Distribution Agreement

In presenting this thesis or dissertation as a partial fulfillment of the requirements for an advanced degree from Emory University, I hereby grant to Emory University and its agents the non-exclusive license to archive, make accessible, and display my thesis or dissertation in whole or in part in all forms of media, now or hereafter known, including display on the world wide web. I understand that I may select some access restrictions as part of the online submission of this thesis or dissertation. I retain all ownership rights to the copyright of the thesis or dissertation. I also retain the right to use in future works (such as articles or books) all or part of this thesis or dissertation.

Signature:

_____ _____

Ellen Margaret Smith Date

Vitamin D and the Regulation of Iron Metabolism: Implications for Anemia of Inflammation

By

Ellen Margaret Smith
Doctor of Philosophy

Nutrition and Health Sciences

_____
Vin Tangpricha, M.D., Ph.D.
Advisor

_____
Jessica A. Alvarez, Ph.D., R.D.
Committee Member

_____
Jose N. Binongo, Ph.D.
Committee Member

_____
Usha Ramakrishnan, Ph.D.
Committee Member

_____
Thomas R. Ziegler, M.D.
Committee Member

Accepted:

_____
Lisa A. Tedesco, Ph.D.
Dean of the James T. Laney School of Graduate Studies

_____
Date

Vitamin D and the Regulation of Iron Metabolism: Implications for Anemia of Inflammation

By

Ellen Margaret Smith
B.S., Cornell University, 2010

Advisor: Vin Tangpricha, M.D., Ph.D.

An abstract of
A dissertation submitted to the Faculty of the
James T. Laney School of Graduate Studies of Emory University
in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

in Nutrition and Health Sciences

2016

ABSTRACT

Vitamin D and the Regulation of Iron Metabolism: Implications for Anemia of Inflammation

By Ellen Margaret Smith

Vitamin D deficiency and anemia are highly prevalent in the general population, and several chronic diseases including cardiovascular disease and chronic kidney disease carry increased risk for both conditions. Vitamin D deficiency has been identified as a risk factor for anemia in epidemiologic studies, and *in vitro* studies suggest that vitamin D may reduce cytokine release and hepcidin expression, markers involved in the etiology of anemia of inflammation. The purpose of this dissertation was to 1) examine the association between vitamin D status and anemia in generally healthy adults; 2) evaluate the effect of vitamin D supplementation on markers involved in the etiology of anemia; and 3) test the effect of vitamin D supplementation on hemoglobin concentrations in a population at risk for vitamin D deficiency and anemia.

These aims were addressed via cross-sectional analyses exploring the association between 25-hydroxyvitamin D [25(OH)D] concentrations and anemia/hemoglobin, in a racially diverse cohort of employees of Emory University, and among Vietnamese women of reproductive age. We then used a double-blind placebo-controlled trial of healthy adults randomized to receive a one-time oral dose of 250,000 IU of vitamin D_3 or placebo to test the effects of vitamin D supplementation on plasma pro-inflammatory cytokine, hepcidin, and ferritin concentrations measured at baseline and one week later. Finally, we tested the effect of vitamin D supplementation on hemoglobin and hepcidin concentrations using a double-blind, placebo-controlled trial of critically ill adults randomized to receive 500,000 IU D_3, 250,000 IU D_3, or placebo.

In generally healthy adults, serum 25(OH)D concentrations <20 ng/mL were associated with lower hemoglobin concentrations and increased odds of anemia, particularly anemia of inflammation. High-dose vitamin D_3 supplementation reduced circulating hepcidin concentrations after one week among healthy adults; there were no changes in cytokine or ferritin concentrations. In critically ill adults, treatment with high-dose vitamin D_3 resulted in increased hemoglobin concentrations over time; hepcidin concentrations did not change over time. These results provide preliminary evidence of a role for vitamin D in the regulation of iron metabolism. Larger clinical trials are warranted to fully evaluate the therapeutic efficacy of vitamin D in improving anemia.

