reported a 16% decrease in ESCC for each 1.14 mg/d increase in dietary vitamin D intake before diagnosis, the standard deviation for controls [185]. This study did not examine adenocarcinoma of the esophagus. In both studies, relationships were stronger when assessment of vitamin D status, by UVB irradiance or dietary vitamin D intake, was reported as a categorical variable by tertile (Table 3). Giovannucci and colleagues discovered a statistically significant inverse correlation between predicted serum 25-hydroxyvitamin D and incidence of esophageal cancer in the Health Professionals Follow-up Cohort [183]. The cohort is composed of 51,529 males and has been followed since 1986 with information updates every 2 to 4 years. Investigators used data from this population to construct a model to predict 25-hydroxyvitamin D serum concentrations. Each 25 nmol/L (10 ng/ml) increase in predicted 25-hydroxyvitamin D corresponded to a 63% decrease in esophageal cancer incidence. Finally, Boscoe and Schymura reported a 27% increase in incidence and 36% increase in mortality of esophageal cancer in populations receiving an annual average of 650 kJ/m2-y UV exposure versus 1,540 kJ/m2-y, albeit in non-Hispanic white males only [177]. A weaker relationship was reported in non-Hispanic white females. The authors report that this relationship is proportional so that populations receiving 1,100 kJ/m2-y could be expected to have half of the increased risk displayed by those receiving 550 kJ/m2-y. The authors analyzed a black cohort separately and reported limited data because of the inconsistency of the results, but did note that the esophagus was the only cancer site that displayed a higher relative risk of cancer in the north versus south United States, in males and females, for both incidence and mortality. In this study, relative risks ranged from 1.3 to 1.5 [177].
The 3 studies that reported higher incidence of upper gastrointestinal cancer with higher vitamin D status were Mulholland and colleagues [186], Chen and colleagues [182], and Abnet and colleagues [179]. Most recently, Mulholland and colleagues evaluated the relationship between vitamin D intake and incidence of esophageal adenocarcinoma in a case–control study using an Ireland-based population cohort, called "Factors Influencing the Barrett’s Adenocarcinoma Relationship (FINBAR)" [186]. A positive association was reported between the highest and lowest tertile of vitamin D intake and esophageal adenocarcinoma with odds ratio (OR) 1.99; 95% confidence interval (CI) 1.03–3.86. This association did not persist for normal weight individuals, individuals negative for Helicobacter pylori, or those who never smoked, but the authors reported no interaction between these variables and vitamin D intake [186]. Pretrial 25-hydroxyvitamin D levels were correlated to subsequent development of ESCC in men in a 2007 case-control study by Chen and colleagues [182]. There was no significant correlation between pretrial serum 25-hydroxyvitamin D and development of gastric carcinoma, but in men pretrial 25-hydroxyvitamin D level was positively correlated with ESCC development. Abnet and colleagues [179] examined the association between serum 25-hydroxyvitamin D and esophageal squamous cell dysplasia in the same population used by Chen and colleagues [182]. They found a positive correlation between the two variables in both men and women with relative risk (RR) 1.86; 95% CI 1.35–2.62. The relative risk was greater for women than in men, in contrast to the statistic reported by Chen and colleagues, which found a positive correlation between vitamin D and esophageal squamous cell cancer only in men [182].
### Table 4. Negative and Positive Correlations between vitamin D and upper gastrointestinal cancer reported in nine studies.

<table>
<thead>
<tr>
<th></th>
<th>EC</th>
<th>EAC</th>
<th>EGIAC</th>
<th>ESCC</th>
<th>GC</th>
<th>GNCA</th>
<th>EC</th>
<th>EAC</th>
<th>ESCC</th>
<th>ESCD</th>
<th>GC</th>
<th>GCA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tran et al 2012</td>
<td>0.82&lt;sup&gt;a&lt;/sup&gt;, 0.59&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.83&lt;sup&gt;a&lt;/sup&gt;, 0.55&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.94&lt;sup&gt;a&lt;/sup&gt;, 0.91&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td>Mulholland 2011</td>
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<td>1.99</td>
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<tr>
<td>Abnet et al 2010</td>
<td>No correlation</td>
<td>No correlation</td>
<td>No correlation</td>
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<td></td>
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<tr>
<td>Chen et al 2010</td>
<td>0.42&lt;sup&gt;c&lt;/sup&gt;, 0.89&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.62&lt;sup&gt;c&lt;/sup&gt;, 0.99&lt;sup&gt;d&lt;/sup&gt;</td>
<td>1.12&lt;sup&gt;c&lt;/sup&gt;, 1.00&lt;sup&gt;f&lt;/sup&gt;</td>
<td>1.08&lt;sup&gt;e&lt;/sup&gt;, 0.92&lt;sup&gt;f&lt;/sup&gt;</td>
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<tr>
<td>Lipworth et al 2009</td>
<td>0.84&lt;sup&gt;g&lt;/sup&gt;, 0.58&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td>Chen et al 2007</td>
<td></td>
<td>0.98</td>
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<td></td>
<td></td>
<td></td>
<td>1.07</td>
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<td>1.03</td>
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<tr>
<td>Abnet et al 2007&lt;sup&gt;a&lt;/sup&gt;</td>
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<td></td>
<td></td>
<td>1.86</td>
</tr>
<tr>
<td>Giovannucci et al 2006</td>
<td>0.37</td>
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<tr>
<td>Boscoe and Schymura 2006</td>
<td>1.27&lt;sup&gt;i&lt;/sup&gt;, 1.36&lt;sup&gt;j&lt;/sup&gt;</td>
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</table>

EAC = Esophageal Adenocarcinoma; EC = Esophageal Cancer; EGIAC = Esophagogastric Junction Adenocarcinoma; ESCC = Esophageal Squamous Cell Carcinoma; GC = Gastric Cancer; GCA = Gastric Cardia Adenocarcinoma; GNCA = Gastric Non-Cardia Adenocarcinoma

Statistically significant values are bolded. The absence of a value in a field indicates this statistic was not evaluated by the study.

<sup>a</sup>This OR is for each increase in 107 J/m² of cumulative ambient UVB exposure. See text for further explanation.

<sup>b</sup>This OR is for highest v. lowest tertile. See text for further explanation.

