Impact of Vitamin D and Asthma on Preeclampsia Development:
A Sequential Evidence-Based Approach to Genomic Analysis

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Bibliography
Preface

The biologic evidence regarding the role for vitamin D in immunologic mechanisms and reproductive outcomes is strong. In the US, there is evidence to show that vitamin D insufficiency has increased almost 2-fold over a span of about 10 years, from the late 1980s through the early 2000s (Ginde, Liu et al. 2009). This takes on greater significance as epidemiologic studies have shown a high prevalence of vitamin D deficiency among pregnant women, especially among high-risk groups. Clinical studies establishing an association between vitamin D levels and adverse pregnancy outcomes such as preeclampsia (PE), gestational diabetes, low birth weight, preterm labor, cesarean delivery and infectious diseases have yielded conflicting results. This is likely due to a paucity of randomized trials, heterogeneity of populations studied, and inadequate sample size leading to reduced power and inadequate adjustment for confounding variables among observational studies (Urrutia and Thorp 2012).

Vitamin D has been also been linked to asthma attacks, another proposed risk factor for preeclampsia. Association of vitamin D and severe asthma has been studied in general population (Brehm, Acosta-Perez et al. 2012, Paul, Brehm et al. 2012, Korn, Hubner et al. 2013, Poon, Mahboub et al. 2013). Along with a clear genetic basis in atopic asthma, environmental factors, including early neonatal nutrition, may have an important influence on asthma development and, thus, present an opportunity to prevent or delay the onset of the disease (Greer, Sicherer et al. 2008, Jenerowicz, Silny et al. 2012). Vitamin D may be an important environmental factor influencing asthma outcomes. A recent meta-analysis investigated the adverse perinatal outcomes in women with asthma and concluded that as much as a 50% increase risk of PE development in asthmatic pregnant women (Murphy, Namazy et al. 2011). Interestingly, in preeclampsia, epidemiologic studies have implicated alterations in vitamin D metabolism and low vitamin D status during pregnancy (August, Marcaccio et al. 1992, Seely, Wood et al. 1992, Halhali, Tovar et al. 2000).

The objective of this dissertation is to investigate the potential confounding/modulating effect of vitamin D in the association of asthma and preeclampsia as well as its role in gene expressions in early pregnancy of preeclamptic women. To achieve this goal, a sequential approach from abstracting available literature evidences,
to epidemiologic and genomic analyses is proposed. Accordingly, in the introductory chapter one of this dissertation, we provide an evidence-based literature review on the role of vitamin D in asthma development or its severity, fetal development and healthy pregnancy as well as immunomodulation and conclude that vitamin D could have modulating effect on asthma and play important role in human development. In chapter two, we investigate the association between maternal asthma and serum level of vitamin D in early pregnancy (10-18 weeks) with the risk of preeclampsia development, using data on the outcomes of pregnancy from the “Vitamin D Antenatal Randomized Trial (VDAART, www.vdaart.com)”. VDAART has two primary aims: (1) to determine whether vitamin D supplementation in the pregnant mother is associated with reduced incidence of asthma in the child during the first 3 years of life; and (2) to determine whether a vitamin D dose of 4,400 IU per day versus the standard 400 IU/day in pregnancy is sufficient to maintain mothers’ vitamin D levels in the range of ≥ 75 nmol/L 25(OH)D (Litonjua, Lange et al. 2014). Determining whether sufficient vitamin D supplementation during pregnancy is associated with reduced incidence of preeclampsia is one of the secondary aims of this randomized trial. Accordingly in a secondary analysis, we estimate the risk of preeclampsia development based on the observed cut-offs of vitamin D serum levels during early pregnancy (10-18 weeks). In this study, we apply a purposeful variable selection algorithm (Bursac, Gauss et al. 2008), which estimates the confounding effect of previously, reported preeclampsia risk factors with vitamin D (e.g. asthma, body mass index, race) as well as the predicted risk of preeclampsia.

The potential observation of vitamin D’s association with preeclampsia development in the VDAART trial (chapter two) provides a unique opportunity to use the trial data to investigate the associated gene expression alterations and related functional pathways contributing to the development of preeclampsia in early pregnancy. Microarray expression profiling is useful in identifying susceptibility genes contributing to risk of complex disorders including PE, showing the effect of quantifiable risk factors (Reimer, Koczan et al. 2002, Nishizawa, Pryor-Koishi et al. 2007, Enquobahrie, Meller et al. 2008, Nica and Dermitzakis 2008, Founds, Conley et al. 2009, Sitras, Paulssen et al. 2009, Hoegh, Borup et al. 2010, Enquobahrie, Qiu et al. 2011, Nishizawa, Ota et al. 2011, Meng, Chen et al. 2012). This method has also demonstrated substantial gene expression alterations
during pregnancy (Rochat, Ege et al. 2010). Using this method and the obtained knowledge from the analysis of the earlier chapter, in chapter three, we explore the influence of vitamin D on the expression of genes, potentially involved in the development of preeclampsia. Finally, in chapter four, the importance of our findings in the three previous chapters will be reviewed and the future perspective that this evidence based approach investigation proposes will be discussed.
Chapter I.

Role of Vitamin D in Development, Asthma and Immunomodulation

Abstract

Vitamin D has known effects on lung development and the immune system that may be important in the development, severity and course of allergic diseases (asthma, eczema and food allergy). Vitamin D deficiency is prevalent worldwide and may partly explain the increases in asthma and allergic diseases that have occurred over the last 50-60 years. In this review we explore past and current knowledge on the effect of vitamin D on lung development and immunomodulation and present the evidence of its role in allergic conditions. While there is growing observational and experimental evidence for the role of vitamin D, well-designed and well-powered clinical trials are needed to determine whether supplementation of vitamin D should be recommended in these disorders.
Introduction

Allergic (atopic) diseases result from an interaction between individual genetic susceptibility and exposure to environmental factors. According to twin studies, the genetic contribution to allergic disease has been estimated to be about 50%, with heritability estimates ranging from 36-79% (Jenerowicz, Silny et al. 2012). Since the beginning of the 20th century, allergic disease has shown a continuous upward trend in prevalence such that asthma, atopic dermatitis, and food allergies currently are common chronic conditions in westernized societies (Calderon, Demoly et al. 2012). A multitude of environmental factors, have triggered the steep rise in this trend over the last five decades (Pawankar R, Canonica GW et al. 2011-2012). Accordingly, sensitization rates to one or more common allergens among school children and adults have globally increased and now approach 40%-50% (Pawankar R, Canonica GW et al. 2011-2012). As a result, allergic conditions are the sixth most costly chronic disease category in the United States (1996-2005). Worldwide, health care costs have reached approximately US$ 21, AU$ 7.8 (US$ 7.32), £1 (US$ 1.7) billion annually in the US, Australia and UK, respectively (1996-2005, Gupta, Sheikh et al. 2004, 2007). In Europe, the costs of health care for asthma alone amounts to more than €25 (US$ 34) billion (2003). The number of disability-adjusted life years (DALYs) lost due to asthma worldwide has been estimated to be about 15 million per year. Worldwide, asthma accounts for around 1% of all DALYs lost (Masoli, Fabian et al. 2004).

Along with a clear genetic basis in allergic disease, environmental factors, including early neonatal nutrition, may have an important influence on allergy development and, thus, present an opportunity to prevent or delay the onset of the disease (Greer, Sicherer et al. 2008, Jenerowicz, Silny et al. 2012). Vitamin D may be an important environmental factor. Evolutionarily, early humans evolved in sun-rich environments and the increased efficiency of vitamin D production in the skin is thought to be a major driving factor in human skin depigmentation as humans migrated away from the equatorial areas (Jablonski and Chaplin 2000, Chaplin and Jablonski 2013). 7-Dehydrocholesterol (7-DHC) in the skin is converted to previtamin D₃ after exposure to UVB rays, and is then transformed to vitamin D₃ (cholecalciferol) by a thermally induced isomerization. Vitamin D₃ can also come from the gut, via diet or supplements. Vitamin D₃ in circulation then undergoes hydroxylation...
in the liver to 25-hydroxyvitamin D\textsubscript{3} (25OHD), which then is hydroxylated further in the kidney to its biologically active form 1,25-dihydroxyvitamin D\textsubscript{3} (1,25(OH)\textsubscript{2}D or calcitriol) (Lips 2006, Holick 2007). Henceforth in this review, the term “vitamin D” will refer to 1,25(OH)\textsubscript{2}D, unless otherwise stated. Vitamin D mediates its biological effect through the vitamin D receptor (VDR) which was discovered to be present in a variety of tissues, suggesting the importance of the vitamin D system in various cellular and tissue functions (Kato 2000, Lips 2006, Prentice, Goldberg et al. 2008, Borradale and Kimlin 2009, Plum and DeLuca 2010).

Hence, over the past few years, researchers have paid a great deal of attention to the effect of vitamin D on immunologic mechanisms as one potentially modifiable environmental factor (Hossein-nezhad and Holick 2013, Wacker and Holick 2013). This is mostly due to the advent of the ability to easily measure 25OHD levels and other vitamin D metabolites in serum or plasma and the growing recognition that vitamin D insufficiency (defined as 25OHD levels of less than 30 ng/ml) exists in most populations around the world (Lips 2010). In the US, there is evidence to show that vitamin D insufficiency has increased almost 2-fold over a span of about 10 years, from the late 1980s and early 1990s through the early 2000s (Ginde, Liu et al. 2009). Indeed, it has been proposed that vitamin D insufficiency has contributed to the rise of asthma and allergic disease, (Litonjua and Weiss 2007) and various biological mechanisms for how vitamin D may play a role in the development and treatment of asthma and allergies have been proposed (Litonjua 2012). Historically, Rappaport and colleagues (Rappaport and Reed 1933, Rappaport, Reed et al. 1934) investigated the modifying effect of vitamin D on allergic conditions as early as the 1930s and reported relief of symptoms in a majority of allergic patients treated with viosterol (a vitamin D preparation produced by the irradiation of ergostrol) compared to controls, a finding subsequently supported by several studies (Jakso 1950, Canon 1951, Utz and Hauck 1976, Reeve, Meunier et al. 1980, Litonjua 2012). These studies have also shown that the determinants of vitamin D status are multifactorial and a nonlinear relationship has been shown between serum levels and biological effects (Hypponen, Berry et al. 2009, Litonjua 2012). However, these studies need to be replicated using more rigorous and modern methods. There is controversy over what levels are optimal for overall health. However, the serum level of 25OHD 30–40 ng/mL (75–100 nmol/L) has been suggested as a lower threshold of an optimal serum level for the immune effects of vitamin D (Bischoff-Ferrari, Giovannucci et al. 2006, Holick 2007, Vieth, Bischoff-Ferrari et al. 2007).
According to the lower defined threshold value for bone health (Holick, Binkley et al. 2011, Rosen, Abrams et al. 2012), more than one-third of the population worldwide may have low levels of vitamin D (25OHD < 20 ng/mL [50 nmol/L]) (Hilger, Friedel et al. 2014). This fact highlights the potential for risk modification of low vitamin D on the worldwide increase in rates of allergic sensitization.

Methods

Using a structured approach to identify the source of materials for the review, a systematic search was conducted for relevant peer-reviewed articles in PubMed and the Google Scholar search engine, using the keywords ‘vitamin D and asthma, ‘vitamin D and atopy’, ‘vitamin D and development’, ‘vitamin D and allergy’, ‘vitamin D and pregnancy’, ‘vitamin D and fetal development’, ‘vitamin D and newborn’, vitamin D and immunomodulation’, ‘vitamin D and early life’, ‘vitamin D asthma gene’, ‘vitamin D and gene expression’, vitamin d asthma and gene expression’, and ‘vitamin D pregnancy gene expression’. References of the relevant articles or editorials were also considered for potential bibliographic related references to avoid any missing publication. All searches were limited to research published in English. There was no restriction on time of publication. The main focus of the search was clinical studies.
Results

Vitamin D and immunomodulation related to atopy

Vitamin D has immunomodulatory effects on allergen-induced inflammatory pathways (Hossein-nezhad and Holick 2013) by acting on VDR expressed on a variety of immune cells, including B cells, T cells, dendritic cells and macrophages (Provvedini, Tsoukas et al. 1983, Adorini, Penna et al. 2004). Many of these cells, such as activated macrophages and dendritic cells, are capable of synthesizing biologically active vitamin D from circulating 25OHD (Barbour, Coburn et al. 1981, Adams, Sharma et al. 1983, Adams and Hewison 2008). This mechanism, extrarenal expression of CP27B, enables immune cells to rapidly increase local levels of vitamin D, potentially needed to shape adaptive immune responses (Barbour, Coburn et al. 1981, Adams, Sharma et al. 1983, Baeke, Takiishi et al. 2010). In this context, a clinical study involving mild allergic asthmatics who underwent segmental allergen challenge showed a significant increase in vitamin D receptor binding protein (VDBP) and 25OHD in bronchoalveolar lavage fluid (but not in their serum levels) 24hrs after allergen challenge, thus, suggesting a role for vitamin D mediated immune responses in the local asthmatic late-phase reaction (Bratke, Wendt et al. 2014).

Vitamin D has shown the ability to inhibit both Th1- and Th2-type responses by suppressing both the production of IL-12-generated IFN-γ as well as IL-4 and IL-4-induced expression of IL-13 (Pichler, Gerstmayr et al. 2002). This ability could be of importance since the balance of Th1 and Th2 affects the pattern of immune response. While asthma is thought to be a Th2 dominant condition and is largely characterized by the production of cytokines such as IL-4, IL-5, IL10 and IL-13, and the production of IgE by B cells (Holgate 2008, Holgate and Polosa 2008, Vasiliou, Lui et al. 2014), some experimental and human studies do not support a unidirectional effect (either inhibition alone or enhancement alone) of vitamin D on adaptive T cell responses (Boonstra, Barrat et al. 2001, Staeva-Vieira and Freedman 2002, Mahon, Wittke et al. 2003, Lange, Litonjua et al. 2009, Litonjua 2012). For example, one study investigated the associations of circulating 1,25(OH)₂D on Th1/Th2 serum markers in patients with concomitant nasal polyps and allergic rhinitis. They found a negative correlation between plasma 1,25(OH)₂D with IgE and IL-4 levels, and a positive correlation between
1,25(OH)₂D with IFN-γ (Ozkara, Keles et al. 2012).

The differences in the observations on Th1-Th2 balance among allergic conditions has been attributed to variation in the baseline vitamin D status, timing of exposure to allergen, and chronicity of vitamin D administration relative to sensitization (Litonjua 2012). The type and concentration of allergen as well as the balance of immune responses (Th1 cytokines/Th2 cytokines) to different types of allergens are additional factors contributing to variability in response (Tiemessen, Van Ieperen-Van Dijk et al. 2004, Larche 2007). How the absolute change in vitamin D levels from the baseline vitamin D status over a time period might affect the balance of Th1/Th2 cytokines in response to various allergens is a matter for further investigation. It has been proposed that in the absence of vitamin D signaling, the T cell compartment has a potentially stronger Th1 phenotype and that at pharmacological levels, vitamin D suppresses both Th1 and Th2 immune responses (Cantorna, Zhu et al. 2004, Searing and Leung 2010).

Vitamin D has potent antiproliferative effects on CD4⁺ T cells as well as the ability to inhibit T lymphocyte function, both directly, and via effects on antigen-presenting cells (APCs) (Griffin, Xing et al. 2003, Mahon, Wittke et al. 2003). Th1-cell responses are responsible for some of the pathogenic features in allergic patients particularly chronic features, including epithelial cell apoptosis, smooth-muscle-cell activation and may contribute to mucus secretion (Holgate and Polosa 2008). In addition to inhibiting Th1-associated pro-inflammatory cytokines (Reichel, Koeffler et al. 1987, Griffin, Xing et al. 2003, Mahon, Wittke et al. 2003, Matheu, Back et al. 2003, Adorini, Penna et al. 2004), vitamin D has been shown to act on Th17 cells to suppress the production of IL-17 (Litonjua and Weiss 2007, Louten, Boniface et al. 2009). Additionally, Rausch-Fan et al. has shown that vitamin D modulates cytokine production in human peripheral blood mononuclear cells and allergen-specific Th cell clones; an effect which was time and concentration dependent (Rausch-Fan, Leutmezer et al. 2002).