Vitamin D and the Regulation of Iron Metabolism: Implications for Anemia of Inflammation

By

Ellen Margaret Smith
B.S., Cornell University, 2010

Advisor: Vin Tangpricha, M.D., Ph.D.

A dissertation submitted to the Faculty of the
James T. Laney School of Graduate Studies of Emory University
in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

in Nutrition and Health Sciences

2016

ACKNOWLEDGEMENTS

There is no such thing as a self-made man. We are made up of thousands of others. Everyone who has ever done a kind deed for us, or spoken one word of encouragement to us, has entered into the make-up of our character and of our thoughts, as well as our success.

-George Matthew Adams

There are many people who have contributed to the success of this dissertation research. I would first like to acknowledge my committee for their guidance during my dissertation work. A special thanks to my mentor, Dr. Tangpricha, who has supported me throughout this process while giving me the freedom to explore different aspects of clinical research and try new things. I am incredibly thankful for everything I have learned, and for the opportunities I have had in working with him. I have truly enjoyed working with Dr. Ziegler - his enthusiasm for nutrition research is infectious and I have learned a great deal from him. I thank Dr. Alvarez for being a wonderful role model, mentor, and friend. I am a better writer, presenter, and scientist because of the countless hours she spent working with me. Dr. Ramakrishnan encouraged me to expand my thinking and strengthened my work by offering alternative points of view to consider. I do not think I have found a course more useful than Dr. Binongo's and I am thankful for his encouragement throughout, including his assistance in the analytical aspects of this work.

One conclusion from this work is that I could not have succeeded in the program if not for Nick, Sam, and Ashley. I feel so incredibly lucky to have landed in a cohort with people who are not only brilliant, passionate, wonderful human beings, but who share a similar sense of humor, self-deprecation, and appreciation for free food (well any food really) and reality TV. I am lucky to have you not only as colleagues but as friends.

Finally, thank you to my family who have supported and encouraged me in everything I have done, and who helped me keep everything in perspective when I was feeling overwhelmed. To my husband Austin, thank you for the love, patience, dance parties, and hiking adventures that sustained me throughout this process. I truly could not have done this without you.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1

INTRODUCTION

Vitamin D is a fat-soluble vitamin and seco-steroid hormone necessary for maintaining calcium homeostasis and supporting bone health.[1] First isolated from cod liver oil in 1922 by McCollum as the factor found to cure rickets, research in recent decades on vitamin D has expanded to explore its extra-skeletal functions.[2] Vitamin D has been suggested to have roles in supporting immune health and reducing inflammation, and in cellular differentiation.[3] Vitamin D deficiency is highly prevalent throughout the world and has been linked to an increased risk for various chronic diseases including heart disease, diabetes, certain cancers, and kidney disease[1]. Recently, vitamin D deficiency has also been associated with an increased risk for anemia, particularly anemia of inflammation.[4-13] This chapter provides an overview of vitamin D metabolism, function, requirements, and deficiency, as well as iron homeostasis and anemia. It will also outline the purpose of this dissertation research.

Vitamin D

Sources and metabolism of vitamin D

Vitamin D is available from the diet or supplements as cholecalciferol (vitamin D_3) or ergocalciferol (vitamin D_2). Natural dietary sources of the nutrient are limited, but include vitamin D_3 from animal products including fatty fish, egg yolks, and cheese, and vitamin D_2 from non-animal sources such as mushrooms. Other food products such as milk, orange juice, and breakfast cereal may be fortified with either vitamin D_2 or D_3.[1] Dietary and supplemental vitamin D is absorbed in the small intestine along with other lipids. Vitamin D enters the enterocyte as a component of the micelle and is absorbed

across the basolateral membrane in a chylomicron. The chylomicron enters the lymphatic system and is subsequently released into the bloodstream and transported to the liver.[14]