<sup>c</sup>Rural incidence ratio

<sup>d</sup>Rural mortality ratio

<sup>e</sup>Urban incidence ratio

<sup>f</sup>Urban mortality ratio

<sup>g</sup>This OR is for vitamin D status reported as a continuous variable.

<sup>h</sup>This OR is with respect to the lowest tertile of vitamin D status.

<sup>i</sup>Incidence ratio

<sup>j</sup>Mortality ratio

Abnet and colleagues reported no correlation between serum 25-hydroxyvitamin D levels and upper gastrointestinal cancer, although analysis did yield some statistically significant trends in certain subgroups [180]. This nested case–control design used the Cohort Consortium Vitamin D Pooling Project of Rarer Cancers and examined the relationship between upper gastrointestinal cancer and circulating serum 25-
hydroxyvitamin D in 1,065 cases. In a subgroup analysis of 256 cases of esophageal cancer, no statistically significant trend was found over 6 levels of serum 25-hydroxyvitamin D status. The same was true when the 142 cases of esophageal squamous cell carcinoma and 104 cases of esophageal adenocarcinoma were looked at separately. Likewise, there was no statistically significant trend for any type of gastric cancer, although statistically significant ORs were calculated for certain comparisons between categories of serum 25-hydroxyvitamin D levels [180].

Chen and colleagues reported both positive and negative correlations in a study taking place in China that looked for an ecologic relationship between the geographic distribution of ambient UVB irradiance, measured in milliwatts per meter squared (mW/m2), and incidence and mortality of esophageal and gastric cancer [181]. Esophageal cancer mortality decreased by 8% and incidence by 27% for each 10 mW/m2 increase in UVB irradiance. However, the inverse relationship between UVB irradiance and esophageal cancer mortality and incidence was restricted to rural counties. In these counties, each 10 mW/m2 increase in UVB irradiance predicted an 11% and a 58% decrease in esophageal cancer mortality and incidence, respectively [181]. In contrast, in urban counties each 10 mW/m2 increase in UVB irradiance predicted no change in mortality and a 12% increase in esophageal cancer incidence. In comparison, gastric cancer mortality decreased by 3% and incidence by 13% for each 10 mW/ m2 increase in UVB irradiance. When stratified by county (urban or rural) UVB irradiance was inversely correlated with gastric cancer mortality only in urban counties, and with gastric
cancer incidence only in rural counties; it was positively correlated with gastric cancer incidence in urban counties [181].

**Vitamin D Receptor Expression at the Gastroesophageal Junction and in Esophageal Adenocarcinoma Biopsy Specimens from Patients who Received Neoadjuvant Therapy**

In total, 3 specimens each were examined for VDR staining of normal squamous mucosa, normal gastric mucosa, and Barrett’s mucosa. No subjective difference in staining was observed between the three specimens with respect to each tissue type. The normal squamous mucosa in the lower esophagus stains negative for VDR (Figure 6). Background fluorescence in these regions is similar in intensity to negative controls (Figure 7 d–f). In contrast, submucosal glands and ducts deep to the normal squamous mucosa stain positive for VDR (Figure 6 d–f). The transition between squamous and columnar mucosa at the gastroesophageal junction illustrates the difference in positive immunostaining between these two mucosal subtypes (Figure 7 a–c).

Normal gastric mucosa and the underlying gastric and fundic glands also showed positive immunofluorescence (Figure 8 a–c). Immunofluorescence for VDR appears to be localized in the cytoplasm and perinuclear regions, with nuclear staining absent (Figure 8 d–f). Barrett's mucosa also stains positive for VDR (Figure 9). Glandular structures in the mucosal layer are far less abundant in Barrett's mucosa than in the normal gastric mucosa. As a result, fewer structures deep to the Barrett's epithelial layer stain positive for VDR when compared to normal gastric mucosa. An island of Barrett's
metaplasia interpolated in the normal squamous epithelium of the lower esophagus is displayed in Figure 9 d–f.

Figure 6. VDR expression in the human esophageal tissue. Each image is a representative photograph from 5 subjects from which tissue was acquired. Images A–C are from the normal esophagus, displaying the absence of staining in the squamous mucosa of this tissue. Images D–F are also from the normal esophagus, highlighting VDR staining of a submucosal gland and duct. Images A and D are hematoxylin and eosin. Images B and E are an overlay of VDR immunofluorescence and DAPI (nuclear counterstain). Images C and F are VDR immunofluorescence without DAPI. All images are taken at a 10x objective.
Figure 7. VDR expression in the human gastroesophageal junction. Images A–C are from the gastroesophageal junction illustrating the difference in staining between the proximal squamous mucosa and distal columnar mucosa. Images D–F are from a negative control. Image A shows staining with hematoxylin and eosin. Images B and E are an overlay of VDR immunofluorescence and DAPI (nuclear counterstain). Images C and F are VDR immunofluorescence without DAPI. Image D is with DAPI. All images are taken at a 10x objective.
Figure 8. VDR expression in the normal gastric cardia. Each image is a representative photograph from the 5 subjects from which tissue was acquired. Images A–C are from the normal gastric cardia at a 10x objective. Images D–F are images of the mucus gland duct pictured in Figure F, D–F at a 60x objective. Images A and D are hematoxylin and eosin. Images B and E are an overlay of VDR immunofluorescence and DAPI (nuclear counterstain). Images C and F are VDR immunofluorescence without DAPI.
Figure 9. VDR expression in Barrett's esophagus. Each figure is a representative photograph from the 4 subjects with Barrett's esophagus from which tissue was acquired. Images A–C are an area of complete columnar metaplasia with goblet cells. Images D–F is a region of metaplasia interpolated between normal squamous mucosa. Images A and D are hematoxylin and eosin. Images B and E are an overlay of VDR immunofluorescence and DAPI (nuclear counterstain). Images C and F are VDR immunofluorescence without DAPI. All images are taken at a 10x objective.
A total of 15 biopsy tissues were included in the analysis of VDR staining of esophageal adenocarcinoma tissue. Neoadjuvant therapy regimens for these patients and their survival data are presented in Table 5. Characteristics of the patients from whom biopsies were taken are displayed in Table 6. Tumor characteristics and the mean VDR fluorescence intensity for each specimen are shown in Table 7. Results of the immunofluorescence staining and other post-operative outcomes are shown in Table 8.