Regulatory T cells (Tregs), including naturally occurring and induced Tregs, play an important role in
maintaining immune homeostasis in response to allergen exposure by suppressing Th2 mediated inflammation such as airway eosinophilia, mucous hyper-secretion, and airway hyper-responsiveness (Hawrylowicz 2005, Larche 2007, Robinson 2009). Tregs use multiple suppressive mechanisms, including IL-10 and TGF-beta as well as cytotoxic T lymphocyte antigen 4 and programmed death 1 as surface molecules to regulate downstream immune activation (Akdis, Verhagen et al. 2004). Vitamin D can induce antigen specific IL-10–producing Tregs, that express low levels of the CD4+CD25+ Treg-associated transcription factor FoxP3 (Barrat, Cua et al. 2002, Baris, Kiykim et al. 2014). Consequently, secreted IL-10 inhibits inappropriate allergen specific Th2-driven immune responses and regulates allergic sensitization (Hawrylowicz 2005, Xystrakis, Kusumakar et al. 2006). Similarly, allergen immunotherapy and glucocorticoid therapy for allergy and asthma have shown increased IL-10 synthesis by Tregs (Akdis, Blesken et al. 1998, Hawrylowicz, Richards et al. 2002, Xystrakis, Kusumakar et al. 2006). Vitamin D supplementation of cholecalciferol (140,000 IU) has been also associated with an increased Tregs frequency (%Tregs) in apparently healthy individuals with vitamin D insufficiency after 4 weeks (Prietl, Pilz et al. 2010). However, Chi et al. demonstrated that cord blood 25OHD levels were inversely associated with the proportion of CD25+, CD25bright, and CD25+FoxP3 T-regulatory (Chi et al. 2011). Thus, it is likely that the effect of vitamin D on Tregs is complex and depends on the contextual cellular milieu and factors such as the age of the individual, pregnancy, as well as health and disease status.

Vitamin D and its receptor (VDR) are both essential for development of Natural Killer (NK) cells and the expression of IL-4 and IFN-γ production (Yu and Cantorna 2008). NK cells contribute to the development of T-cell mediated allergic airway inflammation (Korsgren, Persson et al. 1999, Wingett and Nieelson 2003, Ple, Barrier et al. 2010) and are capable of producing numerous pro-inflammatory cytokines such as IFN-γ, TNF-α, GM-CSF, and MIP-1a upon IgE stimulation and exhibit cytotoxicity against IgE-coated target cells through FcγRIII (Karimi and Forsythe 2013). Natural killer T (NKT) cells are a distinct subset of T lymphocytes that can produce both Th1 (IFN-γ) and Th2 (IL-4) cytokines and have been investigated for their role in asthma and allergy (Benlagha, Kyin et al. 2002, Akbari, Stock et al. 2003, Lisbonne, Diem et al. 2003). Du et al. studied the interaction of low vitamin D levels on asthma exacerbations (Du, Litonjua et al. 2012). They identified three
associated common variants in the class I MHC–restricted T cell–associated molecule gene (CRTAM), which is highly expressed in activated human CD8+ and NKT cells. Their findings implicate a mechanism by which vitamin D might prevent asthma exacerbations through CD8+ and NKT cells, particularly during viral infections.

**Effect of vitamin D on development (fetal lung & immune system) and normal function**

*Impact on pregnancy and fetal development*

The Barker hypothesis first posited that environmental influences early in development and during intrauterine life, could increase the risk of chronic disease later in life (Barker and Osmond 1986). Numerous epidemiological studies have shown strong associations between maternal diet and altered risk of chronic disease, particularly asthma (Burke, Leonardi-Bee et al. 2012, Montefort, Ellul et al. 2012, Peters, Boynton-Jarrett et al. 2013). Active vitamin D (1,25(OH)2D) dramatically rises during pregnancy, with levels reaching up to 124% to 135% of normal values (Papapetrou 2010, Brannon and Picciano 2011). As a result, the role of vitamin D during pregnancy has recently come to the forefront as studies have documented an increased risk to maternal and fetal health in relation to low serum levels of 25OHD (Liu and Hewison 2012).

In addition to the well-described skeletal effects of vitamin D, such as calcium homeostasis (Miller, Halloran et al. 1982, Wasserman, Smith et al. 1992) and bone development, it may promote proper fetal implantation and regulates placental development (Halhali, Acker et al. 1991, Rebut-Bonneton and Demignon 1991). Moreover, vitamin D regulates immune responsiveness by enhancing the activity of immunosuppressive Tregs and suppresses pro-inflammatory Interleukin-17 expressing T_h17 cells (Barrat, Cua et al. 2002, Joshi, Pantalena et al. 2011). In addition, vitamin D is well known for its role in regulating the expression of the neutrophil associated calethicidin antimicrobial protein (C-AMP) as well as numerous defensins molecules, particularly beta defensin 1 (Wang, Nestel et al. 2004). Collectively, these findings suggest that vitamin D has immunosuppressive effects in placental tissue and promotes enhanced protection of the mother and fetus against infection.
Studies in animals and humans have shown that circulating levels of maternal 25OHD crosses the placenta freely. Importantly, placental trophoblast cells express the VDR and CYP27B1 (1aOHase) and hence are capable of converting 25OHD into the active hormonal form (Henry and Norman 1984, Liu, Kaplan et al. 2011). Several observational studies suggest that low 25OHD level promotes intra-uterine growth restriction and low birth weight and has modest effects on pre-term birth (Gernand, Simhan et al. 2013, Bodnar, Klebanoff et al. 2014). Thus, mounting evidence indicates that vitamin D is necessary for normal fetal development and organ function, although the molecular mechanism by which fetal vitamin D levels influence the complex fetal development pathways remain unclear (Brannon 2012).

**Association with lung development**

In addition to immunomodulatory effects during fetal development, vitamin D also has known genomic effects and thus the ability to influence fetal lung development (Liu and Hewison 2012, Kho, Sharma et al. 2013). Experimental evidence in rats show that fetal alveolar type II epithelial cells express VDR, suggesting that pulmonary maturation is responsive to vitamin D exposure (Marin, Dufour et al. 1990, Nguyen, Guillozo et al. 1990, Marin, Dufour et al. 1993). In humans, Kho et al. recently examined gene expression profiles during human fetal lung development and identified a number of genes associated with the vitamin D pathway whose expression was developmentally regulated (Kho, Sharma et al. 2013). Although the exact role these vitamin D related genes play in fetal lung development remains to be fully explored, several genes (LAMP3, PIP5K1B, SCRAB2 and TXNIP) were also found to be significantly overexpressed in cells derived from asthmatic children, thus, suggesting a link between vitamin D pathway genes, fetal development and asthma (Kho, Sharma et al. 2013). Studies in mice and human performed by Zosky et al. further support the notion that maternal vitamin D deficiency during fetal lung development may impact early life lung structure and function and increase the risk of chronic lung disease later in life (Zosky, Berry et al. 2011, Zosky, Hart et al. 2014).
Correlation of serum vitamin D of mother and newborn

Studies show a strong correlation between maternal 25OHD levels and cord blood 25OHD levels of neonates. In a recent study of 107 pregnant mother-infant pairs, cord blood levels of 25OHD were shown to be approximately $62 \pm 16\%$ of maternal levels and correlated positively with maternal 25OHD levels ($r=0.83$, $P<0.001$) (Vieth Streym, Kristine Moller et al. 2013). The influence of vitamin D on early childhood health and growth has emerged as an area of great interest (Thorne-Lyman and Fawzi 2012). In this context, infections, including respiratory infections, are an important cause of neonatal childhood morbidity and mortality and, in this regard, several studies have documented a correlation between deficiencies in 25OHD levels in early life with increased rates of respiratory infections (Bergman, Lindh et al. 2013). This is of particular interest, as respiratory infections in the first years of life have been associated with an increased risk of allergic asthma in young children (Sly, Kusel et al. 2010).

Interaction of Asthma and atopy

The prenatal and early life period have been identified as “windows of opportunity” during which immune responses can be permanently programed. The role of vitamin D during this period in the prevention of asthma and allergies in children remains controversial. Positive and negative results of several epidemiologic studies have been published (Litonjua 2012). Reasons for these differing results are numerous, and can be gleaned from patterns of the results that have emerged. Firstly, all the published studies to date that have reported a protective effect of vitamin D have all assessed vitamin D intake in pregnancy from food frequency questionnaires (Camargo, Rifas-Shiman et al. 2007, Devereux, Litonjua et al. 2007, Erkkola, Kaila et al. 2009, Miyake, Sasaki et al. 2010), which are known to be an indicator of long-term diet pattern (Willett and Hu 2007), hence long-term intakes of vitamin D. Next, studies that have assessed either 2nd and 3rd trimester maternal vitamin D status (via 25OHD levels) (Miyake, Sasaki et al. 2010, Cremers, Thijs et al. 2011, Thomas, Fudge et al. 2011, Maslova, Hansen et al. 2013, Wills, Shaheen et al. 2013) or cord blood 25OHD levels (Camargo, Ingham et al. 2011, Liu, Wang et al. 2011, Rother, Wright et al. 2011, Jones, Palmer et al. 2012, Weisse, Winkler et al. 2013, Chawes, Bonnellykke et al. 2014) have not shown a consistently protective effect on asthma and allergies. Since 25OHD
is known to fluctuate by season (Bolland, Grey et al. 2007, Holick and Chen 2008, Kasahara, Singh et al. 2013) and the intraclass correlation coefficient over several years of 25OHD levels is only on the order of 0.3 (Lasky-Su, Lange et al. 2012), it is likely that a one-time assessment of vitamin D status is not sufficient for the question of whether vitamin D can prevent asthma or allergies. Additionally, the ineffective trial of vitamin D supplementation in the 3rd trimester to show effect modification on clinical characteristics of asthma and allergies, underscores the fact that intervention in the 3rd trimester may be too late for these outcomes. The only observational study that showed that maternal vitamin D status was protective for asthma measured 25OHD levels in the first trimester (Zosky, Hart et al. 2014). The studies that used Food Frequency Questionnaires (FFQs) are consistent with this notion since these are markers of long-term intakes encompassing the first trimester. Thus, vitamin D supplementation trials that begin earlier in pregnancy are needed. One such trial, The Vitamin D Antenatal Asthma Reduction Trial (VDAART): a randomized controlled trial of vitamin D supplementation in pregnancy for the primary prevention of asthma and allergies in children, is currently underway (ClinicalTrials.gov: NCT00920621).

**Role of vitamin D in prevention and modification of asthma and atopy**

*After pregnancy and during early life*

1. **Asthma**

Vitamin D may potentially decrease the severity of asthma and allergies through a variety of mechanisms (figure 1) including effects on immune cells, improved handling or prevention of predisposing infections (Sabetta, DePetrillo et al. 2010, Urashima, Segawa et al. 2010, Majak, Olszowiec-Chlebna et al. 2011, Hossein-nezhad and Holick 2013), decreased inflammatory responses, improved lung function (Black and Scragg 2005, Devereux, Wilson et al. 2010, Li, Peng et al. 2011), effects on airway smooth muscle function and mass, reduced airway remodeling (Umland, Schleimer et al. 2002, Hossein-nezhad and Holick 2013) and reversal of steroid resistance (by IL-10 production and modifying ligand-induced down-regulation of glucocorticoid receptors (Xystrakis, Kusumakar et al. 2006). Several studies have investigated the relationship between vitamin D deficiency and asthma exacerbations. A low level of vitamin D at age 6 was associated with increased allergies and asthma at age
14 in one study from Australia and serum 25OHD levels in children of both ages were negatively associated with concurrent allergic phenotypes in males (Hollams, Hart et al. 2011). In 616 Costa Rican children with asthma aged 6-14 years, Brehm et al. found that 28% of the children had insufficient 25OHD levels. On a log scale, 25OHD level was associated with reduced risks of any hospitalization for asthma in the previous year (odds ratio [OR], 0.05) and any use of anti-inflammatory medications in the previous year (OR= 0.18). In addition, 25OHD levels were significantly and inversely associated with total IgE and eosinophil count (Brehm, Celedon et al. 2009). Brehm et al., using collected data in 1024 participants of the Childhood Asthma Management Program (CAMP), a placebo-controlled randomized trial of inhaled budesonide vs. nedocromil, showed that vitamin D insufficiency (25OHD <30 ng/ml) was associated with higher risks for severe asthma exacerbations leading to ED visits or hospitalizations (Brehm, Schuemann et al. 2010). There was a greater effect among children who were randomized to the inhaled budesonide arm, suggesting an interaction between vitamin D and corticosteroid use. Searing et al. (Searing, Zhang et al. 2010) in a cross-sectional study of 100 asthmatic children, showed that 25OHD levels were inversely associated with serum IgE, number of skin prick tests to perennial aeroallergens, lung function, and use of inhaled or oral corticosteroids. In-vitro studies using peripheral blood mononuclear cells showed that vitamin D amplified glucocorticoid induction of mitogen-activated protein kinase phosphatase (MPK)-1 and IL10, which are critical for anti-inflammatory and immunosuppressive effects. In a study of 54 adult asthmatics, subjects with vitamin D insufficiency had lower lung function and increased airway hyperresponsiveness (Sutherland, Goleva et al. 2010). In addition, 25OHD levels in this study were inversely correlated with tumor necrosis factor (TNF)-α expression and positively correlated with dexamethasone-induced MPK-1 expression. Other studies have also found that lower 25OHD levels are associated with poor asthma control (Chinellato, Piazza et al. 2011), lower lung function and the presence of exercise-induced bronchoconstriction (Chinellato, Piazza et al. 2011) in asthmatic children, and the severity of atopic dermatitis (Peroni, Piacentini et al. 2011).

Bosse et al. (Bosse, Maghni et al. 2007) have shown in vitro that vitamin D increases glucocorticoid bioavailability in bronchial smooth muscle cells suggesting a further beneficial role for vitamin D in the
prevention and treatment of asthma. This interaction between vitamin D and corticosteroids was investigated in a recent clinical trial. In “Vitamin D Add-on Therapy Enhances Corticosteroid Responsiveness in Asthma (VIDA)”, Castro et al. investigated if taking vitamin D in addition to a standard asthma medication would prevent the worsening of asthma symptoms or attacks (Castro, King et al. 2014). In their study, adult asthmatics were randomly received an initial dose of 100,000 IU of oral cholecalciferol followed by 4000 IU/day for 28 weeks or placebo (n = 201 vs. 207) while using inhaled ciclesonide (Alvesco).

After a 12-week inhaled corticosteroids stability phase with 320 µg/d of ciclesonide, the dose of ciclesonide was reduced to 160 µg/day for 8 weeks, followed by maintenance dose of 80 µg/day for another 8 weeks. Although they found that vitamin D supplementation had no significant effect on the overall rate of first treatment failure or exacerbation in patients with asthma and low vitamin D levels, at 28 weeks, there was a significant difference in cumulative ciclesonide dosing between the vitamin D and placebo groups (111.3 vs. 126.2 µg/day; P = .02). In addition, there was significant reduction in the overall asthma treatment failure and the exacerbations in subjects that achieved a normal vitamin D level. This trial was likely underpowered for the primary outcomes of first treatment failure, as the authors acknowledged that there was a lower than expected event rate in the control group. Furthermore, several observational studies have already established that the interaction between corticosteroids and vitamin D in asthma is much stronger in children than in adults with asthma (Sutherland, Goleva et al. 2010, Goleva, Searing et al. 2012), thus well-designed and well-powered trials in children are needed.