The primary source of vitamin D for most individuals is through exposure to sunlight.[15] Ultraviolet B radiation from the sun interacts with a compound in the skin called 7-dehydrocholesterol (7-DHC) which is sequentially converted to previtamin D_3 and then cholecalciferol (D_3).[16] Cholecalciferol synthesized in the skin enters the bloodstream bound to vitamin D binding protein (VDBP) for transport to the liver. There, endogenous vitamin D, along with vitamin D from the diet (vitamin D_3 and D_2) is hydroxylated via 25-hydroxylase to form 25-hydroxyvitamin D [25(OH)D]. 25(OH)D is the major circulating form of vitamin D, and the marker used to assess an individual's vitamin D status owing to its long-circulating half-life (approximately 2-3 weeks).[17] For conversion to the active, hormonal form of the vitamin, 25(OH)D is bound to VDBP for transport to the kidney where it is hydroxylated by 1,α-hydroxylase (CYP27B1) to form 1,25-dihydroxyvitamin D [1,25$(OH)_2$D] or calcitriol[16] [**Figure 1.1**]. This form of vitamin D is not used as the primary marker of vitamin D status given its short half-life in the blood of approximately 8 hours; levels are subject to fluctuate based on the calcium levels within the body[17] Vitamin D is catabolized via 25-hydroxyvitamin D 24-hydroxylase (CYP24A1), which hydroxylates 25(OH)D and 1,25$(OH)_2$D at the 24-C position to produce water-soluble and biologically inert metabolites 24,25$(OH)_2$D and 1,24,25$(OH)_2$D, respectively, which may be excreted in the urine.[14,18]

An area of active research with implications in the assessment of vitamin D status, the mechanism of action, and the biological functions of vitamin D involves the

bioavailability of circulating 25(OH)D. As described above, the marker most often used to assess an individual's vitamin D status is 25(OH)D concentration in the blood, the vast majority of which is bound to VDBP and an additional small percentage is bound to other protein transporters such as albumin. However, less than 1% of 25(OH)D circulates unbound, so called "free vitamin D." Where VDBP-bound 25(OH)D is taken up into the kidney for hydroxylation to $1,25(OH)_2D$, some recent studies have suggested that in extra-renal tissues expressing CYP27B1, such as monocytes, free vitamin D may be rapidly taken up by the cell through simple diffusion, and therefore potentially more bioactive than VDBP-bound 25(OH)D in these tissues.[19] Additionally, there are genetic variations in the VDBP that affect the affinity of the protein for 25(OH)D, and there are racial differences in the form of VDBP commonly expressed.[20,21] While the utility and significance of assessment free 25(OH)D requires continued investigation, the recent commercial availability of an assay to measure free 25(OH)D [DiaSource, Louvain-la Nueve, Belgium] may more readily allow for its measurement and consideration in future studies of the mechanism of action and functionality of vitamin D.