The VDR immunofluorescence was more intense in the non-responder group than the complete responder group (Table 8). The difference was even greater when moderately and well-differentiated tumors (histologic grades G1 and G2) were analyzed separately.
Table 6. Characteristics of patients from whom biopsies were taken.

<table>
<thead>
<tr>
<th>All tumor grades</th>
<th>Complete Responders (n=6)</th>
<th>Non-responders (n=9)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>5 (83%)</td>
<td>9 (100%)</td>
</tr>
<tr>
<td>Mean age ± st.</td>
<td>61 ± 5</td>
<td>64 ± 9</td>
</tr>
<tr>
<td>CAD</td>
<td>1 (17%)</td>
<td>1 (11%)</td>
</tr>
<tr>
<td>DM</td>
<td>1 (17%)</td>
<td>2 (22%)</td>
</tr>
<tr>
<td>HT</td>
<td>5 (83%)</td>
<td>6 (67%)</td>
</tr>
<tr>
<td>Smoker</td>
<td>3 (50%)</td>
<td>6 (67%)</td>
</tr>
<tr>
<td>Other CM</td>
<td>2 (33%)</td>
<td>1 (11%)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Grades G1, G2</th>
<th>Complete Responders (n=4)</th>
<th>Non-responders (n=5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>4 (100%)</td>
<td>5 (100%)</td>
</tr>
<tr>
<td>Mean age ± st.</td>
<td>58 ± 2</td>
<td>66 ± 9</td>
</tr>
<tr>
<td>CAD</td>
<td>1 (25%)</td>
<td>1 (20%)</td>
</tr>
<tr>
<td>DM</td>
<td>1 (25%)</td>
<td>1 (20%)</td>
</tr>
<tr>
<td>HT</td>
<td>4 (100%)</td>
<td>3 (60%)</td>
</tr>
<tr>
<td>Smoker</td>
<td>2 (50%)</td>
<td>3 (60%)</td>
</tr>
<tr>
<td>Other CM</td>
<td>1 (25%)</td>
<td>0</td>
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</table>

<table>
<thead>
<tr>
<th>Grade G3</th>
<th>Complete Responders (n=2)</th>
<th>Non-responders (n=4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>1 (50%)</td>
<td>4 (100%)</td>
</tr>
<tr>
<td>Mean age ± st.</td>
<td>67 ± 3</td>
<td>62 ± 9</td>
</tr>
<tr>
<td>CAD</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>DM</td>
<td>0</td>
<td>1 (25%)</td>
</tr>
<tr>
<td>HT</td>
<td>1 (50%)</td>
<td>3 (75%)</td>
</tr>
<tr>
<td>Smoker</td>
<td>1 (50%)</td>
<td>3 (75%)</td>
</tr>
<tr>
<td>Other CM</td>
<td>1 (50%)</td>
<td>1 (25%)</td>
</tr>
</tbody>
</table>

CAD = coronary artery disease; CM = comorbidities; DM = diabetes mellitus; G1 = well differentiated; G2 = moderately differentiated; G3 = poorly differentiated; HT = hypertension.

from poorly differentiated tumors (Table 8). However, there was no statistically significant difference between any of the groups.

The results of the Mann-Whitney test indicated a non-statistically significant difference in ranked VDR fluorescence intensity between the non-responders and complete responders, Z = -0.471, p = 0.637, with tissues in the non-responder group.
Table 7. Tumor characteristics and mean VDR fluorescing intensity.

<table>
<thead>
<tr>
<th>Biopsy</th>
<th>Grade</th>
<th>T</th>
<th>N</th>
<th>M</th>
<th>Stage</th>
<th>Response</th>
<th>R0</th>
<th>Recurrence</th>
<th>Mean Fluorescing Intensity ± st.dev.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>G2</td>
<td>3</td>
<td>1</td>
<td>0</td>
<td>IIIA</td>
<td>N</td>
<td>Y</td>
<td>Y</td>
<td>145.0 ± 35.2</td>
</tr>
<tr>
<td>2</td>
<td>G2</td>
<td>3</td>
<td>1</td>
<td>0</td>
<td>IIIA</td>
<td>N</td>
<td>Y</td>
<td>Y</td>
<td>137.0 ± 31.1</td>
</tr>
<tr>
<td>3</td>
<td>G1</td>
<td>3</td>
<td>1</td>
<td>0</td>
<td>IIIA</td>
<td>N</td>
<td>Y</td>
<td>Y</td>
<td>116.6 ± 30.5</td>
</tr>
<tr>
<td>4</td>
<td>G3</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>IIB</td>
<td>N</td>
<td>N</td>
<td>Y</td>
<td>115.4 ± 35.5</td>
</tr>
<tr>
<td>5</td>
<td>G2</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>IIB</td>
<td>N</td>
<td>Y</td>
<td>N</td>
<td>114.7 ± 32.6</td>
</tr>
<tr>
<td>6</td>
<td>G3</td>
<td>3</td>
<td>1</td>
<td>0</td>
<td>IIIA</td>
<td>N</td>
<td>Y</td>
<td>Y</td>
<td>98.2 ± 33.5</td>
</tr>
<tr>
<td>7</td>
<td>G3</td>
<td>3</td>
<td>1</td>
<td>0</td>
<td>IIIA</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>95.0 ± 32.6</td>
</tr>
<tr>
<td>8</td>
<td>G3</td>
<td>3</td>
<td>1</td>
<td>0</td>
<td>IIIA</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>85.6 ± 24.5</td>
</tr>
<tr>
<td>9</td>
<td>G2</td>
<td>3</td>
<td>1</td>
<td>0</td>
<td>IIIA</td>
<td>N</td>
<td>Y</td>
<td>Y</td>
<td>83.4 ± 23.6</td>
</tr>
<tr>
<td>10</td>
<td>G2</td>
<td>3</td>
<td>1</td>
<td>0</td>
<td>IIIA</td>
<td>C</td>
<td>Y</td>
<td>N</td>
<td>133.8 ± 38.7</td>
</tr>
<tr>
<td>11</td>
<td>G2</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>IIB</td>
<td>C</td>
<td>Y</td>
<td>N</td>
<td>124.4 ± 29.6</td>
</tr>
<tr>
<td>12</td>
<td>G3</td>
<td>3</td>
<td>1</td>
<td>0</td>
<td>IIIA</td>
<td>C</td>
<td>Y</td>
<td>Y</td>
<td>119.7 ± 34.7</td>
</tr>
<tr>
<td>13</td>
<td>G2</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>IIB</td>
<td>C</td>
<td>Y</td>
<td>Y</td>
<td>90.7 ± 29.3</td>
</tr>
<tr>
<td>14</td>
<td>G3</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>IB</td>
<td>C</td>
<td>Y</td>
<td>N</td>
<td>78.0 ± 27.3</td>
</tr>
<tr>
<td>15</td>
<td>G2</td>
<td>3</td>
<td>1</td>
<td>0</td>
<td>IIIA</td>
<td>C</td>
<td>Y</td>
<td>N</td>
<td>59.7 ± 19.3</td>
</tr>
</tbody>
</table>