II. Other atopic diseases: Atopic dermatitis, allergic rhinitis and food allergy

Several clinical, genetic and experimental studies suggest that prior history of atopic dermatitis (AD) and its severity are a major risk factors for the development of allergic rhinitis, asthma and specific sensitization, highlighting the importance of the epidermal barrier in the pathogenesis of these allergic disorders (Zheng, Yu et al. 2011). Low levels of vitamin D appear to be inversely correlated with AD severity, and vitamin D deficiency at birth is associated with higher risk of developing AD. Also, a pilot randomized trial of vitamin D supplementation in children demonstrated a favorable effect on AD symptoms during winter months (Sidbury,
Sullivan et al. 2008). It is possible that this effect was mediated by the induction of endogenous antimicrobial peptides in the skin in AD by oral vitamin D supplementation (Hata, Kotol et al. 2008).

Few studies have investigated vitamin D status in allergic rhinitis and food allergy. Allergic rhinitis has been shown to be a risk factor for developing asthma (Semper, Heron et al. 2003, Ciprandi, Cirillo et al. 2004, Spergel 2005). Ciprandi et al. (Ciprandi, Cirillo et al. 2004) showed that nasal symptoms, airflow, and markers of inflammation directly correlate with lower airway markers including forced expiratory volume in 1 second (FEV\(_1\)). Leynaert et al. (Leynaert, Neukirch et al. 2004) found that approximately 75% of asthmatics report rhinitis; patients with rhinitis have increased risk for asthma and lower airway reactivity compared to patients without rhinitis; and the risk for asthma increases from 2.0% in subjects without rhinitis to 18.8% in subjects with allergic rhinitis when exposed to either pollen or animal dander. Mai et al. (Mai, Chen et al. 2014) also investigated the relationship between serum 25OHD and the incidence of allergic rhinitis in adults. The study included a random sample (N=1351) from an adult population who participated in Nord-Trøndelag Health Study (HUNT). In the 11-year follow-up of the subjects, they showed that 9% of men and 15% of women developed allergic rhinitis. Among men, serum 25OHD level <50 nmol/L at baseline was associated with an increased risk of allergic rhinitis (adjusted OR 2.55, \(p=0.001\)); each 25 nmol/L reduction in 25OHD level was associated with an adjusted OR of 1.84. However, women had lower risk of allergic rhinitis with adjusted OR of 0.83 (\(p>0.05\)) for each 25 nmol/L reduction in serum 25OHD level.

It has been suggested vitamin D deficiency might impair epithelial barrier integrity, that in turn leads to increased and inappropriate mucosal exposure to food antigens and also a pro-sensitization immune imbalance that compromises immunological tolerance (Roider, Ruzicka et al. 2013). Consequently, early correction of vitamin D deficiency might promote mucosal defense, maintain healthy microbial ecology and allergen tolerance, and decrease risk of food allergies in children (Gale, Robinson et al. 2008, Hata, Kotol et al. 2008). Food allergy is a known provoking cause of AD and the prevalence of IgE-mediated food allergy is about 35% in children affected with AD. Kull et al. showed vitamin D in water-soluble form increased the risk of allergic disease in
children of less than 4 years old compared with supplementation of vitamin D given in peanut oil (Kull, Bergstrom et al. 2006). This study justifiably raised questions about the varying results of vitamin D supplementation on risk modification of food allergy and other allergic diseases (Reinholz, Ruzicka et al. 2012). However, the study did not measure baseline nor follow-up vitamin D levels. Sharief et al. (Sharief, Jariwala et al. 2011) showed that higher levels of IgE sensitization were associated with vitamin D deficiency in children and adolescents. Accordingly, 25(OH)D levels of less than 15 ng/mL were associated with evidence of sensitization to various allergens. For example the odds ratios of allergy for peanut, ragweed, and oak were 2.39, 1.83 and 4.75, respectively (Sharief, Jariwala et al. 2011). Similarly, mean 25(OH)D serum levels have been lower in moderate and severe atopic dermatitis (AD) children, suggesting potential benefit of serum level correction using vitamin D supplementation in AD (Peroni, Piacentini et al. 2011). Allen et al. (Allen, Koplin et al. 2013) demonstrated that infants of Australian-born parents with vitamin D insufficiency (25OHD ≤50 nmol/L) had higher risk of peanut (OR, 11.51) and/or egg (OR, 3.79) allergy than those with adequate vitamin D levels. They were also more likely to have multiple food allergies (≥2) rather than a single food allergy (OR=10.48 vs. OR=1.82) (Allen, Koplin et al. 2013). Moreover, Mullins et al. (Mullins, Clark et al. 2011), similar to Vassallo et al. (Vassallo, Banerji et al. 2010), reported significantly higher rates of food allergy in children born in the autumn/winter, suggesting a relationship between relative food allergy rates and monthly sun exposure.
Discussion

There remains a great deal of controversy as to the role of vitamin D in overall health and what represents adequate levels of vitamin D in the blood for human health generally (Weiss and Litonjua 2011) and specifically for each of the reviewed conditions. However, there is sufficient suggestion of a benefit of raising vitamin D levels to encourage more studies in this field. Future interventional and longitudinal cohort studies are needed to establish whether changes in maternal nutrient intake during pregnancy can be used as a healthy low-cost public health measure to reduce the incidence of childhood asthma and atopy (Bozzetto, Carraro et al. 2012). Such studies will be very important in shedding light on the role of vitamin D in fetal development. More studies in established asthma are also necessary. Clinical trials investigating the role of vitamin D in development and modifying the severity of asthma and in controlling exacerbations need to be adequately powered, use the appropriate dose, and need to be of sufficient duration. Furthermore, a focus on children with persistent disease also seems to be appropriate. These trials should perform additional analyses that attempt to identify the appropriate vitamin D level that provides the maximal beneficial effects.

Vitamin D may regulate epigenetic events (Sundar and Rahman 2011) that promote allergic conditions (Wjst 2012). Hence, studies on molecular genetic and epigenetic mechanisms of vitamin D in allergic diseases and disease severity (immunoinflammatory responses, steroid resistance, host defense) particularly in response to high and low vitamin D supplementation would provide mechanistic insights in the management and prevention of allergic diseases. As an example, a study in healthy adults who received either 400 or 2000 IU/d of vitamin D3 for 3 months in winter demonstrated the up or down-regulation of 291 genes by vitamin D intake. That these genes affected as many as 80 different metabolic pathways, including immune modulation and enhanced antioxidant activity, emphasizes the potential importance of vitamin D status on transcriptional regulation (Hossein-nezhad and Holick 2013, Hossein-nezhad, Spira et al. 2013). Thus, these clinical trials will also need to collect the appropriate specimens and consent to perform these genomic studies. These clinical and genomic studies will help to clarify the potential role of vitamin D on both the development and modulation of asthma
and allergies.

VDAART study as an example of such studies provides the unique opportunity of investigating the hypothesis of interest “effect of vitamin D and asthma in preeclampsia development” from both epidemiologic and genomic aspects. The conducted literature evidence-based review in this chapter provides a proof of concept for the association of low vitamin D status with asthma and immunologic mechanisms. More importantly it shows that vitamin D status affects some biological mechanisms required for fetal development during early time of pregnancy. Accordingly, in the next chapter and using a sophisticated methodology, we investigate the interaction of vitamin D status with asthma in the development of preeclampsia (Figure 2).
Figure 1. Potential effects of vitamin D in asthma and atopy development or modification. Several studies support the modification role of vitamin D in asthma and allergy. However, preventive effects of vitamin D in risk reduction of asthma and allergy development during pregnancy and early life requires more well designed longitudinal studies.
Figure 2. The literature review provided the links between asthma, low vitamin D status and preeclampsia. The depiction of the associations is suggestive of a confounding effect of either asthma or vitamin D in the development of preeclampsia. In chapter two we examine this hypothesis using an appropriate statistical model and conduct risk estimation for the identified predictor.
Maternal Vitamin D Deficiency, but Not Asthma Increases Risk of Preeclampsia
-The VDAART Trial-

Abstract

Preeclampsia (PE) complicates from 3 to 7% of pregnancies and has globally remained a major public health problem, often resulting in maternal-fetal or neonatal complications including death. PE is a heterogeneous disorder such that the clinical presentation and outcome could vary depending on different risk factor profiles. Better knowledge of these risk factors and their biological effects during pregnancy would lead to progress in prevention of PE. Low maternal levels of vitamin D (25OHD) and maternal asthma exacerbations have both been reported to be associated with increased risk of PE. However, their simultaneous potential independent effects have not been studied. We, therefore, investigated the relation of maternal asthma and serum level of vitamin D in early pregnancy in association with PE using the data on the outcomes of pregnancy from the Vitamin D Antenatal Asthma Reduction Trial (VDAART).

819 subjects with complete data on the outcome of pregnancy (PE) and potential risk factors were analyzed. A completed questionnaire at enrollment and monthly thereafter in addition to medical record review provided information on maternal health characteristics and variables of interest. A group of experienced obstetricians and gynecologists from three clinical centers validated PE diagnoses. Vitamin D (25OHD) was measured at 10-18 weeks of gestation. The Asthma Activity Test (ACT) was used to assess asthma symptoms during pregnancy. A purposeful variable selection algorithm was applied to investigate the risk factors of interest (vitamin D, asthma, uncontrolled asthma) and the effect of potential confounders.
Seventy-four pregnant women developed a PE event (9%). The frequency of PE was not affected by the history of asthma (45% and 40% among women with PE and without, respectively). Higher odds of uncontrolled asthma events were observed in women with PE (OR: 1.31; 95% CI: 1.01-1.65). However, after adjustment for maternal vitamin D serum level, race, total number of pregnancies, body mass index, study site and time, the effect was not significant (aOR: 1.22; 95% CI: 0.91-1.6). Lower vitamin D status was found to be associated with increased risk of PE, such that a 20-ng/ml decline in 25OHD concentration showed a 2.7 fold increase in odds of PE (aOR: 2.7, 95% CI: 1.4-5.5), with the least imposed risk being at serum concentrations of 40-50 ng/ml.

Maternal vitamin D level in early pregnancy is a major modifiable risk factor of PE during early pregnancy. A vitamin D serum level of 40-50 ng/ml might provide the optimal concentration for the greatest risk reduction of PE. Early detection of low vitamin status and adequate vitamin D supplementation as a safe and effective preventive measure for PE and its maternal-fetal consequences are recommended. Future randomized controlled trials of vitamin D to prevent PE are warranted.
Introduction

Hypertension is a common finding in pregnancy that complicates 10% or more of pregnancies (Samadi and Mayberry 1998, Wagner, Barac et al. 2007, Craici, Wagner et al. 2008, Lindheimer, Taler et al. 2010, Hutcheon, Lisonkova et al. 2011, Burris, Rifas-Shiman et al. 2014) and has been shown to be increasing in US (Wallis, Saftlas et al. 2008, Berg, Mackay et al. 2009, Kuklina, Ayala et al. 2009, Ananth, Keyes et al. 2013). The incidence of hypertensive disorders during pregnancy (HDP) has increased from 67.2 per 1000 deliveries in 1998 to 81.4 per 1000 deliveries in 2006, along with an enhanced burden of severe obstetric morbidity (Kuklina, Meikle et al. 2009). According to the Hospital Cost Utilization Project (HCUP) Nationwide Inpatient Survey (NIS), the healthcare cost of HDP was staggering $4.94 billion in the United States (US) with approximately 245,160 pregnant women admitted to the hospital, with an average stay of 3.6 days in 2012. The average per-person charge for such hospital admissions totaled $20,160, up from $6664 in 1997.

Preeclampsia (PE) is the most common hypertensive disease of pregnancy, showing an incidence that varies by geographic region and ethnicity (Caughey, Stotland et al. 2005). Geographic variation in the prevalence of PE in the US is widespread (Wallis, Saftlas et al. 2008) such that PE complicates from 3 to 7% of pregnancies with a 1.5 to 2-fold higher incidence in first pregnancies (Smith, Walker et al. 1997, Lie, Rasmussen et al. 1998, Sibai, Lindheimer et al. 1998, Li and Wi 2000, Basso, Christensen et al. 2001, Skjaerven, Wilcox et al. 2002, Hernandez-Diaz, Toh et al. 2009, Hutcheon, Lisonkova et al. 2011, Lisonkova and Joseph 2013, Thornton, Dahlen et al. 2013), however, in other areas of the world, such as Africa and Latin America, the incidence was reported to be up to 20% (Stewart, Kampman et al. 2009, Bilano, Ota et al. 2014). The disease not only affects pregnancy outcome such as altered fetal growth, but also predisposes both mother and child to long-term health complications such as cardiovascular disease (Irgens, Reisaeter et al. 2001, Smith, Pell et al. 2001, Ray, Vermeulen et al. 2005, Bellamy, Casas et al. 2007, Craici, Wagner et al. 2008). The maternal and fetal morbidity and mortality associated with preeclampsia and in particular with the adverse consequences of pre-term delivery are a major

The risk of PE development varies by the underlying mechanism. Due to its heterogeneous nature, the pathophysiology could vary depending on different risk factor profiles. (Conde-Agudelo and Belizan 2000, Duckitt and Harrington 2005, Bhattacharya, Campbell et al. 2009, Bilano, Ota et al. 2014). This complexity leads to a spectrum of clinical presentations and variable adverse outcomes (Lisonkova and Joseph 2013). In the US, overall PE incidence rose from 3.4% in 1980 to 3.8% in 2010 (Ananth, Keyes et al. 2013). This noticeable variation over time has led to speculation that the change in population level distribution of risk factors may have influenced this trend. Accordingly, better knowledge of these risk factors and their biological effects during pregnancy could lead to significant progress in prevention of PE.

Asthma prevalence increased from 7.3% in 2001 to 8.4% in 2010. In adulthood, females had traditionally and consistently higher rates of asthma than males. In 2011, females were about 14.0% more likely than males to ever have been diagnosed with asthma (Hickey, O'Connor et al. 1987). Some epidemiologic studies have provided evidence on the association of vitamin D (25OHD) levels with asthma prevalence in general population samples (Brehm, Acosta-Perez et al. 2012, Paul, Brehm et al. 2012, Korn, Hubner et al. 2013, Poon, Mahboub et al. 2013, Mirzakhani, Al-Garawi et al. 2014). Furthermore, several studies have shown that maternal asthma and its activity during pregnancy might increase the risk of PE (Stenius-Aarniala, Piirila et al. 1988, Perlow, Montgomery et al. 1992, Stenius-Aarniala, Riikonen et al. 1995, Demissie, Breckenridge et al. 1998, Wen, Demissie et al. 2001, Sobande, Archibong et al. 2002, Triche, Saftlas et al. 2004, Acs, Puho et al. 2005, Tamasi, Bohacs et al. 2005, Kallen and Otterblad Olausson 2007), yet, some other researchers’ observations do not support these findings (Dombrowski, Bottoms et al. 1986, Schatz, Zeiger et al. 1995, Dombrowski, Schatz et al. 2004, Dombrowski, Schatz et al. 2004, Tata, Lewis et al. 2007). A recent meta-analysis sought to investigate adverse perinatal outcomes in women with asthma, concluded that there could be a 50% increase risk of PE development in asthmatic pregnant women (Murphy, Namazy et al. 2011). While
these studies considered modifiable factors for risk prediction of PE development, lack of adjustment for vitamin D status, as a contributor to both PE development and asthma exacerbations, is a notable weakness.

In the general population, 25-hydroxyvitamin D (25OHD) deficiency has been linked to hypertension, the cardinal feature of PE (Martins, Wolf et al. 2007, Forman, Curhan et al. 2008, Melamed, Michos et al. 2008, Hutchinson, Grimnes et al. 2010). Furthermore, epidemiologic studies of PE, have implicated alterations in vitamin D metabolism during clinical disease in late pregnancy; including low serum 1, 25-dihydroxyvitamin D$_3$ (1,25(OH)$_2$D, the bioactive form of vitamin D) (August, Marcaccio et al. 1992, Seely, Wood et al. 1992, Halhali, Tovar et al. 2000, Lechtermann, Hauffa et al. 2014). More importantly, several studies demonstrated low levels of 25(OH) D in early pregnancy in women who subsequently developed PE, suggesting that the deficiency predates disease onset and might contribute to its pathogenesis and severity (Mostello, Catlin et al. 2002, Bodnar, Catov et al. 2007, Baker, Haeri et al. 2010). A meta-analysis of these studies concluded that low maternal serum vitamin D concentration increases risk of PE (Hypponen, Cavadino et al. 2013). In the US and similar to the trend in PE occurrence rate, there is evidence to show that low vitamin D status has increased approximately two fold from the late 1980s and early 1990s through the early 2000s (Ginde, Liu et al. 2009, Mirzakhani, Al-Garawi et al. 2014).