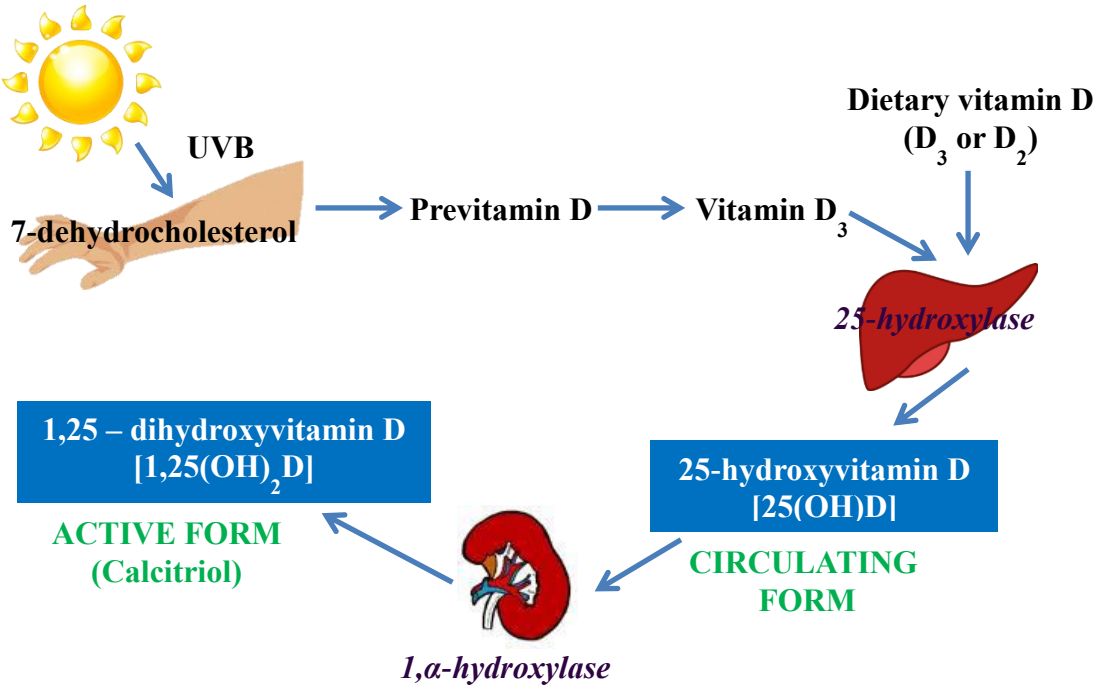


Figure 1.1: Vitamin D metabolism

Functions of vitamin D

Vitamin D exerts its action through genomic and non-genomic processes. Upon conversion to the active form, the classic mechanism of vitamin D action is as follows: $1,25(OH)_2D$ is bound to VDBP and transported to the target cell. $1,25(OH)_2D$ enters the target cell where it binds to its nuclear receptor, the vitamin D receptor (VDR). The $1,25(OH)_2D$-VDR complex binds to a retinoic acid X receptor (RXR) and the $1,25(OH)_2D$-VDR-RXR complex interacts with a vitamin D response element (VDRE) on the DNA of the target cell to affect the rate of transcription.[16]

In addition to the classic genomic action of vitamin D outlined above, vitamin D has also been demonstrated to exert effects through non-genomic pathways. This action involves the binding of $1,25(OH)_2D$ to a membrane VDR and the rapid induction of cellular signaling cascades involved in a wide range of physiologic processes including immune regulation, bone metabolism, and muscle function.[22,23]

The primary and best characterized function of vitamin D is to maintain calcium homeostasis and support proper bone mineralization. Calcitriol is synthesized in the kidney in response to low serum calcium and elevated concentrations of parathyroid hormone (PTH), and acts to stimulate calcium absorption in the small intestine through up-regulation of a number of calcium transport proteins.[18] Low calcium may also stimulate calcitriol and PTH to activate RANKL and promote osteoclast formation and bone resorption to increase calcium concentrations in the blood. Where PTH is a positive regulator of $1,25(OH)_2D$, fibroblast growth factor (FGF)-23, a phosphaturic hormone expressed in bone cells, is a negative regulator of $1,25(OH)_2D$, acting with a cofactor called Klotho to suppress the expression of CYP27B1 and induce the expression of CYP24A1, promoting the catabolism of $1,25(OH)_2D$.[24]

It is now known that CYP27B1 is expressed in numerous tissues throughout the body, including immune cells. Because of this, much attention has been given to the investigation of extra-skeletal functions of vitamin D in the past few decades. Vitamin D is thought to be immunoprotective through roles in supporting innate and adaptive immune responses.[3] A mechanism for vitamin D in innate immunity is as follows: in the presence of a pathogen, toll-like receptors on monocytes signal, through paracrine/intracrine mechanisms, the local hydroxylation of 25(OH)D to form$1,25(OH)_2D$. $1,25(OH)_2D$ binds to its VDR and complexes with RXR and binds to the VDRE on the cathelicidin antimicrobial peptide (*CAMP)* gene to promote the transcription of the antimicrobial peptide cathelicidin, LL-37.[25,26]