C = complete responder; N = non-responder; G1 = well differentiated; G2 = moderately differentiated; G3 = poorly differentiated; Y= Yes; N= No
Table 8. Immunofluorescence staining intensity of VDR, and post-operative outcomes.

<table>
<thead>
<tr>
<th>All tumor grades</th>
<th>Complete Responders (n=6)</th>
<th>Non-responders (n=9)</th>
</tr>
</thead>
<tbody>
<tr>
<td>VDR IF intensity: average of the means ± st. dev.</td>
<td>101.0 ± 26.8</td>
<td>110.1 ± 20.3</td>
</tr>
<tr>
<td>R0 resection</td>
<td>6 (100%)</td>
<td>6 (67%)</td>
</tr>
<tr>
<td>Recurrence</td>
<td>2 (33%)</td>
<td>6 (67%)</td>
</tr>
<tr>
<td>Survival in months ± st. dev.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Disease Free</td>
<td>17 ± 13</td>
<td>18 ± 16</td>
</tr>
<tr>
<td>Overall</td>
<td>23 ± 17</td>
<td>22 ± 15</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Grades G1, G2</th>
<th>Complete Responders (n=4)</th>
<th>Non-responders (n=5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>VDR IF intensity: average of the means ± st. dev.</td>
<td>102.1 ± 29.3</td>
<td>119.3 ± 21.4</td>
</tr>
<tr>
<td>R0 resection</td>
<td>4 (100%)</td>
<td>5 (100%)</td>
</tr>
<tr>
<td>Recurrence</td>
<td>1 (25%)</td>
<td>4 (80%)</td>
</tr>
<tr>
<td>Survival in months ± st. dev.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Disease Free</td>
<td>19 ± 15</td>
<td>21 ± 18</td>
</tr>
<tr>
<td>Overall</td>
<td>29 ± 18</td>
<td>28 ± 16</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Grade G3</th>
<th>Complete Responders (n=2)</th>
<th>Non-responders (n=4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>VDR IF intensity: average of the means ± st. dev.</td>
<td>98.9 ± 20.9</td>
<td>98.6 ± 10.8</td>
</tr>
<tr>
<td>R0 resection</td>
<td>2 (100%)</td>
<td>1 (25%)</td>
</tr>
<tr>
<td>Recurrence</td>
<td>1 (50%)</td>
<td>2 (50%)</td>
</tr>
<tr>
<td>Survival in months ± st. dev.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Disease Free</td>
<td>11 ± 6</td>
<td>14 ± 11</td>
</tr>
<tr>
<td>Overall</td>
<td>11 ± 6</td>
<td>16 ± 10</td>
</tr>
</tbody>
</table>

G1 = well differentiated; G2 = moderately differentiated; G3 = poorly differentiated

indicating greater ranked staining intensity on average compared to tissues in the complete responder group. Results of the Kruskal-Wallis test indicated a non-statistically significant difference in staining intensity with respect to tumor grade, $\chi^2 (2) = 1.181$, p =
Mean rank of staining intensity for tumor grades 1, 2, and 3 were 10, 8.8, and 6.5, respectively. The results of a simple linear regression analysis indicated that rank of intensity of staining did not significantly predict overall or disease free survival, F(1,13) = 1.064, p = 0.321, adjusted $R^2 = 0.005$, and F(1,13) = 1.461, p = 0.248, adjusted $R^2 = 0.032$, respectively.

Moderately and well-differentiated tumors had greater average mean fluorescence intensity (111.7) than poorly differentiated tumors (98.7). The specimen that showed most intense immunofluorescence for VDR was a moderately differentiated esophageal adenocarcinoma in the non-responder group (Figures 10 a–c). The specimen that stained least intensely was a moderately differentiated esophageal adenocarcinoma in the complete responder group (Figures 10 d–f). Representative specimens of high fluorescent intensity and low fluorescent intensity from the non-responder group are displayed in Figure 11. Representative specimens of high fluorescent intensity and low fluorescent intensity from the complete responder group are shown in Figure 12.
Figure 10. Representative micrographs of the specimen that fluoresced most intensely (specimen 1) and least intensely (specimen 15) at 40x objective. Both specimens were moderately differentiated adenocarcinoma. Specimen 1 (A–C) was a non-responder and specimen 15 (D–F) from a complete responder. Images A and D are H&E. Images B and E are an overlay of VDR immunofluorescence and DAPI (nuclear counterstain). Images C and F are VDR immunofluorescence without DAPI.
Figure 11. Analysis of immunofluorescence in specimens from the non-responder group. Specimen 2 (A–C) displayed the second strongest intensity in the non-responder group. Specimen 9 (D–F) displayed the lowest intensity in the non-responder group. Images A and D are H&E. Images B and E are an overlay of VDR immunofluorescence and DAPI (nuclear counterstain). Images C and F are VDR immunofluorescence without DAPI. Both were moderately differentiated.
Figure 12. Analysis of immunofluorescence of tissue specimens from the complete responder group. Specimen 11 (A-C) is a moderately differentiated esophageal adenocarcinoma and displayed the second strongest intensity in complete responder group. Specimen 14 (D–F) is a poorly differentiated esophageal adenocarcinoma and displayed the second lowest intensity in the complete responder group. Images A and D are H&E. Images B and E are an overlay of VDR immunofluorescence and DAPI (nuclear counterstain). Images C and F are VDR immunofluorescence without DAPI.
DISCUSSION

Vitamin D in the Pathogenesis of Barrett’s Esophagus and Esophageal Adenocarcinoma