Pregnancy has been implicated by several studies as a high-risk state for vitamin D deficiency (Karras, Anagnostis et al. 2014, Karras, Anagnostis et al. 2014). While there is controversy over what level of vitamin D is optimal for overall health (Urrutia and Thorp 2012), the current recommendations address vitamin D serum level for adults and their bone health, not during pregnancy and PE risk. Accordingly, some experts have suggested that vitamin D deficiency should be defined as circulating 25OHD levels less than 32 ng/ml (80 nmol/L) (Hollis and Wagner 2005, Practice 2011). The 2011 US Institute of Medicine (IOM) defines 20ng/ml (50nmol/L) as sufficient level (Gitlin 2006). Using this level, prevalence of vitamin D deficiency during pregnancy has been estimated to be from 20%-84% worldwide (Karras, Anagnostis et al. 2014). Thus, documenting the potential association between low vitamin D status and the rate of PE while identifying the optimal preventive vitamin D serum concentration during pregnancy might be of high public health importance. Previous studies have demonstrated higher levels of vitamin D serum levels for risk reduction of PE in both early and late pregnancies (Wei, Audibert et al. 2012,
Bodnar, Simhan et al. 2014). However, a well-timed optimal serum level of vitamin D to provide the biological effect for risk reduction of PE and its potential effect on other reported PE risk factors during pregnancy has not been investigated (Practice 2011).

We, therefore, aimed to investigate the relation between serum level of vitamin D in early pregnancy (10-18 weeks) and maternal asthma with risk of PE development using the data on the outcomes of pregnancy from the Vitamin D Antenatal Asthma Reduction Trial (VDAART). Furthermore, we intended to estimate the risk of PE based on the observed levels of serum vitamin D in early pregnancy.
VDAART (www.vdaart.com) is sponsored by The National Heart, Lung, and Blood Institute (NHLBI) and was registered at ClinicalTrials.gov (NCT00920621).

The Institutional Review Boards (IRB) of the participating Clinical Centers approved VDAART and written consent was obtained from all the participating pregnant women at the first enrollment visit (Litonjua, Lange et al. 2014). Throughout the study, the selected Data and Safety Monitoring Board (DSMB) consisting of 10 members monitored the trial.

Study group

The study group consisted of pregnant women with singleton pregnancies at 10-18 weeks who were enrolled in a multicenter, randomized trial for assessment of the defined outcome of pregnancies after receiving either vitamin D (cholecalciferol, 4000 IU/day; equivalent to 100 µg/day) or placebo (Litonjua, Lange et al. 2014). All the pregnant participants received prenatal vitamins containing 400 IU (10 µg/day) of cholecalciferol; thus, the vitamin D treatment arm received a total of 4400 IU/day (110 µg/day) and the placebo arm received 400 IU/day (10 µg/day). The pregnant women’s clinical course was followed prospectively from randomization (10-18 weeks) to the end of the pregnancy (Litonjua, Lange et al. 2014).

Exclusion criteria for VDARRT enrollment included smoking, chronic hypertension, diabetes mellitus, multiple gestation pregnancy, parathyroid and thyroid disease, kidney stones and sarcoidosis, intake of vitamin D supplements more than 2000 IU/day, and taking illicit drugs in last 6 months (Litonjua, Lange et al. 2014).

Data

We performed a secondary analysis of the collected data from the VDAART study (Litonjua, Lange et al. 2014). The enrollment visit occurred at the subjects' pre-natal visit between 10 and 18 weeks of gestation. A completed questionnaire at enrollment and monthly thereafter, in addition to medical record review, obtained
information on maternal health characteristics, past medical history, non-study medications, delivery information and clinical course or any new disease diagnosis during pregnancy as well as subjects’ characteristics. Serum vitamin D levels were measured at 32–38 weeks gestation, in addition to the enrollment visit at 10-18 weeks. Overall, VDAART randomized 881 pregnant women (Litonjua, Lange et al. 2014).

Primary Endpoint: Preeclampsia diagnosis and validation

The main goal of this study was investigating the association of asthma and vitamin D status in early pregnancy on PE development. Among the enrolled pregnant women, the occurrence of adverse events (AEs) and serious adverse events (SAEs) was monitored by each Clinical Center through the monthly maternal health questionnaire and the monthly obstetrical medical record review. PE occurrence was pre-defined as an SAE for monitoring in the study design and diagnosed by the physicians’ during clinical care of pregnant women at each study site through the trial. Thirty-eight cases of PE were reported as SAEs in the trial. In addition, a review of medical records after all women delivered was conducted and relevant information was extracted using a structured form by study staff at each clinical center. Subsequently, all the members of a special expert panel, consisting of obstetricians and gynecologists (OB-GYNs) from the study sites, reviewed the forms that contained the extracted medical records, and this resulted in thirty-six additional cases of PE (Litonjua, Lange et al. 2014). Accordingly, only PE cases diagnosed by the Clinical Center OB-GYNs and confirmed by the qualified clinician expert panel were used in this data analysis.

The diagnosis of preeclampsia was based on the presence of high blood pressure (either systolic blood pressure $\geq 140$ mmHg or diastolic blood pressure $\geq 90$ mmHg) with a second measurement within 6 hours of the first measure after 20 weeks of gestation. Other complementary criteria in the establishment of diagnosis were presence of proteinuria, elevated liver enzymes, low count platelet, headache or visual disturbances (ACOG 2013).

Main predictors: Maternal serum vitamin D and asthma status
I. Vitamin D assay

Serum assays of 25OHD were performed at the Channing Division of Network Medicine. Circulating 25OHD was determined using the DiaSorin Liaison® machine, which uses a chemiluminescence immunoassay (CLIA) (Ersfeld, Rao et al. 2004), to determine plasma concentrations of 25 OH. For quality control, the laboratory used US National Institute of Standards and Technology (NIST) level 1 samples in each run.

II. Maternal asthma and severity during pregnancy

Completed questionnaires at enrollment and monthly maternal health questionnaires thereafter as well as monthly medical record review provided the information on absence or presence of maternal asthma and monitored asthma severity throughout the study and until delivery. The analysis excluded asthmatic pregnant women who did not complete the follow-up questionnaires.

A subject was considered to have asthma if, at initial interview, she reported physician-diagnosed asthma at any time in her life. Henceforth, the monthly questionnaires prospectively collected all the information characterizing exacerbations of asthma, if any. Asthma Control Test (ACT), a 5-item questionnaire included in monthly questionnaires, was used to assess the multidimensional perspective of asthma control from activity limitation, shortness of breath, night symptoms, use of rescue medication and self-perception of asthma control (Nathan, Sorkness et al. 2004, Schatz, Sorkness et al. 2006). As asthma control varies in different clinical settings due to its multidimensional and heterogeneous nature, the validated cutoff of ACT score ≤ 16 during pregnancy was considered as the threshold for any occurrence of uncontrolled asthma (Monteiro de Aguiar, Rizzo et al. 2014). Accordingly, occurrence (ACT score ≤ 16) or non-occurrence (ACT score > 16) of asthma exacerbation was abstracted from the monthly questionnaires of each asthmatic subject. Thereafter, number of asthma exacerbation events in each asthmatic woman was defined as the total number of ACT scores less than 16 during her study time (from enrollment until delivery) which calculated from her abstracted ACT scores. Non-asthmatic women's exacerbation was considered to be null.
Independent variables

The risk factors of PE other than main predictors were extracted from the study questionnaires and data records. These variables included: number of pregnancies, gestational diabetes, race (African American [AA] vs. non-African American), site of enrollment, treatment arm of the trial (placebo vs. vitamin D treatment), body mass index (BMI) at enrollment and duration of treatment (time on study) prior to PE development. BMI at enrollment was based on measured height and maternal weight at the initial visit.

Statistical analysis

R version 3.12 and JMP version 11 (SAS Inc, Cary, NC) were used for all analyses (Prentice 2011, Young, McNanley et al. 2012). The package “effects”, an R package, was used to plot the probability curve and effect estimate of serum level of vitamin D in prediction of preeclampsia (Fox 2009, http://www.jstatsoft.org/v32/i01/).

A purposeful variable selection algorithm was applied for investigating the risk factors and the variables collected as potential confounders of PE events (Bursac, Gauss et al. 2008), as previously elucidated. Any variable having a significant univariate test with p-values of $\leq 0.25$ was fitted in a model based on backwards elimination. In the iterative process of variable selection, covariates were removed from the model if they were non-significant ($p>0.1$) and not a confounder (a change in any remaining parameter estimate $\geq 15\%$ to the full model). Finally, variables with initially excluded based on the univariate test were further assessed for confounding with the included variables and were similarly added back to the model if the regression coefficients change by $\geq 15\%$. This approach has shown the advantage of risk factor modeling as well as prediction. In addition to significant covariates, the applied method in variable selection has the capability of retaining important confounding variables, resulting potentially in a richer model (Bursac, Gauss et al. 2008).

The measured vitamin D blood level was also categorized to assess the risk of PE across the intervals ($<15$ ng/mL, $15 \leq \text{Vit D} <30$ ng/mL, and $\geq 30$ ng/mL). A Spearman Rank-Order Correlation was run to determine the relationship between continuous variables (i.e. vitamin D and BMI). We also examined if the effect of
maternal vitamin D serum levels on the risk of PE occurrence was different across the corresponding subjects’ BMIs, i.e. their interaction (Saneei, Salehi-Abargouei et al. 2013, Vimalesswaran, Berry et al. 2013, Vimalesswaran, Cavadino et al. 2013, Ekwaru, Zwicker et al. 2014). A post hoc subgroup “sensitivity” analysis was also conducted among asthmatic pregnant women to investigate consistency of findings for the relation of low vitamin D status and asthma exacerbation.

Except as specified previously in the initial selection of variables using the purposeful selection algorithm, p-values <0.05 were considered significant. All statistical tests were two-sided. The data are presented as mean and 95% confidence intervals (CI) or median and 95% central range, as required.

Results
The baseline characteristics of the women are shown in Table 1. After exclusion of 72 subjects (5 women for lack of baseline vitamin D measurements and 57 due to no return for the follow-up questionnaires after enrollment, i.e. overall 8% of total study subjects), 819 subjects with complete data were included in this secondary analysis. The range of maternal age was 18-39 years old with a mean of 27.4 ± 5.5. The mean of gestational weeks at enrollment was 14.2 ± 3 and there was no difference among women with PE or without. The overall incidence of PE was 9% and the incidence was similar in the women receiving vitamin D treatment or placebo (4.4% and 4.6%, respectively).

According to the univariate analysis, increased frequency of uncontrolled asthma, maternal age, AA race, study site, higher BMI, and shorter duration of study time (treatment duration) were associated with a higher risk of PE. After execution of the purposeful selection algorithm on all of the variables from Table 2, those remaining in the final best-fit logistic regression model included vitamin D serum level, frequency of uncontrolled asthma, race, total number of pregnancies, study site, BMI and time on the study. However, only serum vitamin D level remained significant. Total number of pregnancies and BMI also were associated with PE events. For the risk factors and confounders, including those found to be statistically significant in the final regression model, the distribution by occurrence or no occurrence of PE is shown in Table 1.

Women who developed PE had lower vitamin D level at baseline as compared to non-preeclamptic women (median of 17 ng/ml vs. 22 ng/ml, respectively, Table 1). The preeclamptic women had baseline vitamin D level in the range of 6-36.5 ng/ml vs. 4.4-81 ng/ml for non-preeclamptic women. The median vitamin D level at 32-38 weeks showed improvement to 24.5 and 32 ng/ml in preeclamptic women and women without preeclampsia, respectively.

Receiver operating curves of maternal vitamin D in prediction of PE occurrence showed substantial improvement of the area under the curve after adjustment for frequency of uncontrolled asthma during pregnancy, BMI, total number of pregnancies, race and duration of treatment/time on study (ΔAUC≈0.1, p=0.0004, Figure 1). Eventually, a 20-ng/ml decline in 25(OH)D concentration showed a 2.7 fold increase in
odds of PE (aOR: 2.7, 95% CI: 1.4-5.5). As demonstrated in figure 2 a strong, inverse relation between serum 25(OH)D levels at 10-18 weeks of gestation and risk of PE was observed for both the unadjusted and adjusted prediction models. Accordingly, the vitamin D serum concentrations of 40-50 ng/ml showed the lowest predicted risk of PE development in the pregnant women under the study at 10-18 weeks of gestation (Figure 2).

Across the categories of vitamin D, women with serum 25(OH)D levels in the range of 15-30 ng/ml had 53% lower odds of PE development (OR: 0.47; 95% CI: 0.28-0.8; p=0.004) as compared to women with serum 25(OH)D levels lower than 15 ng/ml. In continuation of this significant trend, the increase of maternal 25(OH)D levels above 30 ng/ml from the range of 15-30 ng/ml reduced the odds of PE 2.6 fold (OR: 0.18, 95% CI: 0.1-0.4; p<0.001, Table 3).

The prevalence of asthma among the study population was 36% (45% and 40% for women with PE and without, respectively). Women with a report of 3 or more uncontrolled asthma episodes were more common among preeclamptic women than those without PE. While women with more frequent asthma exacerbations during their pregnancy showed a higher incidence of PE (OR: 1.31; 95% CI: 1.01-1.65, p=0.039), after adjustment for vitamin D, BMI, race, gravidity, and treatment duration, the effect did not remain significant (aOR: 1.22, 95% CI: 0.91-1.6, p=0.16).

In subgroup univariate analysis of asthmatic pregnant women (NPE=33 and Nnon-PE=295), vitamin D (OR=0.95, CI: 091-0.99; p=0.019), but not asthma exacerbation (OR=1.3, CI:097-168; p=0.063) was the predictor of PE occurrence. In the bivariate analysis (vitamin D plus asthma exacerbation) vitamin D remained as the main predictor of PE (vitamin D: OR=0.953, CI: 091-0.99, p=0.024; asthma exacerbation: OR=1.26, CI=0.94-1.65, p=0.1). Addition of any strong confounder or predictor identified in the primary analysis did not substantially change the observations. However, the size of subgroup did not provide adequate power for subgroup effect estimation with all the covariates in the primary analysis.
Women who developed PE had higher BMI (32 ± 8 kg/m², 57% above 30 kg/m²) at 10-18 weeks as compared to those who did not develop PE (28.6 ± 7.5 kg/m², 32% beyond 30 kg/m²). Accordingly, higher risk of PE events (OR: 1.05, 95% CI: 1.02-1.08; p=0.0003) was observed for each unit increase of BMI. The effect of BMI remained significant when multi-level multivariate logistic-regression analysis was applied (aOR: 1.04; 95% CI: 1.01-1.08; p=0.009). There was a weak, negative correlation between vitamin D status and BMI that was statistically significant (r= -0.3, p<0.001). No statistically significant multiplicative interaction between vitamin D effect and BMI values or confounding effect on main predictors was observed (p=0.57). Increased number of previous pregnancies was associated with lower risk of PE occurrence (aOR: 0.73; 95% CI: 0.6- 0.9; p=0.0028).