Vitamin D may support adaptive immune responses through anti-proliferative effects on T- and B-cells. $1,25(OH)_2D$ has been shown to act on T helper cells to

influence cellular proliferation and cytokine production, specifically to inhibit the Th1 phenotype and associated pro-inflammatory cytokines including interferon (IFN)-γ and tumor necrosis factor (TNF)-α, and promote the Th2 phenotype, which includes the anti-inflammatory cytokine IL-10. Regulatory T cells, which can suppress the action of other T cells so as to prevent an excessive immune response as may be seen in some chronic autoimmune diseases, have also been shown to be induced by $1,25(OH)_2D$.[3] Vitamin D has also been found to have direct effects on B-cells, suppressing B-cell proliferation involved in the pathophysiology of autoimmune disorders including multiple sclerosis and systemic lupus erythematous.[3,27]

The function of vitamin D in the immune system is relevant not only to infectious disease but to chronic disease as well. Indeed, several chronic diseases are characterized by chronic inflammation. In addition to the mechanisms described above, vitamin D has been shown to have specific anti-inflammatory actions by lowering pro-inflammatory cytokines involved in the pathophysiology of several chronic diseases such as chronic kidney disease (CKD), cardiovascular disease (CVD), and inflammatory bowel disease (IBD)[28], through inhibitory or down-regulatory effects on p38 phosphorylation, NFκB, and toll-like receptors[16,29-31].

Vitamin D requirements

The Recommended Dietary Allowance (RDA) for vitamin D is 600 IU per day for people aged 1-70 years, and 800 IU for individuals older than 70 years of age[32] [**Table 1.1**]. However, there is currently some controversy regarding what concentrations constitute an adequate level of 25(OH)D in the blood to support vitamin D functioning in

the body. The Institute of Medicine (IOM) guidelines state that for the average, healthy individual, 25(OH)D concentrations ≥ 20 ng/mL are sufficient to maintain adequate bone and overall health, concentrations between 20 and 12 ng/mL are generally inadequate for bone and overall health, and concentrations < 12 ng/mL are associated with vitamin D deficiency and may put individuals at risk for bone mineralization disorders.[32] The recommendations of the Endocrine Society differ slightly, and are focused on those at risk for chronic disease.[33] These guidelines define vitamin D deficiency as 25(OH)D concentrations < 20 ng/mL and consider this level inadequate to support both skeletal and extra-skeletal functions of vitamin D. Concentrations between 20 and 30 ng/mL are considered insufficient, and concentrations ≥ 30 ng/mL are considered sufficient to support adequate vitamin D functioning throughout the body.

Vitamin D deficiency and toxicity

Vitamin D toxicity, though rare, may occur in the setting of excessive supplemental vitamin D intake, and manifests as hypercalcemia and hyperphosphatemia. Symptoms include fatigue, confusion, nausea, vomiting, constipation, anorexia, polydipsia, polyuria, or muscle weakness.[17] Vitamin D intoxication does not result from sun exposure as prolonged sun exposure results in the formation of inactive photoproducts including lumisterol and tachysterol, and the degradation of previtamin D_3 and vitamin D_3.[1] The tolerable upper intake for vitamin D as designated by the IOM is 4,000 IU daily.[32] In contrast, the Endocrine Society considers intakes up to 10,000 IU per day to be generally safe.[33]

Though vitamin D toxicity is rare, there are certain groups who may be at higher risk than others. Those with hypercalcemic disorders including hyperparathyroidism and chronic granulomatous disease may be at risk for hypercalcemia with pro-longed high-dose vitamin D.[1] Therefore, individuals should be screened for hypercalcemia and hypercalciuria prior to high-dose vitamin D therapy, and serum and/or urinary calcium should be monitored while on vitamin D therapy to avoid toxicity.