Over the last 20 years, the immunomodulatory role of 1,25-dihydroxyvitamin D in non-musculoskeletal diseases has been increasingly appreciated. However, so far, there is very limited information addressing the role of 1,25-dihydroxyvitamin D in the context of Barrett’s esophagus and esophageal adenocarcinoma. As was demonstrated, considerable variation in VDR staining amongst esophageal adenocarcinoma biopsy specimens is observed, but no statistically significant relationship. In addition, expression of VDR appears to be limited to columnar epithelium at the gastroesophageal junction and in Barrett’s esophagus with an absence of staining in squamous epithelium of the esophagus. In another study examining the level of expression of VDR mRNA, no significant difference was observed between biopsies of normal esophagus, esophagitis, Barrett’s esophagus or esophageal adenocarcinoma [70]. In cell cultures, there was decreased expression of VDR mRNA in esophageal adenocarcinoma compared with Barrett’s esophagus that approached but did not reach statistical significance [70]. Also, a trend for an increased number of apoptotic cells in Barrett’s esophagus-derived cultures was observed after treatment with either a VDR inhibitor or a VDR stimulator [70]. Evidence that vitamin D and its hydroxylated forms may induce apoptosis in prostate, breast, and colon cancer cells makes these statistically nonsignificant results difficult to dismiss [188], but the paradox of increased apoptosis by both VDR inhibition and stimulation limits confidence in these findings.
At this point, it is difficult to draw conclusions on the extent to which 1,25-dihydroxyvitamin D can impact the immune cell population, cytokine milieu, cell signaling, and gene expression in the pathogenesis of Barrett’s esophagus and esophageal adenocarcinoma. So far, only deductive assumptions have been used to characterize the CD4+ T cell population in the esophagus based on cells and cytokines associated with a particular inflammatory response (i.e., Th1 or Th2) rather than by cell typing. Th17 cells release major cytotoxic mediators, the presence of which has not been characterized in the diseased esophagus. Dendritic cells and macrophages have been investigated, but their role in esophageal disease is uncertain. This may be due to, at least in part, incomplete characterization with respect to their types: immature or mature dendritic cells, M1 and M2 macrophage polarization. Furthermore, despite some progress characterizing the type of T cell response in Barrett’s esophagus and esophageal adenocarcinoma, the implications for metaplasia and neoplasia remain unclear, especially the impact that a Th2-skewed response could have on the progression of disease. Considering that lymph node-positive esophageal adenocarcinoma portends poor prognosis [16], the immune cell population in nodes of patients with esophageal adenocarcinoma requires exploration. Finally, elucidation of the immune cell population during each stage of pathogenesis – reflux esophagitis, nondysplastic Barrett’s esophagus, dysplastic Barrett’s esophagus, and esophageal adenocarcinoma – compared with normal esophageal tissue will help determine the role of these cells in disease.

Investigation into the cellular signaling pathways involved in Barrett’s esophagus and esophageal adenocarcinoma has been equally inconclusive, offering ample
correlational statistics but no concrete elucidation of mechanisms as they pertain to metaplasia and proliferation of esophageal tissue. Furthermore, reported associations between cell signaling pathways and Barrett’s esophagus pathogenesis have even conflicted at times, such as the case with Wnt and HH discussed here. A clearer understanding of the pathogenesis is needed in order to generate more auspicious hypotheses regarding the role of vitamin D and its hydroxylated forms in prevention or treatment of the disease.

Part of the ability of Barrett’s mucosa to resist the harsh reflux environment and become malignant is afforded by resistance to apoptosis, a property that may be afforded by aberrant NF-κB and IL-6–JAK3–STAT3 signaling. The ability to recover an appropriate rate of cell death in this context could mitigate the disease process, and vitamin D may have a role in regulating this ability. The strong implication of NF-κB [169] and IL-6–JAK3–STAT3 [172, 174] signaling in Barrett’s esophagus and esophageal adenocarcinoma and the observation that 1,25-dihydroxyvitamin D or its analogs inhibit these signaling pathways in other disease models [176, 189] suggests a potentially fruitful line of investigation. Culturing esophageal squamous and glandular epithelial cells in acidified bile salts while measuring IL-6, STAT, and NF-κB, in the presence of and without 1,25-dihydroxyvitamin D, may be a fortuitous starting point.

In general, progress on the cellular and molecular pathogenesis of Barrett’s esophagus and esophageal adenocarcinoma has generated abundant data but very little information on mechanisms of pathogenesis and the exact role of vitamin D. Additional
studies are warranted to draw conclusions about the potential of 1,25-dihydroxyvitamin D as an immunomodulator of the disease process, and the current evidence does not militate against generation of biologically plausible hypotheses. Currently, in vivo characterization of the immune cell population throughout the stages of Barrett’s associated disease and the implications of NF-κB and IL-6-JAK3–STAT3 signaling and the effect of 1,25-dihydroxyvitamin D on these pathways are possible areas of high-yield research. 1,25-dihydroxyvitamin D can modulate the immune response by increasing the expression and activity of molecules, such as prohibitin, which inhibits the functional activity of importin-α3 and importin-α4, the molecules required to import the homo- or heterodimer of RelA and RelB to the nucleus [190]. In addition, 1,25-dihydroxyvitamin D might also inhibit the activity of CDX2 that is induced by NF-κB in esophageal mucosa with Barrett’s esophagus. Careful studies designed both in ex vivo human tissues and relevant in vivo models would hopefully establish a causal versus correlative role of vitamin D in the pathogenesis of esophageal diseases.

Epidemiology of Esophageal Adenocarcinoma and Vitamin D

The most apparent limitation to the existing literature is the methods used to estimate vitamin D status. Only 3 of 9 publications used serum 25-hydroxyvitamin D as a measure, the most accurate way to estimate vitamin D status [191]. A single blood sample obtained in the spring or fall offers a reasonable estimate of the average serum 25-hydroxyvitamin D over a 1-year period [192]. However, using a single serum sample still has its limitations, possibly underestimating statistical relationships [192]. As Giovannucci and colleagues pointed out, one-time serum 25-hydroxyvitamin D
measurements can be transiently high or low [183]. Tran and colleagues contest that they may not account for the impact of vitamin D on esophageal carcinogenesis, which may take place over a lifetime and exhibit a latency period with respect to this impact [178].