Table 1. Characteristics of VDAART pregnant women studied by preeclampsia event

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Preeclampsia (N=74)</th>
<th>No preeclampsia (N=745)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

43
<table>
<thead>
<tr>
<th></th>
<th>&lt;15 (ng/ml)</th>
<th>15-29.9 (ng/ml)</th>
<th>≥30 (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>History of asthma (yes, %)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Uncontrolled asthma (number of event, %)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>30</td>
<td>29</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>4</td>
<td>6</td>
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</tr>
<tr>
<td>3</td>
<td>3</td>
<td>3</td>
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<tr>
<td>≥4</td>
<td>5</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Gestational age (weeks)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean (SD)</td>
<td>14.1 (3)</td>
<td>14.2 (3)</td>
<td></td>
</tr>
<tr>
<td>Median</td>
<td>13.36</td>
<td>13.86</td>
<td></td>
</tr>
<tr>
<td>Range</td>
<td>10-18</td>
<td>10-18</td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean (SD)</td>
<td>27.5 (5.53)</td>
<td>25.9 (5.11)</td>
<td></td>
</tr>
<tr>
<td>Range</td>
<td>18-37</td>
<td>18-39</td>
<td></td>
</tr>
<tr>
<td>Vitamin D at baseline (ng/ml)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean (SD)</td>
<td>18.4 (7.9)</td>
<td>23.4 (10.3)</td>
<td></td>
</tr>
<tr>
<td>Median</td>
<td>16.65</td>
<td>22.4</td>
<td></td>
</tr>
<tr>
<td>Range</td>
<td>6-36.5</td>
<td>4.5-81</td>
<td></td>
</tr>
<tr>
<td>95% central range</td>
<td>6.4-35.2</td>
<td>6.7.46-3</td>
<td></td>
</tr>
<tr>
<td>Status (category, %)</td>
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<tr>
<td>&lt;15 (ng/ml)</td>
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<td>20</td>
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</tr>
<tr>
<td>15-29.9 (ng/ml)</td>
<td>52.7</td>
<td>56.78</td>
<td></td>
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<tr>
<td>≥30 (ng/ml)</td>
<td>8.11</td>
<td>23.22</td>
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<tr>
<td>Vitamin D at 32-38 weeks of gestation (ng/ml)</td>
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<td></td>
</tr>
<tr>
<td>Mean (SD)</td>
<td>28.72 (14.6)</td>
<td>33.4 (14.6)</td>
<td></td>
</tr>
<tr>
<td>Median</td>
<td>24.5</td>
<td>32</td>
<td></td>
</tr>
<tr>
<td>Range</td>
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<td>6-90</td>
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</tr>
<tr>
<td>95% central range</td>
<td>6.74-56.6</td>
<td>9.1-68.46</td>
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<td>Gravidity</td>
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</tr>
<tr>
<td>Mean (SD)</td>
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<td>3 (2)</td>
<td></td>
</tr>
<tr>
<td>Median</td>
<td>2</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Range</td>
<td>1-6</td>
<td>1-13</td>
<td></td>
</tr>
<tr>
<td>Status (category, %)</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Primigravida</td>
<td>39</td>
<td>35</td>
<td></td>
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<tr>
<td>Secundigravida</td>
<td>30</td>
<td>25</td>
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</tr>
<tr>
<td>Tertigravida</td>
<td>18</td>
<td>20</td>
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<tr>
<td>&gt;Tertigravida</td>
<td>13</td>
<td>20</td>
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<td>Site (%)</td>
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<td>1</td>
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<td>35</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>20</td>
<td>31</td>
<td></td>
</tr>
<tr>
<td></td>
<td>57</td>
<td>34</td>
<td></td>
</tr>
<tr>
<td>Trial arm (group, %)</td>
<td>Treatment</td>
<td>Placebo</td>
<td></td>
</tr>
<tr>
<td>-----------------------------</td>
<td>-----------</td>
<td>---------</td>
<td></td>
</tr>
<tr>
<td></td>
<td>49</td>
<td>50.2</td>
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</tr>
<tr>
<td></td>
<td>51</td>
<td>49.8</td>
<td></td>
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</table>

| Gestational diabetes (yes, %) | 7 | 6 |

<table>
<thead>
<tr>
<th>Body mass index at baseline (kg/m²)</th>
<th>32.1 (8.36)</th>
<th>28.6 (7.5)</th>
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<tbody>
<tr>
<td>Mean (SD)</td>
<td>31.4</td>
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<td>Median</td>
<td>18.5-54</td>
<td>16-62</td>
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<tr>
<td>Range</td>
<td>18.6-52.7</td>
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<tr>
<td>95% central range</td>
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<table>
<thead>
<tr>
<th>Status (category, %)</th>
<th>1.4</th>
<th>2.3</th>
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<tbody>
<tr>
<td>&lt;18.5 mg/kg²</td>
<td>20.3</td>
<td>33.3</td>
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<td>18.5-24.9 mg/kg²</td>
<td>20.3</td>
<td>26.2</td>
</tr>
<tr>
<td>25-29.9 mg/kg²</td>
<td>57</td>
<td>32.2</td>
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<tr>
<td>≥30 mg/kg²</td>
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<td></td>
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</table>

<table>
<thead>
<tr>
<th>Race (category, %)</th>
<th>57</th>
<th>42.3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-Hispanic-African American</td>
<td>43</td>
<td>57.7</td>
</tr>
<tr>
<td>Non Hispanic-Non-African American</td>
<td>29.7</td>
<td>41.07</td>
</tr>
<tr>
<td>White</td>
<td>5.3</td>
<td>0.8</td>
</tr>
<tr>
<td>American Indian or Alaska</td>
<td>4</td>
<td>4.56</td>
</tr>
<tr>
<td>Asian</td>
<td>0</td>
<td>1.47</td>
</tr>
<tr>
<td>Native Hawaiian</td>
<td>4</td>
<td>9.8</td>
</tr>
<tr>
<td>Other</td>
<td></td>
<td></td>
</tr>
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</table>

<table>
<thead>
<tr>
<th>Gestational age at delivery (weeks)</th>
<th>37.7 (3)</th>
<th>38.8 (2.7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>38.45</td>
<td>39.2</td>
</tr>
<tr>
<td>Median</td>
<td>26-41</td>
<td>18-42</td>
</tr>
</tbody>
</table>

* Standard deviation

b The number of previous pregnancies
### Table 2. Unadjusted and adjusted associations between selected characteristics of VDARRT study population and preeclampsia development.

<table>
<thead>
<tr>
<th>Risk factors</th>
<th>Univariate analysis</th>
<th>Multivariate analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>OR(^a) (95% CI(^b))</td>
<td>P-value</td>
</tr>
<tr>
<td>Asthma</td>
<td>1.23 (0.75-1.98)</td>
<td>0.4</td>
</tr>
<tr>
<td>Uncontrolled asthma (frequency)</td>
<td>1.31 (1-1.65)</td>
<td>0.034*</td>
</tr>
<tr>
<td>Maternal age</td>
<td>0.95 (0.9-0.99)</td>
<td>0.0160*</td>
</tr>
<tr>
<td>Gestational age</td>
<td>1.24 (0.42-2.96)</td>
<td>0.7</td>
</tr>
<tr>
<td>Vitamin D serum level (ng/ml) at baseline</td>
<td>0.94 (0.92-0.97)</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>Gravidity</td>
<td>0.86 (0.71-1.01)</td>
<td>0.096</td>
</tr>
<tr>
<td>Site</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Reference</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>0.98 (0.47-2)</td>
<td>0.96</td>
</tr>
<tr>
<td>3</td>
<td>2.53 (1.43-4.7)</td>
<td>0.002*</td>
</tr>
<tr>
<td>Trial arm</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Placebo</td>
<td>Reference</td>
<td>-</td>
</tr>
<tr>
<td>Treatment</td>
<td>0.94 (0.58-1.52)</td>
<td>0.8</td>
</tr>
<tr>
<td>Gestational diabetes</td>
<td>1.23 (0.42-2.96)</td>
<td>0.66</td>
</tr>
<tr>
<td>Body mass index at baseline (kg/m(^2))</td>
<td>1.05 (1.02-1.08)</td>
<td>0.0003*</td>
</tr>
<tr>
<td>Race</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-African American</td>
<td>Reference</td>
<td>-</td>
</tr>
<tr>
<td>African American</td>
<td>1.8 (1.11-2.92)</td>
<td>0.018*</td>
</tr>
<tr>
<td>Time on study (^d)</td>
<td>0.99 (0.98-0.999)</td>
<td>0.036*</td>
</tr>
</tbody>
</table>

\(^a\) Confidence interval (CI)  
\(^b\) Odds ratio (OR)  
\(^c\) Adjusted odds ration (aOR)  
\(^d\) Number of asthma exacerbations (uncontrolled asthma) during study time  
\(^e\) Defined as number of days on study (delivery date minus enrollment date)

### Table 3. Maternal risk of preeclampsia according to the defined vitamin D level categories at 10-18 weeks of gestation

<table>
<thead>
<tr>
<th>Vitamin D level (ng/ml)</th>
<th>Odds ratio</th>
<th>Confidence interval</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;15</td>
<td>Reference</td>
<td>0.47</td>
<td>0.004*</td>
</tr>
<tr>
<td>15 ≤ Vitamin D &lt;30</td>
<td></td>
<td>0.28-0.8</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>≥30</td>
<td></td>
<td>0.1-0.4</td>
<td>-</td>
</tr>
</tbody>
</table>

**Trend test p-value**

- 0.001*
<table>
<thead>
<tr>
<th>Predictor</th>
<th>AUC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unadjusted</td>
<td>0.6431</td>
</tr>
<tr>
<td>Adjusted</td>
<td>0.7370</td>
</tr>
</tbody>
</table>

**Figure 1.** Receiver operating curves (ROC) of maternal vitamin D in prediction of preeclampsia

Blue curve: Area under curve of unadjusted model for maternal vitamin D in risk prediction of preeclampsia development

Red curve: Area under curve of adjusted model for maternal vitamin D in risk assessment of preeclampsia development ($\Delta$ adjusted-unadjusted AUC=0.093, $p=0.0004^*$)
Figure 2. Dose-response association between maternal serum 25-hydroxyvitamin D concentration at 10-18 week of gestation and corresponding predicted probability of preeclampsia derived from a logistic regression model. The inner ticks on top and bottom x axes depict pregnant women with and without preeclampsia, respectively (N=74 and N=745). The grey zone demonstrates 0.95 confidence bands. The minimum predicted risk for the population under study falls at the concentrations of 40-50 ng/ml of serum vitamin D.

Discussion
Evaluating a large number of pregnant women randomized to investigate the effect of vitamin D treatment on the outcome of their pregnancies, we found that presence of low vitamin D status early in pregnancy was significantly associated with higher rate of PE. Our findings not only corroborate and add to previous research conducted to assess the risk of PE in pregnancies with low vitamin D status (August, Marcaccio et al. 1992, Seely, Wood et al. 1992, Halhali, Tovar et al. 2000, Mostello, Catlin et al. 2002, Bodnar, Catov et al. 2007, Baker, Haeri et al. 2010, Lechtermann, Hauffa et al. 2014), but also suggest a higher serum level of vitamin D than the current recommendation for adults might be required to minimize risk of PE during pregnancy. Furthermore, our investigation suggests that vitamin D has a substantial role in previously observed association of asthma severity with PE.

The rate of PE in the VDARRT study is somewhat higher than those reported by other studies; however, this can be explained, at least in part, by the fact that the protocol for the VDARRT trial required intensive, active surveillance for both SAEs and outcomes of pregnancy including PE by the study site staff and site clinicians. Moreover, VDARRT subjects were more likely to be nulliparous (35.3% and 60.6 primi- or secundigravida, respectively) and of AA race (43.6%) than in the general population, possibly placing them at increased risk of PE (Skjaerven, Wilcox et al. 2002). Consistently, our data show that an increase in the number of prior pregnancies reduces the risk of PE in the current pregnancy. Most of epidemiologic studies, if not all, have reported higher incidence of PE among AAs (Sibai, Gordon et al. 1995, Dekker 1999, Roberts and Cooper 2001, Triche, Saftlas et al. 2004, Caughey, Stotland et al. 2005, Bodnar, Catov et al. 2007, Tanaka, Jaamaa et al. 2007, Lisonkova and Joseph 2013). Low vitamin D status and obesity, which makes vitamin D less bioavailable (Wortsman, Matsuoka et al. 2000), are also more prevalent among AAs (Harris 2006, Flegal, Carroll et al. 2012, Ng, Scott et al. 2014). As a result, the prevalence of low vitamin D status in AAs has been estimated to be up to 50% (Holick 2004) with levels of 25(OH)D approximately half those of Caucasians (Matsuoka, Wortsman et al. 1995, Aloia, Mikhail et al. 1998, Looker, Dawson-Hughes et al. 2002, Nesby-O'Dell, Scanlon et al. 2002, Bodnar, Simhan et al. 2007).
 Appropriately, in unadjusted analysis of our data, the AAs had a higher risk of PE. However, after adjustment for vitamin D, the association was not observed anymore. This finding takes on greater significance considering that women with chronic hypertension and diabetes, two prominent risk factors for PE, both relatively more common among AAs, were excluded from the VDAART trial (Chesley 1984, Saftlas, Olson et al. 1990, Samadi, Mayberry et al. 1996). Therefore, our study suggests low vitamin D status might be the major contributing factor in previously observed associations between AA race and PE.

While we did not observe increased risk of PE by physician-diagnosed asthma, our unadjusted higher risk of PE by the effect of increased number of asthma exacerbations during pregnancy is consistent with some other reports (Stenius-Aarniala, Piirila et al. 1988, Perlow, Montgomery et al. 1992, Stenius-Aarniala, Riikonen et al. 1995, Demissie, Breckenridge et al. 1998, Wen, Demissie et al. 2001, Sobande, Archibong et al. 2002, Triche, Saftlas et al. 2004, Acs, Puho et al. 2005, Tamasi, Bohacs et al. 2005, Kallen and Otterblad Olausson 2007). However, our adjusted risk assessment of PE is in line with other researchers’ findings on observing no association (Dombrowski, Bottoms et al. 1986, Schatz, Zeiger et al. 1995, Dombrowski, Schatz et al. 2004, Dombrowski, Schatz et al. 2004, Tata, Lewis et al. 2007). There are two important points to be considered regarding these investigations. Firstly, the majority of these studies had a retrospective case-control design and used ICD9 codes to identify the asthmatic and preeclamptic subjects. Secondly, while these studies variably considered different risk factors or potential confounders, none of them included the measurement of maternal vitamin D status in their study. The effect of vitamin D on asthma has been widely studied (Litonjua and Weiss 2007, Mirzakhani, Al-Garawi et al. 2014). In a recent study, Confino-Cohen et al. investigated this association in a cohort of 308 000 adults with vitamin D status. According to their study, while there was no significant association between vitamin D status and physician-diagnosed asthma, low vitamin D status was associated with 25% higher odds of asthma exacerbations. The authors also found an inverse linear association between the proportion of asthmatics with exacerbations and vitamin D levels (Confino-Cohen, Brufman et al. 2014). Tama’si et al. observed a higher proliferation of IFN-γ+ and IL-4+ producing T cells (Th1 and Th2) in asthmatic pregnant women than the control values of non-pregnant asthmatics. The authors speculated that
low serum vitamin D levels led to a yet-to-be-defined immunological connection between asthma and pregnancy that might potentially impair both maternal airway symptoms as well as fetal development (Tamasi, Bohacs et al. 2005). This is consistent with data that 1,25(OH)2D directly affects both Th1 and Th2 cells and modulates the production of IFN-γ+ and IL-4+, the two cytokines involved in acute asthma severity. In addition vitamin D controls regulatory T-cells (Tregs), which are pivotal for T-balance (Pichler, Gerstmayr et al. 2002, Cantorna, Zhu et al. 2004, Adams, Liu et al. 2007, Steinman 2007, Mirzakhani, Al-Garawi et al. 2014). Furthermore, the potential effects of vitamin D deficiency on reducing respiratory infections as a trigger of acute asthma severity and better response to inhaled corticosteroids should also be added to the effect modulation of vitamin D on asthma and its severity (Lange, Litonjua et al. 2009, Huang, Purpodis et al. 2013, Mirzakhani, Al-Garawi et al. 2014). Accordingly, our findings suggest that low vitamin D status during pregnancy explains previous reports on the association of maternal frequency of asthma exacerbations with risk of PE.