In contrast to vitamin D toxicity, inadequate vitamin D status is highly prevalent in the U.S. population. Data from the National Health and Nutrition Examination Survey (NHANES) 2009-2010 suggests that over 25% of adults have 25(OH)D concentrations < 20 ng/mL, and the prevalence is even higher for certain demographic groups.[34] For example, nearly ⅔ of Non-Hispanic Blacks had 25(OH)D concentrations < 20 ng/mL in the 2009-2010 survey.

There are several well known risk factors for vitamin D deficiency.[1] Limited sun exposure such as through sunscreen use, clothes cover, and living in a northern latitude can lead to vitamin D deficiency. Skin pigmentation is another risk factor as higher melanin content in people with darker skin limits 7-DHC conversion to vitamin D_3, thus preventing endogenous production of vitamin D.[35] Older adults may be at risk for vitamin D deficiency as 7-DHC content of the skin decreases with age, again limiting endogenous production of vitamin D.[35] Obesity may result in inadequate circulating 25(OH)D concentrations as vitamin D can become sequestered within adipose tissue and unavailable for biological functioning.[36] Low dietary vitamin D intake, especially in the context of limited sun exposure may lead to vitamin D deficiency.[37] Finally lipid malabsorption such as in those post-bariatric surgery, those with inflammatory bowel

disease, and those with cystic fibrosis, is a risk factor for vitamin D deficiency if insufficient quantities of dietary or supplemental vitamin D are absorbed.[38]

Prolonged vitamin D deficiency can be clinically manifested as bone mineralization disorders: rickets in children and osteomalacia in adults.[1] Research into the extra-skeletal functions of vitamin D has also suggested that inadequate vitamin D status may be associated with cardiovascular disease, chronic kidney disease, certain cancers, and diabetes.[1] Recently, vitamin D deficiency has also been linked to anemia, as will be discussed in the subsequent chapters[4-13]

Vitamin D deficiency can be treated or prevented in at risk groups through vitamin D supplementation. Owing to the long-circulating half-life of 25(OH)D, supplementation may be given daily, weekly, or monthly.[39] High-dose vitamin D has been shown to be efficacious and generally safe in rapidly correcting vitamin D deficiency in both healthy and diseased individuals.[40,41] There is no universally accepted regimen, but in the United States, 50,000 IU of vitamin D is typically given once weekly for 8-12 weeks as an oral capsule to replete 25(OH)D concentrations.[42,43] While both vitamin D_3 and vitamin D_2 are available as supplements and have demonstrated efficacy in repleting 25(OH)D concentrations, there is evidence that vitamin D_3 may be more efficacious in raising 25(OH)D concentrations in the blood, and is therefore the form used in the supplementation studies included in this dissertation.[44]

Iron Metabolism and Anemia

Iron is a trace element found in all cells of the body with many important biological functions including oxygen transport, immune function, cognitive development, and energy metabolism.[45] Iron deficiency is the most common nutrient

deficiency in the world, and is the leading cause of anemia[46]. Other contributors to anemia include hemoglobinopathies, blood loss, inflammation, and other nutrient deficiencies including folate, vitamin B_{12}, vitamin B_6, copper, and as this dissertation will suggest, vitamin D.[47]

Sources and metabolism of iron

There are two types of iron in the diet, heme iron from animal sources, and non-heme iron from plant sources, fortified food, and supplements.[48] Heme iron is bound in a porphyrin ring and good sources include organ meats, red meat, shellfish such as clams and oysters, and poultry. Natural sources of non-heme iron include legumes, nuts, and cooked green leafy vegetables. Fortified sources of non-heme iron include breakfast cereals and breads.[49]