Furthermore, these studies pose the issue of temporality. The methods used in the published data did not allow for a calculation of the amount of time elapsed between evaluation of serum 25-hydroxyvitamin D and diagnosis of upper gastrointestinal cancer. This was exemplified in the study of Chen and colleagues [182]. After obtaining pre-trial serum 25-hydroxyvitamin D levels, subjects were followed over a 5-year period, establishing a prospective timeline between the serum 25-hydroxyvitamin D measurement and development of upper gastrointestinal cancer. This is a strength of the study; however, neither follow-up serum samples nor samples at the time of cancer mortality or incidence were assayed. Only the initial serum 25-hydroxyvitamin D level was used as a predictor with possibly considerable variability in the time between this measurement and the identification of disease among subjects. Giovannucci and colleagues [183] made attempts to address this by tracking surrogate measurements of serum 25-hydroxyvitamin D and analyzing for correlations over time.

One point worthy of mention in the study of Abnet and colleagues [179] is the method used to identify squamous cell dysplasia. Investigators used a staining test that had a range of specificity of 40% to 95% for the detection of higher-grade dysplasia or early neoplasia, and an even lower specificity for dysplasia of lower grades. This wide range of specificity along with the fact that subjects with any grade of dysplasia were
included allows for the potential of false positive results. This could exaggerate the relative risk or lead to a falsely increased relative risk if a sufficient number of subjects with 25-hydroxyvitamin D levels in the upper quartiles had misclassified esophageal disease.

Two studies used food frequency questionnaires (FFQs) to estimate a subject’s vitamin D status, a method purported by one group of authors to have high reproducibility and validity [185]. Evidence suggests that surrogates such as dietary intake and vitamin supplementation are poor predictors of vitamin D status. A model using physical inactivity, skin pigmentation, dietary intake, body mass index (BMI), and region could account for only 28% of the variability in serum 25-hydroxyvitamin D levels [183]. In this model, physical inactivity and skin pigmentation were the best predictors. This is logical when one considers that approximately 80% to 90% of vitamin D may be obtained from synthesis in the skin [24]. In a study of a Sydney, Australia population, variables typically considered as surrogates of sun exposure, physical activity and smoking, were documented as significant predictors of serum 25-hydroxyvitamin D levels [193]. Interestingly, the same study did not yield sun exposure itself as a significant predictive factor of vitamin D status.

It is likely that factors predicting vitamin D status differ according to race. In a Chicago, Illinois study, predictors of vitamin D status were different for men of European versus African descent. In European American men, the strongest predictors were season and lifetime sun exposure followed by income and BMI. In African American men,
dietary and supplemental vitamin D intake were major predictors [194]. A global report on hypovitaminosis D concluded that not only skin pigmentation but also cultural differences such as certain clothing practices significantly influence vitamin D status [195]. The impact of skin color on the studies presented in this review is likely limited, however, considering most of the cohorts examined were largely non-black. Studies that did not report on race were conducted in Irish, Italian, and Chinese populations and presumably consisted of a negligible percentage of black subjects considering the demographics of these countries [179, 181, 182, 185, 186]. Furthermore, two of the studies not reporting on race used 25-hydroxyvitamin D levels obviating the need to use skin color as predictor of vitamin D status. Boscoe and Schymura analyzed non-Hispanic white and black cohorts separately to control for the impact of skin color on vitamin D status [177].

Predictors of vitamin D status and vitamin D status itself may also differ by gender; indeed 2 studies reviewed here reported correlations that differed between men and women [177, 182]. In a study conducted in the Netherlands, gender and season were the major predictors of vitamin D status. Men tended to have higher serum 25-hydroxyvitamin D levels than women. When parsed by gender, physical activity and season remained as correlates in men, whereas physical activity and estradiol levels were the main determinants in women [196].

Additional evidence supports winter season, low vitamin D dietary and supplement intake, high BMI, physical inactivity, and low milk and calcium intake as
major determinants of low vitamin D status [197], highlighting the myriad of opinions about predictors of vitamin D and the complexity of using surrogate markers to predict vitamin D status. In addition, FFQs allow for the potential of recall bias. This is exemplified in the report by Mulholland and colleagues in which study participants were asked to report dietary habits and BMI for a 12-month period beginning 5 years before the administration of the FFQ [186]. This should be considered when interpreting these results.

Three studies examined the correlation of UV exposure to incidence and mortality of esophageal cancer. Interestingly, all three studies reported inverse correlation between UV exposure, a proposed surrogate for vitamin D status, and esophageal cancer. However, this method imposes some limitations. Measurements of ambient UVB irradiance may not reliably approximate serum 25-hydroxyvitamin D levels or even UVB exposure. In addition, Chen and colleagues [181] did little to control for significant confounding variables, including smoking, alcohol intake, and BMI. But, these investigators reported both positive and negative correlations between UVB exposure and upper gastrointestinal cancer. Esophageal cancer mortality and incidence was inversely correlated to UVB irradiance only in rural counties, a restriction that could reflect the increased amount of UVB exposure that the agrarian worker presumably gets. This would strengthen the inference that higher vitamin D status may help limit esophageal cancer mortality and incidence, as the authors point out. However, it could also reflect other lifestyle factors associated with the agrarian lifestyle that could protect against
esophageal cancer mortality and incidence, confounding its relationship to UVB exposure.

The inconsistency of the relationship should be reiterated: UVB irradiance was inversely correlated with esophageal and gastric cancer incidence in rural counties, but positively correlated in urban counties, and an inverse correlation to gastric cancer mortality was only present in urban counties. These differences could be a consequence of different neoplastic process between esophageal and gastric cancer; or statistically significant relationships could have been found serendipitously because of the increased probability of making a type I error when conducting numerous tests for significance. Nevertheless, there may be merit to using UVB irradiance as a surrogate for vitamin D status. Even at high latitudes, season influenced vitamin D more than diet, ethnicity, and vitamin intake suggesting that sun exposure is the major determinant of vitamin D status [198].

The study by Tran and colleagues [178] was also limited by the possibility that UVB irradiance does not accurately predict vitamin D status. However, investigators were able to more accurately estimate UVB exposure by approximating individual lifetime exposure to UVB and collecting data on many confounding variables. In addition, they examined UVB exposure at different age periods for each subject in an attempt to evaluate the contribution of estimated vitamin D status to the prevention of esophageal cancer over one’s lifetime. This is a strength afforded by this study design and a limitation of study designs that assess serum 25-hydroxyvitamin D status at one
particular instance, thus failing to account for the possibility of a latent period for esophageal carcinogenesis.