Our findings reemphasize the importance of low vitamin D status at the early stages of pregnancy as a major risk factor in women who subsequently develop PE (August, Marcaccio et al. 1992, Seely, Wood et al. 1992, Halhali, Tovar et al. 2000). In a nested case-control study using the data from a prospective cohort study, Bondar et al. followed pregnant women (55 PE cases vs. 219 controls) from less than 22-week gestation to delivery (Bodnar, Catov et al. 2007). The mean of serum vitamin D in their study in women who developed PE was 18.23 (95% CI: 15.5-21.4 ng/ml) and 23.3 (95% CI: 19-24 ng/ml). In our study, these values comparably amounted to 18.4 (95% CI: 10.5-26.3 ng/ml) and 23.4 (13.3-33.5), respectively. The authors showed that after confounder adjustment, a 20-ng/ml decline in 25(OH)D concentration doubled the risk of PE (aOR: 2.4, 95% CI: 1.1-5.4) that is comparable to our estimate (aOR: 2.7, 95% CI: 1.4-5.5). In accordance with their findings, we observed a similar monotonic dose-response relation between serum 25(OH)D concentrations at less than 18-weeks’ gestation and risk of PE (figure 2). Our investigation demonstrated almost a 3-fold risk reduction of PE by improving 25(OH)D concentrations beyond 30 ng/ml. Accordingly, the dose-response curve of vitamin D for the risk prediction of PE suggests higher concentrations of vitamin
D are need through the pregnancy than what has been currently advised for adults and is suggestive of the range of 40-50 ng/ml vitamin D concentrations during 10-18 weeks of pregnancy. This range of concentration might be desirable for imposing the minimal risk of PE development, particularly in women with high-risk profile for PE occurrence during their pregnancies. The US Endocrine Society guideline recommends a 25(OH)D level equal to 100 ng/ml (250 nmol/l) as a safety margin to minimize the risk of hypercalcemia that is far above the suggested optimal vitamin D concentration for the prevention of PE in this study (Holick, Binkley et al. 2011).

In this study, the risk of PE showed no significant reduction in women who were enrolled in the treatment arm of vitamin D (4000 IU/day) and the association of low vitamin D status at baseline for this group (treatment arm) and PE development remained significant. Additionally, the median of serum vitamin D at 32-38 weeks of gestation in the women enrolled under treatment arm who subsequently developed PE was 40 ng/ml and 39 ng/ml in those who did not developed PE. These observations along with substantial risk reduction of PE for vitamin D serum levels above 30 ng/ml highlights the importance of surveillance for low vitamin D status, with measurement of 25(OH)D concentrations very early in pregnancy or at conception and providing restorative supplementation. Our study also confirms others’ reports on an inverse association of BMI and vitamin D status (Saneei, Salehi-Abargouei et al. 2013, Vimalessaran, Berry et al. 2013, Vimalessaran, Cavadino et al. 2013, Ekwaru, Zwicker et al. 2014) and its association with PE (Sibai, Gordon et al. 1995, O’Brien, Ray et al. 2003, Bodnar, Ness et al. 2005, Bhattacharya, Campbell et al. 2007, Wei, Audibert et al. 2012, Ananth, Keyes et al. 2013, Bilano, Ota et al. 2014). This might matter the most as in pregnancy, substantial gradual weight gain is a physiologic and natural process, which might exacerbate any prepregnancy low vitamin D status (Bodnar, Catov et al. 2007) and consequently exacerbate the effect of low vitamin D status in development of PE. Accordingly, adjustment of vitamin D supplementation, particularly in second or third trimester might be required.
Our study shows the current recommendation of 400-600 IU/day for pregnant women with low vitamin D status and subsequent serum levels, as also previously studied (Vieth, Chan et al. 2001, Heaney, Davies et al. 2003, Hollis and Wagner 2004, Hollis, Johnson et al. 2011), will not provide sufficiently high 25(OH)D concentrations to effectively reduce the risk of PE. Our data also confirms prior results (Hollis, Johnson et al. 2011) that vitamin D supplementation of 4000 IU/day for replenishment of low vitamin D status might not provide optimal concentrations of vitamin D to effectively reduce the risk of PE at early pregnancy. This fact is suggestive of considering the baseline vitamin D measurement for correction of the deficiency at early stages of pregnancy and providing individualized sufficient supplementation to achieve the appropriate serum concentrations (Heaney, Davies et al. 2003). Previous studies have suggested 2000-10000 IU/day to achieve a robust concentration of serum 25(OH)D during pregnancy (Vieth, Chan et al. 2001, Heaney, Davies et al. 2003, Hollis and Wagner 2004) that was reported to be safe for short-term supplementation (Heaney, Davies et al. 2003, Hollis and Wagner 2004). Due to inter-individual variability in response to vitamin D supplementation, reassessment of vitamin D serum level after supplementation is recommended (Vieth 1999, Aloia, Patel et al. 2008, Hollis, Johnson et al. 2011, Lewis, Laing et al. 2013, Waterhouse, Tran et al. 2014).

There are some limitations to our study to be considered. The major limitation is that our study was not originally designed to investigate all known risk factors of PE. Exclusion of subjects who smoked, or who had chronic hypertension and/or diabetes limits our ability to assess these factors and how they interact with vitamin D and asthma in risk assessment of PE. Our dosing and timing of administration of vitamin D also limit our conclusions. Finally, we were studying families with a history of asthma and allergies, and our findings might be different in a general population sample.

VDAART study highlights the previous investigations on the significance of vitamin D as a major and important modifiable risk factor of PE during pregnancy (Flegal, Carroll et al. 2012, Ogden, Carroll et al. 2012). Early detection of low vitamin status and adequate vitamin D supplementation with monitoring as a safe and effective preventive instrument for risk reduction of PE and the maternal-fetal consequences are
recommended. Future longitudinal study with large sample size is required to investigate the appropriate doses of vitamin D in early pregnancy to achieve the optimal serum concentration of vitamin D to prevent PE.

The findings of the conducted research in this chapter provides a substantial ground for our next research plan to investigate how vitamin D status might influence the PE development through perturbation of functional gene expressions and their potential interplay in a biological network. We hypothesize that low vitamin D status during early pregnancy affect the biological functions which predates the occurrence of PE. Accordingly we will explore the target genes affected by vitamin D and inspect which integrating part of the vitamin D associated genes might be linked to development of PE.
Chapter III.

Vitamin D gene expression signatures in early pregnancy for susceptibility to preeclampsia

Abstract

Vitamin D insufficiency has been epidemiologically recognized as a risk factor for preeclampsia, a leading cause of maternal, fetal and neonatal morbidity and mortality. In the Vitamin D Antenatal Asthma Reduction Trial (VDAART) and consistent with previous investigation, we showed that low vitamin D concentrations during early pregnancy (10-18 weeks) increases risk of preeclampsia. Vitamin D supplementation has been also shown to effect transcriptional responses to both vitamin D status and supplementation during pregnancy. Accordingly, we conducted a microarray study to investigate the vitamin D associated peripheral gene expression profile of VDAART pregnant women (10-18 weeks) destined to develop preeclampsia.

25-OH vitamin D was measured in maternal serum obtained from 163 pregnant women (53 preeclampsia, 110 unaffected controls matched by age, gestational age and race) at 10-18 weeks of gestation. mRNA expression in peripheral blood was quantified and analyzed using the Bioconductor package "RankProd" and according to both vitamin D (<30 vs. ≥30 ng/mL) and disease status. The intersection of the sets of vitamin- and disease-associated genes was taken as the basis for a replication analysis in a cohort of 30 pregnant women (14 preeclampsia, 16 unaffected) to identify a replicated vitamin D gene signatures for susceptibility to preeclampsia (replicated vitamin D gene expression signature for PE “rVDGSPE”). The members of the rVDGSPE were then analyzed for network structure using MetaCore™ and rVDGSPE genes were mapped in the molecular interaction network to infer their topological features and connectivity.
Two hundred ninety eight differentially expressed genes (131 down regulated) were identified as replicated VDG SPE in early pregnancy. The 30 identified networks of the master network implied by the full signature were enriched mostly in immune response and immune system regulation genes indicating earlier inflammatory responses and dysregulation of immune responses in preeclamptic women. MMP-9 and CREB1 were key hubs influenced by VDR through several regulatory pathways and in connection with VDG SPE.

Differential expression of several hundred genes predates the presence of preeclampsia. Vitamin D serum levels influence the regulation of 15 these networked genes directly and several others through the network hubs during early pregnancy, mostly involved in immune responses. CREB1 might be a key transcript factor in this response and vitamin D has a direct effect on its expression and hence regulation of the preeclampsia associated genes.
Introduction

Preeclampsia (PE) is a complex disease with both genetic and environmental components involved in its pathogenesis. The underlying molecular mechanism of PE, however, remains unknown. PE complicates from 3 to 7% of pregnancies. The maternal and fetal morbidity and mortality associated with PE and, in particular, with the adverse consequences of pre-term delivery are a major health burden (Friedman, Schiff et al. 1995, Sibai, Gordon et al. 1995, Rigo, Beke et al. 1996, Goldenberg and Rouse 1998, Goldenberg and Rouse 1998, Xiao, Sorensen et al. 2003, Basso, Rasmussen et al. 2006). The disease not only affects pregnancy outcome such as altered fetal growth, but also predisposes both mother and child to long-term health complications such as cardiovascular disease (Irgens, Reisaeter et al. 2001, Smith, Pell et al. 2001, Ray, Vermeulen et al. 2005, Bellamy, Casas et al. 2007, Craici, Wagner et al. 2008).

Expression profiling using microarrays has shown to be useful in identifying susceptibility genes contributing to risk of complex disorders including PE, showing the effect of quantifiable risk factors (Reimer, Koczan et al. 2002, Nishizawa, Pryor-Koishi et al. 2007, Enquobahrie, Meller et al. 2008, Nica and Dermitzakis 2008, Founds, Conley et al. 2009, Sitras, Paulssen et al. 2009, Hoegh, Borup et al. 2010, Enquobahrie, Qiu et al. 2011, Nishizawa, Ota et al. 2011, Meng, Chen et al. 2012) as well as gene expression alterations during pregnancy (Rochat, Ege et al. 2010). Using this method it has been shown that genes such as the obesity-related, cytokine-receptor, apoptosis-related and endothelial genes might be involved in the development of PE (Maynard, Min et al. 2003, Venkatesha, Toporsian et al. 2006). Owing to possible multifactorial causes involved in PE (Sheppard and Khalil 2010), an investigation of perturbations in “gene expression” caused by known environmental risk factors of PE could generate a large amount of information on potential early biomarkers, lead to an unraveling of the mechanism by which environmental factors that influence the early stages of PE development and allow potential exploration of preventive measures.
Among all known PE risk factors, epidemiologic studies have suggested that low vitamin D status is a major risk factor for PE and, more importantly, have suggested that vitamin D supplementation during early pregnancy lowers the risk of PE (Hypponen, Cavadino et al. 2013). Additionally, vitamin D supplementation’s effect on gene expression has been investigated (Enquobahrie, Williams et al. 2011, Hossein-nezhad, Spira et al. 2013). It has been demonstrated that improvement in vitamin D status will significantly affect the expression of genes that have with a wide variety of biologic functions linked to cell proliferation, autoimmune disorders and cardiovascular disease, all which have been associated with vitamin D deficiency (Enquobahrie, Williams et al. 2011, Hossein-nezhad, Spira et al. 2013).

In a recent study, we investigated the association of maternal serum level of vitamin D during early pregnancy with PE using the data on the outcomes of pregnancy from the Vitamin D Antenatal Asthma Reduction Trial (VDAART) (Litonjua, Lange et al. 2014). Our results corroborated prior studies and reemphasize the preventive role of vitamin D in risk reduction of PE during early pregnancy. As a follow-up study, it is of potential importance to perform gene expression profiling influenced by vitamin D among women who developed PE compared to those without PE during their pregnancy.

In this study, we, therefore, performed a two stage-supervised algorithm in a nested case control design to identify vitamin D gene signatures for susceptibility to PE (VDGSPE). First, we explored the module of interacting genes associated with vitamin D status during early pregnancy independent of the outcome of pregnancy, and then examined the sub-module that overlapped with the separately identified PE susceptibility genes. Furthermore, to validate our results, we replicated the overlapping genes of this sub-module in an independent dataset, and further elucidated the biologic pathways involved with the vitamin D sub-module.
Figure 1. Epidemiologic studies have shown the association of low vitamin D status with risk of preeclampsia in early pregnancy. Our findings, in the VIDAART trial, corroborate these observations. There are evidences on the effect of vitamin D status and supplementation on gene expression in healthy and pregnant subjects, investigated by bioinformatics methods. These methods have also demonstrated involvement of some functional biological pathways in preeclampsia. Accordingly, investigating genes whose expression changes due to the level of vitamin D serum concentration and determining their association with preeclampsia seems a logical step to identifying biological pathways that contribute to preeclampsia development.
Method

Study population

Participants of the preeclampsia microarray study comprised a nested case-control group (N=164) selected among participants of the Vitamin D Antenatal Asthma Reduction Trial (VDAART, www.vdaart.com). The study was sponsored by The National Heart, Lung, and Blood Institute (NHLBI) and was registered at ClinicalTrials.gov (NCT00920621). The Institutional Review Boards (IRB) of the participating Clinical Centers approved VDAART and written consent was obtained from all the participating pregnant women at the first enrollment visit (Litonjua, Lange et al. 2014). The VDAART study consisted of pregnant women with singleton pregnancies at 10-18 weeks who were enrolled in a multicenter, randomized trial for assessment of the defined outcome of pregnancies after receiving either vitamin D (cholecalciferol, 4000 IU/day; equivalent to 100 µg/day) or placebo (Litonjua, Lange et al. 2014). All of pregnant participants received prenatal vitamins containing 400 IU (10 µg/day) of cholecalciferol; thus, the vitamin D treatment arm received a total of 4400 IU/day (110 µg/day) and the placebo arm received 400 IU/day (10 µg/day). The pregnant women’s clinical course was followed prospectively from randomization (10-18 weeks) to the end of the pregnancy and for investigation of predefined outcomes of pregnancies, including PE, preterm birth and term deliveries (Litonjua, Lange et al. 2014). Among these subjects, 53 women who developed preeclampsia as well as 111 women with normal pregnancy were selected for microarray gene expression analysis on the blood samples obtained during their early pregnancies (10-18 weeks). Cases and controls were matched for age race and study center so that there were no significant differences (P > 0.05) in age, race, and maternal gestation in early pregnancy between the 53 PE cases and 111 normal controls in this study.

Exclusion criteria for VDARRT enrollment included smoking, chronic hypertension, diabetes mellitus, multiple gestation pregnancy, parathyroid and thyroid disease, kidney stones and sarcoidosis, intake of vitamin D supplements more than 2000 IU/day, and taking illicit drugs in last 6 months (Litonjua, Lange et al. 2014).
**Preeclampsia diagnosis**

PE occurrence was pre-defined as an SAE for monitoring in the VDAART study design and diagnosed by the physicians’ during clinical care of pregnant women at each study site throughout the trial. Thirty-eight cases of PE were reported as SAEs in the trial. In addition, a review of medical records after all women delivered was conducted and relevant information was extracted using a structured form by study staff at each clinical center. Subsequently, all the members of a special expert panel, consisting of obstetricians and gynecologists (OB-GYNs) from the study sites, reviewed the compiled forms that contained the extracted medical records, and this resulted in 36 additional cases of PE.

The diagnosis of preeclampsia was based on the presence of high blood pressure (either systolic blood pressure $\geq 140$ mmHg or diastolic blood pressure $\geq 90$ mmHg) with a second measurement within 6 hours of the first measure after 20 weeks of gestation. Other secondary criteria in the establishment of diagnosis were presence of proteinuria, elevated liver enzymes, low count platelet, headache or visual disturbances (ACOG 2013). Accordingly, only PE cases diagnosed by the Clinical Center OB-GYNs and confirmed by the qualified clinician expert panel were used in this data analysis. On this basis and availability of samples, fifty-three out of 74 cases diagnosed with PE were included for gene expression analysis.