Iron is absorbed in the small intestine where non-heme iron is reduced from the ferric (Fe^{3+}) form to the ferrous (Fe^{2+}) form via duodenal cytochrome b (Dctyb) on the apical membrane of the enterocyte.[50] Fe^{2+} then enters the enterocyte via divalent metal transporter 1 (DMT1). Once inside the enterocyte, Fe^{2+} may complex with ferritin, the iron storage protein, for cellular storage, or it can be absorbed across the basolateral membrane and enter the circulating pool of iron. To cross the basolateral membrane, Fe^{2+} exits the enterocyte via the cellular iron exporter, ferroportin. It is subsequently oxidized to Fe^{3+} via hephaestin and bound to transferrin, the iron transport protein for transport in circulation to target tissues.[51] Less is understood about the mechanism of heme iron absorption, but it likely involves a heme importer on the surface of the apical membrane, a heme oxygenase enzyme inside the enterocyte which may extract Fe^{2+} from

the heme complex, and possibly a heme iron exporter on the basolateral membrane through which it is absorbed.[45,50]

Absorption of iron is highly regulated and determined by iron status. In general, approximately 10-18% of the iron consumed is absorbed, but in times of iron sufficiency only about 10% is absorbed. Conversely, during iron deficiency, approximately 35% of iron consumed is absorbed.[49,52]

Heme iron is better absorbed than non-heme iron and, in fact, enhances the absorption of non-heme iron when the two forms are consumed together.[49,53] Other enhancers of non-heme iron include ascorbic acid and citric acid. Inhibitors of non-heme iron are phytates, compounds found in plants which bind and form insoluble complexes with the iron, preventing its absorption; other inhibitors are polyphenols and calcium.[49]

Iron enters the body through absorption in the small intestine and is subsequently transported in circulation in the ferric (Fe^{3+}) form bound to transferrin.[45] Iron in circulation can be transported to the liver for storage or to other tissues to carry out its various functions which are discussed in the following section.[54] It may also be transported to the bone marrow to support erythropoiesis and hemoglobin synthesis. Iron status and absorption is tightly controlled such that the amount of iron absorbed per day is roughly equivalent to the amount obligatory iron losses (through sweat, urine, feces) per day.[55] This is because iron is recycled within the body. Senescent red blood cells are engulfed by macrophages and the iron is recycled back into circulation to support further erythropoiesis and bodily functions.[54] The majority of iron found in the body is in the functional form (hemoglobin, myoglobin, iron-containing enzymes), and the rest is found

as storage iron (ferrous iron, Fe^{2+}) bound to the iron storage protein ferritin or transport iron (ferric iron, Fe^{3+}) bound to transferrin.[55]

As noted previously, iron status is tightly controlled and regulated at both the cellular and systemic levels. Iron content at the cellular level is regulated through iron regulatory proteins which bind to iron response elements on genes coding for iron importers, exporters, and transporters, thereby affecting the transcription of iron proteins which influence cellular iron content.[54,56] Global iron content is regulated by hepcidin, a 25-amino acid peptide hormone secreted from the liver, which controls iron absorption and iron recycling.[57-59] Hepcidin has a short half-life in circulation (on the order of minutes), and is transcriptionally regulated in response to iron status, erythroid regulators, and inflammation.[60] It operates by binding to and inducing the internalization and degradation of the cellular iron exporter, ferroportin, on the surface of cells of the reticuloendothelial system including enterocytes, macrophages, and hepatocytes.[61] This action blocks the release of storage iron from hepatocytes, limits iron absorption from enterocytes, and prevents the release of recycled iron from macrophages, effectively limiting the amount of iron in circulation.[51] Hepcidin is responsive to body iron content: in times of iron deficiency, hepcidin is down-regulated to increase iron absorption and the amount of iron in circulation.[62] Conversely, when iron is high, hepcidin is up-regulated to prevent further iron absorption and limited the amount of iron in circulation. The signaling pathway related to iron-responsive hepcidin regulation involves bone morphogenic proteins (BMPs) and their receptors. Specifically, BMP-6 acts to stimulate hepcidin transcription in response to elevations in iron stores.[63] Other proteins that may be involved in iron-responsive hepcidin regulation include transferrin receptors 1 and 2,

and membrane proteins HFE and transmembrane serine protease 6 (TMPRSS6) which sense extracellular iron status and may be involved in signaling related to hepcidin expression.[51]