Two studies published in the last decade, a meta-analysis and a systematic review, examined the risk of subsequent cancer after diagnosis of skin cancer, essentially using prior skin cancer as a surrogate for UV irradiance. After previous diagnosis of squamous cell carcinoma, basal cell carcinoma, or non-melanoma skin cancer, Grant reported relative risks for developing gastric cancer and esophageal cancer of 0.67 and 0.60, respectively [184]. Wheless and colleagues [187] reported no association between previous diagnosis of skin cancer and subsequent esophageal cancer. This review included the data published in the study by Grant [184].

The discrepancy in the results of the investigators may be explained by the suggestion that the absence of a skin cancer diagnosis does not preclude adequate exposure to UVB light. If this is the case—that subjects with adequate UVB exposure are significantly represented in the group without a skin cancer diagnosis—then the inverse correlation reported by Grant [184] would attenuate. Nevertheless, if in fact sun exposure is linked to high vitamin D status, the findings by Grant [184] support the hypothesis that vitamin D plays a role in the prevention of cancer. In addition, Grant [184] astutely excluded melanocytic skin cancers, which are associated with intermittent and blistering sun exposure at an early age [199] and the presence of melanocytic nevi [200], factors that may correlate less closely with overall sun exposure.
Despite a particular interest in adenocarcinoma of the esophagus, all upper gastrointestinal cancers in this review because of the limited information on the topic. However, it has been suggested that adenocarcinoma of the esophagus and gastric cardia share many similar risk factors that they may be considered together, and may even be of the same etiology [201]. Other sources suggest otherwise [202], and this could be one of the limitations of this interpretation of the literature.

The mechanism by which vitamin D may impact carcinogenesis in the upper gastrointestinal tract, in particular adenocarcinoma of the esophagus, is uncertain, but may involve the immunomodulatory role of vitamin D in the regulation of immune cells involved in reflux-related esophageal disease including CD4\(^+\) T cells [114-118, 121], macrophages [115, 136, 137], and dendritic cells [58, 144-146], and key signaling pathways including Wnt [32, 155], Hedgehog [164-166], NF-\(\kappa\)B [189], and IL-6–JAK–STAT [110, 176]. The discrepancy between the role of vitamin D in cancers of lower gastrointestinal tract, including colorectal cancer where there is strong evidence of a protective effect [203, 204], and upper gastrointestinal cancers, including esophageal adenocarcinoma where the relationship is still unclear, may be explained by different pathogeneses of these two diseases. Esophageal adenocarcinoma is thought to arise from a metaplasia–neoplasia sequence as a consequence of chronic inflammation induced by bile and acid reflux [95], whereas it is generally accepted that colorectal cancer progresses through an adenoma–carcinoma sequence [205]. The role of inflammation in these two disease states is also likely different and could impact the response to vitamin D status.
In summary, the current literature is limited in many cases by the method used to assess vitamin D status, lack of specific data for the types of upper gastrointestinal cancer including subtypes of esophageal cancer, and failure to establish a temporal relationship between vitamin D status assessment and presentation of upper gastrointestinal cancer. The most weight should be placed on the 3 studies using serum 25-hydroxyvitamin D to assess vitamin D status as this limits confounding and misclassification. However, still there was no consensus relationship among these three datasets. Furthermore, there is merit to ecologic studies and studies examining UV exposure that attempt to estimate an individual’s vitamin D status over a lifetime. This may better predict the impact of vitamin D status on esophageal cancer if long-term vitamin D status is more relevant to carcinogenesis than current or recent vitamin D status. Future studies should aim to combine individual data about lifetime sun exposure, surrogate markers for vitamin D status, and serum 25-hydroxyvitamin D levels, ideally at multiple intervals throughout the study period.

It is possible that the lack of a consistent relationship reported across the nine studies reviewed is a consequence of study design. Inaccurate and imprecise assessment of vitamin D status could certainly attenuate, exaggerate, or obscure relationships, but this would require methods that systematically under assessed or over assessed vitamin D status. It is likely that studies using measurements other than serum 25-hydroxyvitamin D to assess vitamin D status are both inaccurate and imprecise. In addition, each different subtype of upper gastrointestinal cancer—including esophageal adenocarcinoma and squamous cell carcinoma, which have distinctly different pathologies—may exhibit a
different relationship with vitamin D levels and should be assessed separately. In conclusion, no consistent relationship between vitamin D status and upper gastrointestinal cancers is currently evident, but studies using sun exposure as a main measurement consistently report lower rates of esophageal cancer with higher levels of UV irradiance.

**Vitamin D Receptor Expression at the Gastroesophageal Junction and in Esophageal Adenocarcinoma Biopsy Specimens from Patients Who Received Neoadjuvant Therapy**

The vitamin D receptor can be grouped with a cluster of nuclear receptors that have important functions in the enteric tract [206]. In mice, VDR displays extensive expression in the lower digestive tract, colon, and small intestine [62], and it is expressed in normal human colon [68]. Vitamin D receptor mRNA has also been detected in the human esophagus [70]. Dietary vitamin D intake is considered potentially protective against malignancies of the colon, skin, prostate, and breast [207]. Although the primary function of 1,25(OH)\(_2\)D\(_3\) is to regulate calcium absorption and control bone mineralization, upon binding to VDR, 1,25(OH)\(_2\)D\(_3\) induces cell cycle arrest, differentiation, and apoptosis in normal and transformed cells as well as alters the MAP kinase and Wnt/β-catenin signaling pathways involved in carcinogenesis [207-209]. The extent of VDR expression may correlate with the neoplastic process. High VDR expression in prostate tumors is associated with a reduced risk of lethal cancer, suggesting a role of the vitamin D pathway in prostate cancer progression [210], and immunohistochemistry of basal cell carcinoma indicates an increase in VDR staining intensity compared to normal cells [211].
The most apparent finding with respect to VDR expression in the esophagus was the restriction of immunofluorescent staining to the columnar epithelium and glandular structures; the squamous epithelium of the normal esophagus universally stained negative for VDR. Barrett's mucosa showed immunopositivity for VDR unlike the normal squamous epithelium of the esophagus. Staining of the columnar mucosa and glandular structures is consistent with findings in other tissues that share architectural similarities with the mucosa of the esophagus and gastric cardia. The columnar and glandular epithelia display the strongest immunohistochemical staining in the murine placenta [212], and the glandular epithelium of the tubuloalveolar glands of the neoplastic prostate glands also displays immunohistochemical staining for VDR [210]. In the latter, there is considerable variability in the intensity of staining. This is consistent with the variability in the immunofluorescent staining that was observed in esophageal adenocarcinoma and may be associated with the neoplastic process.