**Blood collection and vitamin D assay**

Peripheral blood samples were collected at enrollment (10-18 weeks of gestation). Serum assays of 25OHD were performed at the Channing Division of Network Medicine. Circulating vitamin D (25OHD) was determined using the DiaSorin Liaison® machine, which uses a chemiluminescence immunoassay (CLIA) (Ersfeld, Rao et al. 2004), to determine plasma concentrations of 25 OH. This assay is co-specific for 25-hydroxyvitamin D3 and 25-hydroxyvitami D2. The LIAISON 25 OH Vitamin D assay is a direct competitive chemiluminescence immunoassay (CLIA) for quantitative determination of total 25OHD in serum. During the
incubation, 25-hydroxy vitamin D was dissociated from its binding protein, and competed with the isoluminol labeled analogue for binding sites on the antibody. After the incubation, the unbound material was removed with a wash cycle. Subsequently, the starter reagents were added and a flash chemiluminescent reaction was initiated. The light signal was measured by a photomultiplier as relative light units (RLU) and was inversely proportional to the concentration of 25-hydroxyvitamin D presented in calibrators, controls, or samples. The inter-assay and intra-assay Coefficient of Variability of this assay were 11.2% and 8.1%, respectively. For quality control, the laboratory used US National Institute of Standards and Technology (NIST) level 1 samples in each run.

**Demographic Characteristics**

Clinical characteristics of participants, i.e. age, gestational age, and race were compared using appropriate t-test or chi square with p ≤ 0.05 set as level of significance for each of discovery and replication cohorts, separately.

**RNA isolation and microarray processing**

Total RNA was isolated from whole blood using the paxgene Blood RNA Kit (Qiagen®) according to manufacturers protocol. The Ambion Globin Clear kit (Ambion®) was used to remove alpha and beta globin mRNA from the sample to increase the sensitivity of gene expression assays through improving the detection rate of expressed genes. The RNA was quantified using Nanodrop 8000 and checked for high integrity before preparation of cDNA. The integrity of RNA samples was assessed using the Agilent 2100 Bioanalyzer, and purity of the samples was confirmed using NanoDrop spectrophotometer and RNA Integrity Number (RIN ≥ 8).

Gene expression was assessed using Affymetrix Human Gene 1.0 ST Array. Biotinylated-cRNA prepared according to manufactures protocol and hybridization was processed according to protocol for the GeneChip® Hybridization Control Kit
After image production and processing, an expression set with 33297 probes and 164 samples collected at 10-18 weeks of gestation (enrollment) were generated, representing the 53 PE subjects and 111 unaffected normal pregnant women.

In order to check the quality of arrays, quantiles of expressions were examined before and after normalization. Background adjustment, log2 transformation and quantile normalization were performed using the Robust Multiarray Analysis (“rma”) function in R BioConductor's library affy to minimize nonbiological variability among arrays (Gautier, Cope et al. 2004). Principal component (PC) analysis of samples and their expressions was done and one sample was identified as an extreme outlier and removed. The PCs were rechecked after removal of outliers for consistency of expression after exclusion. Thereafter, phenotypes of interest were merged with the expression set. Probes were annotated using the annotation package for pd.hugene.1.0.st.v1 and confined to annotated probes of autosomal chromosomes (19137 probes).

Surrogate variable analysis (SVA) was applied using the “SVA” package for identifying and removing any potential batch effects (Leek, Johnson et al. 2012). Thereafter, IQR filter (values more than median of expressions) was implemented in R using “genefilter” package (Bioconductor) to remove expressions showing little changes within arrays (Zavorotnyy and Zwanzger 2011). This process resulted in an expression set of 15,309 probes belong to 163 samples collected at 10-18 weeks of pregnancy, representing 53 PE cases and 110 women.

**Identifying vitamin D differential expressions for susceptibility to PE**

Identification of differentially expressed genes were analyzed using the publicly available open source statistical computing and graphics software tool "R" (http://www.r-project.org). In conjunction with this
platform, several Bioconductor tools for detailed gene expression analysis (http://www.bioconductor.org) were also used. Differential expression analysis was carried out using the Bioconductor package "RankProd" which implements the Rank Product (RP) method for identifying differentially expressed genes (Breitling, Armengaud et al. 2004, Huuhka, Anttila et al. 2005). It offers several advantages over linear modeling, including the biologically intuitive fold-change (FC) criterion, fewer assumptions under the model, and increased performance with noisy data and/or low numbers of replicates (Breitling and Herzyk 2005, Hong, Breitling et al. 2006). Accordingly, this approach applies a non-parametric statistic derived from biological reasoning that detects items that are consistently highly ranked in a subset of genes found among the most strongly up or down-regulated genes. Differentially expressed (up- or down- regulated) genes are identified through permutation analysis, after setting the percentage of false prediction (pfp) threshold for which the false discovery rate (FDR) is less than 0.05. The number of permutations used was set as the default value (Hong, Breitling et al. 2006).

To identify the differentially expressed genes associated with vitamin D status, 30-ng/mL serum level of vitamin D (the current recommendation for vitamin D level during pregnancy) was considered as the cut-off for the pregnant women in 10-18 weeks of gestation (Hollis and Wagner 2005, Practice 2011). In this approach all women with a vitamin D level less than 30 ng/mL were deemed to be pregnancies with low vitamin D status and susceptible to developing PE. We applied RankProd to detect genes differentially expressed between vitamin D categories. We used an FDR ≤ 0.05 as a cutoff to define the significance of differential expressed genes.

We also applied the same method to identify differentially expressed genes among VDAART study pregnant women who developed PE vs. those whose outcomes of pregnancy were not affected. Significant overlaps of the vitamin D associated module genes during early pregnancy with genes identified to be involved in disease status (PE vs. No PE) were selected for replication in our second case control study. Figure 2 provides a summary on the analysis approach.
Identification of DE genes across vitamin D Categories (vitamin D <30, and ≥30 ng/ml)

RankProd method
fdr<0.05

2701 vitamin D gene signatures
1323 up-regulated genes
1378 down-regulated genes

Identification of PE susceptibility genes (PE: 53, Normal: 110)

RankProd method
fdr<0.05

1607 PE gene signatures
1087 up-regulated genes
520 down-regulated genes

1166 Vitamin D gene signatures for susceptibility to PE
503 up-regulated genes
663 down-regulated genes

Replication Cohort (N=30)

rVDGSPE

PE: 14, Normal: 16
p value <0.05: 298 genes
167 up-regulated transcripts
131 down-regulated transcripts

Pathway analysis of the replicated VDGSPE

Figure 2. Method and Result flow chart and summary of findings on gene expression analysis through discovery and replication stages.

DE: differentially expressed; VDGS: vitamin D gene signatures; PEGS: preeclampsia gene signatures; VDGSPE: vitamin D gene signatures for susceptibility to PE.
Microarray validation by real-time PCR

To ensure internal validity, the most highly up- or down-regulated genes, as well as some genes of biological interest, based on the replication results, were chosen for validation by QRT-PCR. QRT-PCR was performed.

To identify candidate genes with more biological relevance for validation, we examined the direct interactions between rVDGSPE genes using Metacore™ platform to identify islands of directly interconnected objects. Then, the identified islands were investigated for common genes of rVDGSPE known to be involved in PE (Figure 3 A, B, C). Accordingly, CXCL10 (IP10), MMP-9, OLR1, CLU, ITGB3, and NOD2 were selected for validation by QRT-PCR.

Replication population

In order to improve the quality of inference from our discovery population, the vitamin D gene signatures linked to the development of PE were examined using maternal peripheral blood gene expression in an independent cohort. The replication cohort was a nested case control from the participants of the Omega study (1996-2007), a prospective study designed to examine the risk factors of pregnancy complications. Participants were nulliparous women who initiate prenatal care before 20 weeks gestation at Swedish Medical Center (SMC) affiliated clinics in Seattle, Washington. The replication for this report was conducted among preeclampsia cases (N=16) and controls (N=16) from Omega cohort members enrolled during the period of July 2003 to May 2008 (Enquobahrie, Qiu et al. 2011).

QC on the replication data and identification of genes for pathway analysis

The gene expression of subjects were profiled using Affymetrix GeneChip Arrays (Enquobahrie, Qiu et al. 2011). Quantiles of raw expression and pcs across arrays were examined before and after background adjustment normalization and log2 using ‘rma’ function in the R library ‘affy’. 2 samples’ expressions were identified as outliers and removed and quantile normalized expressions and background adjusted as well as pcs were re-examined. The results were compared with running the function ‘QCreport’ function from R library
‘affyQCRreport’. The microarray expression data were annotated using “hgu133plus2” available on Bioconductor. The annotated expression from autosomal chromosomes was included in the analysis. The final expression dataset contained 40003 rows of background adjusted, log2 transformed, rma summarized, and quantile normalized expression data of 14 PE cases and 16 controls. RP method was applied on the vitamin D gene signatures for susceptibility to PE. Those genes showing p values < 0.05 in the replication were considered for further investigation in Network Analysis.

Gene-gene interactions and functional profiling of VDGSPE

Functional annotations were carried out using MetaCore™ in which ranked gene were imported. Accordingly, the MetaCore™ platform was used on the replicated VDSGPE (rVDGSPE) module for generation of biological networks using the “Analyze Network” and to generate a list of key disease-related networks, direct gene-gene interactions or analysis of connections through the shortest directed paths within the modules and their functional involvement, including other genes enriched in the networks. Probe lists of modules of interest were converted to Entrez IDs and along with their p-values uploaded to the MetaCore™ website (https://portal.genego.com) and a network enrichment analysis performed. Analyze network creates a master network described by MetaCore™ and divides it into smaller networks that can each be built separately. Each network contains nodes from modules of interest that are labeled as seed nodes. In this approach, the networks are prioritized based on the number of fragments of canonical pathways in the network and relative enrichment with the rVDGSPE genes. Networks are ranked by a p-value and FDR and interpreted in terms of Gene Ontology. The “FDR” value of 0.05 was considered as the significance threshold.
Results

Table 1 summarizes the clinical characteristics of the study groups including the discovery and replication cohorts. No differences were observed between maternal gestational age at enrollment, age, and distribution by race in both cohorts (all $p >0.05$, minimum $p=0.12$). The vitamin D serum level was significantly lower in pregnant women in the VDAART discovery cohort who developed preeclampsia compared to those who did not ($p=0.004$). Overall, in this cohort and according to the current guidelines for the recommended serum vitamin D level during pregnancy, 125 and 38 subjects had insufficient (<30 ng/mL) and sufficient vitamin D status ($\geq 30$ ng/dL), respectively.

Table 1. Clinical characteristics of participants in the cohorts of study

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Discovery cohort (VDAART)</th>
<th>Replication cohort (Omega)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PE (N=53)</td>
<td>No PE (N=110)</td>
</tr>
<tr>
<td>Gestational age (weeks)</td>
<td>10-18</td>
<td>10-18</td>
</tr>
<tr>
<td>Race (number)</td>
<td>20</td>
<td>44</td>
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<tr>
<td></td>
<td>27</td>
<td>61</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>5</td>
</tr>
<tr>
<td>Serum 25OHD (ng/mL)</td>
<td>19.2 ± 8.2</td>
<td>24.4 ± 14.5</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>6.9-36.5</td>
<td>5.1-80</td>
</tr>
</tbody>
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Gene expression profiling

Vitamin D (25OHD) status during early pregnancy (<30 ng/mL [N=125] vs. ≥30 ng/dL [N=38]) resulted in differential expression of 2701 genes, including 1323 up-regulated and 1378 down-regulated genes in VDAART subjects . These vitamin D gene signatures (VDGS) are representative of a potential role of vitamin D in different biological functions that might be affected by vitamin D status and according to the defined cut-off (vitamin D status) at early pregnancy, include pathways related to the risk of PE development (Figure 2 and 4).
The pregnant women who developed PE in the VDAART gene expression study (N=53) showed 1607 gene that were differentially expressed. These gene signatures of PE (GSPE) showed functional relationship in association with PE by 1087 up- or 520 down-regulations Accordingly, these genes could be representative of functional pathways that might be differentially expressed in a pregnant women destined to develop PE, including vitamin D related functional pathways. The intersection of PE-associated differentially expressed genes and vitamin D gene expression signatures presented 1223 unique genes that were expressed through 540 up-regulated and 693 down-regulated genes, namely vitamin D gene signatures for susceptibility to PE (VDGSPE). In the replication cohort, 298 genes of these genes were differentially expressed between the PE subjects and controls (p<0.05). Of these, 216 genes showed up-regulation and 162 genes were down regulated (Supplement 1). This replicated subset of VDGSP was subsequently used for network and functional profiling (rVDGSPE).

Network analysis and functional profiling

The enrichment analysis of biological processes using Metacore™ (https://portal.genego.com/) suggested the involvement of VDGSP genes in several processes the immune system process (FDR=2.755e-21), immune responses (2.011e-17), response to stress (2.478e-11), regulation of leukocyte migration (4.275e-6), inflammatory response (2.251e-7), immune defense responses (4.330e-20), response to hypoxia (2.457e-3), and platelet activation (5.727e-7) and degranulation (1.4e-10) (Figure 5, Supplement 3). Approximately, 500 potential biological functions and disease categories in the MetaCore™ classified database incorporated the genes of interest in VDGSP (all FDR<0.05) of which the majority of these biologic process or disease categories were immune or autoimmune related. The top ranked objects analyzed in disease networks were: MHC class II beta chain, Galpha (q)-specific peptide GPCRs, HLA-DQB1, and IL8 and the majority of 298 rVDGSPE genes were involved in the top scored networks. Of note some network objects such as MHC class II beta and HLA-DQB1 were common key objects among the majority of networks (Figure 7).

Applying disease network-building algorithms to the rVDGSPE, MetaCore™ identified several networks corresponding to known disease networks. Of these, the most prominent networked rVDGSPE genes were
related to the genes involved in systemic lupus erythematosus (FDR=1.495e-17), immune system diseases (2.869e-14), autoimmune diseases (7.511e-14), platelet activation (5.727e-7), response to stimulus (1.854e-5), pregnancy induced hypertension (2.043e-4), and inflammation (4.497e-12, 8, supplement 3). Twenty-seven of the rVDSGPE genes were related to pregnancy complications (ranked 71 among the networks, 2.570e-6 and according to the Metacore database), with a subset of 12 known PE associated genes (UTS2, GSTM1, HP, CXCL10, HLA-DQB1, MMP-9, OLR1, F5, CLU, ITGB3, NOD2, S100B; all p <0.05, maximum p=0.039, Supplement 4).

By the network analysis algorithm, 30 correlated networks were identified that were enriched with seed nodes generated from the root list “VDGSPE” (Supplement 5). “cAMP responsive element binding protein 1 (CREB1)” was the key component of these networks (Supplement 5). Analyzing the direct interactions between “rVDGPSE” objects, we identified matrix metalloproteinase 9 (MMP-9) as a major hub with direct interactions with CLU, IP10, MMP-9, THBS1, LCN2, HP, MMP8, IP10, LGA52, ACKR1, SUB1, IL8, and NID1. MMP-9 was the convergence hub in shortest directed paths among the master network rVDGSPE objects with 143 edges and similarly CREB1 with 100 edges was the main divergence hub. The seed node “MMP-9” with other objects of the network “CREB1 transcription factor (TF) vitamin D (1,25- dihydroxyvitamin D3) receptor (VDR)” formed the core hub of the network (Figure 9 and 10). Similar interactions were observed in tracing the pathway between the observed core elements in peripheral blood with placenta (Figure 9).
Figure 3. A. Drawing the direct interactions between rVDGSPE genes. B. Identifying islands of directly interconnected objects from the seed node list of rVDGSPE. C. Identifying common genes of the identified islands involved in preeclampsia (darker blue circles with red dot)
Figure 4. Graphical displays of the estimated false discovery percentage and the number of identified genes in the VDAART expression study using the output from RP under the defined cut-off, 0.05; identified genes are marked in red.

A. Differentially expressed genes for vitamin D status during early pregnancy (<30 ng/mL vs. ≥30 ng/dL, class 2 representing low vitamin status).

B. Differentially expressed genes for disease status (preeclampsia vs. normal pregnancy, class 2 representing the outcome of pregnancy as preeclampsia). Of note, both graphs show relatively similar distribution of p values under the comparative classes.