The absence of staining in the squamous mucosa of the esophagus is in contrast to what is seen in the squamous epithelium of the epidermis and cervix where the nuclei stain positive throughout the thickness of the epithelial layer and strongest in the basal cell layer [211, 213]. Cytoplasmic staining is also observed but to a lesser degree [214]. Certain nuclear receptors like the VDR shuttle between the cytoplasm and the nucleus even in the absence of their ligands [215], and in the unliganded state VDR distributes evenly between the cytoplasm and the nucleus [216]. Upon ligand binding, the steady state shuttling between the cytoplasm and the nucleus favors nuclear localization [215].
Despite this, the immunofluorescent staining used here appeared to show mainly cytoplasmic staining with almost complete sparing of the nuclei. This is also in contrast to VDR immunohistochemistry of the epidermis, which showed mainly nuclear staining [211, 213, 214]. However, the epidermal mucosa is composed of keratinized epithelium whereas the esophageal mucosa is composed of non-keratinized epithelium, and immunohistochemistry was utilized as an assay as compared to immunofluorescence. These are two differences that may partially explain the discrepancies observed in studies of the VDR expression in the epidermis with this study.

Here, high-grade tumors demonstrate lower levels of expression of VDR by immunofluorescence. This could be due to the low-grade tumor differentiation and marked cellular pleomorphism observed in these tumors at histological examinations, which are possibly associated with a loss of expression of VDR. A similar reduction in VDR mRNA and protein has been observed with progressive tumor dedifferentiation in colon adenocarcinoma [208], supporting the hypothesis that VDR is downregulated in poorly differentiated tissue and that lower expression indicates a poor prognosis [217].

Similar to the observations in healthy gastric mucosa and Barrett’s mucosa, VDR protein expression in adenocarcinoma biopsy tissue was present in the cytoplasm but not in the nucleus. These findings are similar to those in prostate and colon tissues that have evaluated the presence of VDR protein using immunohistochemistry [208, 210, 218], but in contrast to observations in the epidermis, which show mainly nuclear staining [211, 213, 214]. Absence of nuclear expression of VDR protein may be due to a shift in localization
of this protein from nucleus to cytoplasm as neoplastic transformation takes place. Hendrickson and colleagues postulated that the VDR antigens may be processed quickly in the nucleus after functioning and cytoplasmic staining may be a surrogate of VDR actions that are ultimately mediated in the nucleus [210]. A substantial proportion of cytoplasmic VDR is co-localized with endoplasmic reticulum, the Golgi complex, and microtubules. Cytoplasmic VDR mediates non-genomic actions of VDR including regulation of calcium and chloride channel activities, protein kinase C activation, and phospholipase C activity occurring through cytoplasmic signaling pathways such as protein kinase and mitogen-activated protein kinase [219, 220].

The major obstacle to improved outcome in operable esophageal cancer is the inability to predict response to chemoradiation therapy. The expression of excision repair cross-complementation group 1 (ERCC1), glutathione S-transferase P1, and thymidylate synthase have been associated with clinical outcome in patients with esophageal and gastric cancers [221]. If VDR expression is associated with response to neoadjuvant therapy, VDR expression could act as a biomarker to predict clinical outcome in these patients. The results presented here show that higher intensity of VDR staining in pretreatment biopsies is associated with a lack of response to chemoradiation therapy. This is the first investigation to evaluate relationship of VDR expression in pretreatment biopsies with response to neoadjuvant therapy.

There were significant limitations to this investigation. The study was inadequately powered to show statistical significance of the effect sizes observed. This
was in part due to the considerable variability of staining intensity observed within groups, as well as heterogeneity within each sample. This variability is consistent with the variability reported in immunohistochemical staining of prostate tumors [210], and is possibly a result of an inherent heterogeneity of cellular division, differentiation, and gene expression concomitant to the uncontrolled proliferation characteristic of neoplasia. This heterogeneity was not observed in healthy stomach and in Barrett’s esophagus, although this was assessed subjectively. Additionally, the lack of comparison with adjacent normal tissue from the same patient prevents complete interpretation of the staining intensity.

Another limitation to this investigation is the use of immunofluorescence to quantify the extent of VDR expression. Although every effort is made to avoid it, the potential to interject bias is present when adjusting contrast and exposure times for photography of the prepared slides. Also, the use of Image J software as a method to quantify staining intensity has not been validated. Furthermore, there are many steps to immunohistochemical and immunofluorescent techniques that have the potential to contribute to inaccurate assessment of a specimen. The time from tissue collection to fixation, preparation of the tissue to be fixed, fixation times, antigen retrieval, and the type and concentration of antibody are all steps in the methodology with the potential to influence mischaracterization of the level of expression one is intending to detect [222]. Assessment of staining intensity should include a positive control, ideally multiple of varying staining intensities, with which to compare study specimens. Although we did have a positive control, it was not included in the experimental batch of tissue specimens.
because fresh tissue was not available. Quantifying the intensity of staining is also difficult, and it may not be appropriate to draw conclusions regarding the level of protein being expressed based on the intensity of immunofluorescence [223].

In summary, this investigation suggest that: (i) VDR expression is present in normal gastric mucosa, Barrett’s esophagus mucosa, and esophageal adenocarcinoma, but not normal squamous mucosa of the esophagus; (ii) the level of expression may decline with tumor de-differentiation; and, (iii) VDR expression may serve as a prognostic marker for responsiveness to neoadjuvant therapy, with higher staining intensity portending a complete response to neoadjuvant treatment. Considering the data obtained, it is plausible that the extent of VDR expression is related to tumor progression and response to therapy, but it is equally as plausible that these data are merely random.
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