Figure 5. MetaCore™ identification of the known biological functions within the replicated differentially expressed genes (rVDSGPE) using GO process. Top 10 processes have been shown (supplement 2).
Figure 6. The top 10 identified diseases sharing network objects with rVDGSPE module using Metacore™ (supplement 3).

Figure 7. Interactions of MHC class II genes via HLADOB (one of genes in rVDGSPE module) with CREB1 and VDR.
Figure 8. The central hub of the rVDGSE module consists of CREB1, MMP-9, interacting with VDR-VDR/RXR-alpha, as defined by the number of network edges. Thick cyan lines indicate the fragments of canonical pathways. The seed nodes not in direct interaction with MMP-9 are hidden. CREB1 has the maximum number of edges in the network (109) and is regulated by VDR through STAT1 and other alternative pathways.
**Figure 9.** The demonstration of regulatory pathways among VDR, CREB1, MMP9 and STAT1. VDR might express an inhibitory effect on MMP9 through binding with STAT1. STAT1 inhibits MMP9 by transcript regulation. Additionally VDR inhibits CREB1, a major hub of rVDGSPE module, by direct binding mechanism. Green arrows demonstrate activation and red arrows inactivation.
Figure 10. Traced placental active pathways extrapolated in Metacore™. Blue edges show the similarity of placental pathways with those of peripheral blood and interactions among VDR, CREB1, MMP-9 and STAT1.
**Discussion**

Few studies have investigated the gene expression profiling of maternal peripheral blood throughout pregnancy in women who developed PE and have demonstrated several differentially expressed genes (Dahlstrom, Esbensen et al. 2010, Enquobahrie, Qiu et al. 2011, Rajakumar, Chu et al. 2011). While our findings are consistent with these reports in identifying genes involved in a systematic inflammatory response, we additionally demonstrated that vitamin D insufficiency, might affect the function of many key PE related genes, e.g. MMP-9, CLU, IL-8, and MHC class II beta chain and introducing CREB1 as a center for interaction of PE related genes.

In complex disorders, it is more plausible that single gene expression often does not perform as well as signatures extrapolated from clinically relevant sets of genes. Maternal health features could play an important role in intrauterine growth and fetal development. In this view, the role of vitamin D has been investigated in influencing the development of diseases (e.g., PE, gestational diabetes) during pregnancy as well as the origin of childhood and adult disorders (e.g., asthma and allergy) (Kovacs 2008, Wei, Qi et al. 2013, Mirzakhani, Al-Garawi et al. 2015).

In this study, we first identified differentially expressed genes (VDGS) among women with low 25(OH)D compared with those with high 25(OH)D concentrations during early pregnancy (Enquobahrie, Williams et al. 2011). Accordingly, VDGS is representative of the genes whose differential expressions might be potentially involved in the development of vitamin D-associated diseases during pregnancy (e.g., PE, gestational diabetes) and/or development of childhood and adult disorders (e.g., asthma and allergy) (Kovacs 2008, Wei, Qi et al. 2013, Mirzakhani, Al-Garawi et al. 2015). The overlaps of VDGS with PEGS (VDGSPE) allowed us to target the potential vitamin D associated genes specifically involved in PE and identify the most generalizable ones through the replication set (rVDGSPE). Consequently, the rVDGSPE enrichment analysis is suggestive of highly functional gene modules related to the systematic inflammatory responses, which implicates the emergence of distinctive immune response changes compared to normal early pregnancy. This observation is consistent with
previous reports that such immune response dysregulations predate the presence of PE symptom occurrence in the second trimester (Germain, Sacks et al. 2007, Founds, Conley et al. 2009, Enquobahrie, Qiu et al. 2011).

One of the prominent immune response patterns in PE is $T_h1/T_h2/T_h17$ imbalance with $T_h1/T_h17$ predominance versus more prominent T regulatory (Treg)-$T_h2$ activities that favor an immune-tolerance state to fetal components (Saito and Sakai 2003, Perez-Sepulveda, Torres et al. 2014). These types of immune response alterations, evidences of inadequate tolerance induction, increased risk of PE due to level of exposure to paternal seminal fluid prior to conception, as well as the identification of autoantibodies that activate the angiotensin type 1 receptor (AT1) on a variety of cell types, have implicated PE as a pregnancy-induced autoimmune condition (Saito, Sakai et al. 2007, Xia and Kellems 2009, Saftlas, Rubenstein et al. 2014). TNF-$\alpha$, IL-12, L-18, TGF-$\beta$, and IFN-$\gamma$ are the main cytokines responsible for the chronic inflammatory response in PE during pregnancy (Germain, Sacks et al. 2007, Dragojevic-Dikic, Marisavljevic et al. 2010). There is evidence that anti-angiogenic factors, products of oxidative stress and circulating syncytiotrophoblast micro particles released from the placenta are stimulants for the release of theses cytokines and subsequent PE-associated systematic inflammatory responses (Germain, Sacks et al. 2007). Vitamin D could have effects on the both innate and adaptive immune responses involved in PE. Vitamin D augments the development of immature “tolerogenic” dendritic cells (DC)” after antigen encounter through toll-like receptors (TLRs) and more importantly might help in maintenance of the induced “tolerogenic” phenotype. This effect suppresses DC maturation, macrophage differentiation and cytokine profile predominance (Hewison 2010). Furthermore, T cell antigen activation increases the T cell” vitamin D receptor (VDR) expression and a shift of $T_h1$ to $T_h2$. Vitamin D has also been implicated in exerting some effects on inflammation and autoimmune responses through the regulation of $T_h17$ cells and the induction of Tregs (Chang, Chung et al. 2010, Hewison 2010, Joshi, Pantalena et al. 2011). CXCL10/IP-10 is a powerful chemokine driving Th1-mediated inflammation and has been proposed to be a key link between inflammation and angiogenesis. Higher concentrations of CXCL10/IP-10 have been reported in preeclamptic women (Gotsch, Romero et al. 2007), and, we consistently identified higher expression of CXCL10 in VDAART preeclamptic women. Vitamin D is able to counteract CXCL10 production and release (Scolletta, Colletti et al. 2013).
Furthermore, we identified several networks enriched with the rVDGSPE genes, in which CREB1 was a key component to their interactions (Figure 6 and 7). Vaiman et al. reported overrepresentation of CREB1 along with NFkB in placenta of preeclamptic women. CREB inhibits NFkB, as well (Wen, Sakamoto et al. 2010). Reactive oxygen species (ROS) serve as signaling molecules that induce transcription of several genes, including CREB1 and Nuclear factor-kappaB (NFkB), which are important in oxygen sensing, cell differentiation, and proliferation (Rudov, Balduini et al. 2014). Of note, PE has been also linked to oxidative stress (Roberts and Lain 2002, Thompson and Al-Hasan 2012).

The CREB1 gene encodes a TF that is a member of the leucine zipper family of DNA binding proteins. This protein binds as a homodimer to the cAMP-responsive element, an octameric palindrome. The protein is phosphorylated by several protein kinases, and induces transcription of genes in response to hormonal stimulation of the cAMP pathway. Vitamin D exerts an inhibitory effect on the functionality of CREB1 (Nakane, Ma et al. 2007, Yuan, Pan et al. 2007). This effect takes on greater significance as CREB contributes to the regulation of placenta growth factor (PLGF) gene expression. Moreover in cytotrophoblast cells CREB modulates human chorionic gonadotropin (hCG) gene-expression by a direct protein-protein interaction with AP-2α (Vaiman, Calicchio et al. 2013).

Among the genes in rVDGSPE, MMP-9 had the most direct interaction with other genes in the module that have been reported in association with PE e.g. S100B (Schmidt, Tort et al. 2004), CLU (Blumenstein, McCowan et al. 2012), CCL16 (Makikallio, Kaukola et al. 2012). MMP-9 is also connected with several other PE associated genes such as OLR1 (Zuniga, Ormazabal et al. 2014), ITGB3 (Chappell and Morgan 2006), F5 (Serrano 2006) and NOD2 (van Rijn, Franx et al. 2008) through shortest pathways analysis (Figure 8). MMP-9 is a zinc dependent proteinase involved in inflammation, tissue remodeling, processing of cytokines, matrix-bound growth factors mobilization and trophoblastic invasion and placentation. It has shown that the maternal plasma concentration of MMP-9 is higher in preeclamptic women during first trimester (Poon, Nekrasova et al. 2009). Timms and colleagues measured the circulating MMP-9 and discovered that vitamin D deficiency is associated...
with elevated levels of circulating MMP-9 and inflammatory immune responses, which vitamin D supplementation substantially reduced MMP-9 concentration and inflammation markers (Timms, Mannan et al. 2002). NF-kB TF is also required for MMP-9 expression (Bond, Chase et al. 2001, Rhee, Lee et al. 2007, Chou, Sheu et al. 2010), a process that is suppressed by vitamin D. To achieve this effect, the vitamin D receptor (VDR) directly interacts with IκB kinase β (IKKβ) to block NF-kB activation (Sun, Kong et al. 2006, Chen, Zhang et al. 2013). Importantly, NF-kB has been shown to be more active in the placentas of preeclamptic women (Vaughan and Walsh 2012).

There are other additional biochemical mechanisms through which vitamin D might interactively regulate the function of the VDGSPE module and its hub components through VDR. Rajakumar and colleagues reported the downregulation of signal transducer and activator of transcription 1 (STAT1) and upregulation of MMP-9 in maternal gene expression of preeclamptic women in their peripheral blood (Rajakumar, Chu et al. 2011). STAT1 inhibits MMP-9 (Ramana, Chatterjee-Kishore et al. 2000), and this process may be augmented by VDR interaction which prolongs STAT1 activation and increases its induced transcription (Figure 8) (Vidal, Ramana et al. 2002). STAT1 and NF-kB collaboratively regulate the expression of many inflammatory genes (Vidal, Ramana et al. 2002). Nucleotide-binding oligomerization domain containing 2 (NOD2), a down-regulated gene in rVDGSPE and previously linked to PE might play a role in the immune response through activating the NF-kB protein. Vitamin D through ligand-bound VDR directly induces NOD2 gene transcription (Wang, Dabbas et al. 2010).

There are some considerations in the interpretation of our findings. In the previous study on the association of asthma and vitamin D with PE (chapter 2), we have shown that a vitamin D concentration increase to 40-50 ng/mL might minimize the risk for PE development during pregnancy. The single vitamin D cut-off of 30 ng/mL in this study reflects the current recommendation for vitamin D in adults. Adding this fact to a larger sample size in both discovery and replication cohorts could help in the identification of a more robust set of differentially expressed genes (rVDGSPE). While we gained substantial information in using peripheral blood
gene expression of PE subjects during early pregnancy and showed the similarity of placental pathways, the study of placenta in early pregnancy and full term might provide more knowledge on potential biological mechanisms of how vitamin D impacts the outcome of pregnancy “preeclampsia”.
Chapter IV.
Conclusion and future perspectives

The applied evidence-based approach in this research had some unique features that enabled us to streamline our study design in investigating the association of vitamin D status with preeclampsia development as well as how vitamin D status could affect the previously reported association of asthma with preeclampsia. The conducted literature review (chapter 1 and the introduction and chapter 2) provided the epidemiologic evidence on the association of “Asthma and Vitamin D status”, “Vitamin D and Preeclampsia” and “Asthma and Preeclampsia (Murphy, Namazy et al. 2011, Hyponnen, Cavadino et al. 2013). However, while the “Asthma and Preeclampsia” studies considered additional modifiable factors for risk prediction of preeclampsia development, we demonstrated that lack of adjustment for vitamin D status, as a contributor to both preeclampsia development and asthma exacerbations, was a notable weakness.

Subsequently, we hypothesized that vitamin D was the major predictor of preeclampsia and a confounder in previously observed association between “Asthma and Preeclampsia”. Using the data on the outcomes of pregnancy from the Vitamin D Antenatal Asthma Reduction Trial (VDAART), we showed that maternal vitamin D level in early pregnancy, but not asthma or its exacerbation during pregnancy, is an important modifiable risk factor of PE during early pregnancy (chapter 2). Furthermore, our study showed the current recommendation of 400-600 IU/day for pregnant women with low vitamin D status and subsequent serum levels, as also previously studied (Vieth, Chan et al. 2001, Heaney, Davies et al. 2003, Hollis and Wagner 2004, Hollis, Johnson et al. 2011), will not provide sufficiently high 25(OH)D concentrations to effectively reduce the risk of PE. Our data also suggested that serum vitamin D higher than the current recommendation of 30 ng/mL is required to minimize the risk preeclampsia occurrence such that a vitamin D serum level of 40-50 ng/mL might provide the optimal concentration for the greatest risk reduction of preeclampsia during pregnancy. Accordingly, our investigation recommends early detection of low vitamin status and adequate vitamin D supplementation to effectively reduce the risk of preeclampsia and its maternal-fetal consequences.
Vitamin D supplementation has been also shown to affect the expression of several genes. As a follow-up study, it was of vital importance to perform a gene expression profiling influenced by vitamin D among women who developed preeclampsia compared to those without preeclampsia during their pregnancy (chapter 3). Therefore in the continuation of our sequential evidence based approach, we examined the gene expression profile of a subset of VDAART cohort to identify vitamin D gene expression signatures for susceptibility to preeclampsia (VDGSPE). Our results are suggestive of differential expression of several hundred genes that predate the presence of preeclampsia. Vitamin D serum levels influence the regulation of several of these networked genes during early pregnancy. Our findings support a network model for genes involved in preeclampsia. The identified genes have interactive regulatory effects on their expression such that they build a functional network with many sub-regulatory networks. Notably, the network model would account for how previously identified genes associated with preeclampsia might interact each other. Importantly, we identified several genes (298 replicated VDGSPES) and key transcript factors (e.g. CREB1) that their expression might have a key regulatory role in development of preeclampsia and vitamin D might have a direct effect on the regulation of these preeclampsia-associated genes. The network analysis on the 298 differentially expressed genes yielded some novel insights for the pathophysiology of preeclampsia and identification of potential biomarkers during early pregnancy of women destined to develop preeclampsia. The activity of several functional networks corresponding to biological process in immune responses and autoimmune diseases is suggestive of involvement of dysregulated global inflammatory response that predate the clinical presentation of preeclampsia in the second or third trimester. The identified key network genes and transcript factors such as MMP-9 and CREB1 might play an important role in the regulation of observed inflammatory responses under the influence of VDR.

The obtained insights in this dissertation not only highlights the role of vitamin D in development and preeclampsia but also helps in designing a future larger studies to further investigate the role of vitamin D during pregnancy. Such longitudinal clinical trials with larger sample size will be required to investigate the appropriate doses of vitamin D in early pregnancy to achieve the optimal serum concentration of vitamin D to prevent
preeclampsia. These appropriately designed studies will help in making our results generalizable as well as adding the gene expression profiling to more robustly investigate the regulatory role of VDR and vitamin D supplementation on the network components that their interactive biological functions might be responsible for development or prevention of preeclampsia. Substantial information could be obtained in using peripheral blood gene expression of pregnant women during early pregnancy such as identification biomarkers in women who might be at higher risk of preeclampsia. However simultaneous gene expression profiling of placenta in early pregnancy and full term might provide more knowledge on potential biological mechanisms of how vitamin D impacts the outcome of pregnancy “preeclampsia” and is recommended. High prevalence of vitamin D deficiency during pregnancy, estimated to be from 20%-84% worldwide (Karras, Anagnostis et al. 2014), reemphasizes the importance of further investigation to define the optimal vitamin D serum concentration during pregnancy and to demystify the associated biological mechanisms involved in preeclampsia related to low vitamin D status.
Bibliography


Confino-Cohen, R., et al. (2014). "Vitamin D, asthma prevalence and asthma exacerbations: a large adult population-based study." Allergy 69(12): 1673-1680.


Mirzakhani, H., et al. (2014). "Vitamin D and the development of allergic disease: how important is it?" *Clin Exp Allergy*.

Mirzakhani, H., et al. (2014). "Vitamin D and the development of allergic disease: how important is it?" *Clin Exp Allergy* (00) 01-12.