Hepcidin expression is also affected by hypoxia and erythroid regulators.[62] In states of hypoxia, hepcidin is down-regulated to allow more iron to be absorbed and increase circulating iron to increase erythropoiesis and improve oxygen delivery. Erythroferrone (ERFE), a hormone produced in erythroblasts in response to erythropoietin, was recently discovered as an erythroid regulator of hepcidin which suppresses hepcidin transcription after blood loss.[64]

Finally, hepcidin is up-regulated in response to inflammation, specifically cytokines interleukin-6 (IL-6) and interleukin-1β (IL-1β), such that iron becomes sequestered within cells of the reticuloendothelial system and circulating iron is reduced.[65] This is a protective mechanism to limit iron bioavailability for invading pathogens; however, as discussed in the subsequent chapters, the chronic inflammatory stimulus which accompanies many chronic diseases may lead to pathologic dysregulation of iron homeostasis which results in anemia of inflammation.[66]

Functions of iron

Iron has numerous important and diverse functions in the body as a component of heme proteins and iron metalloenzymes.[45] Heme proteins hemoglobin and myoglobin are required for oxygen transport and metabolism. Other examples of functions of heme proteins include proteins involved in signal transduction, cytochromes involved in the electron transport chain necessary for ATP production and cellular respiration, and

peroxidases and catalases responsible for the clearance of reactive oxygen species.[45,67] Iron metalloenzymes and other non-heme iron proteins are necessary for several metabolic functions including amino acid metabolism and protein synthesis, carbohydrate metabolism, and DNA synthesis.[45,67]

Iron requirements

The Dietary Reference Intakes (DRIs) for iron in different age groups and sexes are based on obligatory losses (fecal, urinary, dermal), menstrual losses, fetal requirements in pregnancy, requirements for growth, and tissue and storage iron.[68] The RDAs for iron at different ages and physiological states are given in **Table 1.1**.[69] The tolerable upper limits of intake are 40 mg/day for individuals younger than 14 years and 45 mg/day for those 14 years and older, based on amounts associated with gastrointestinal distress.[69]

Table 1.1: Recommended Dietary Allowances for Vitamin D and Iron

	Vitamin D (IU/d)		**Iron (mg/d)**	
Age (y)	**Male**	**Female**	**Male**	**Female**
0 – 0.5	400*	400*	0.27*	0.27*
0.5 – 1	400*	400*	11	11
1 – 3	600	600	7	7
4 – 8	600	600	10	10
9 – 13	600	600	8	8
14 – 18	600	600	11	15
19 – 50	600	600	8	18
51 – 70	600	600	8	8
>70	800	800	8	8
Pregnancy	N/A	600	N/A	27
Lactation	N/A	600	N/A	9**

*Adequate Intake
**RDA for lactating females age 19-51; RDA for lactating females age 14-18: 10 mg/d

Iron deficiency and toxicity

Iron deficiency is highly prevalent worldwide and is a major contributor to anemia; approximately half of all anemia cases are attributed to iron deficiency.[70] Iron status may be assessed using several biomarkers, the most common of which are blood ferritin concentration, transferrin saturation [(serum iron/total iron binding capacity) x 100], soluble transferrin receptor concentrations (sTfR), and erythrocyte protoporphryin concentrations. Cut-offs used to define iron deficiency in adults in the general population are serum ferritin < 12 μg/L, transferrin saturation < 16%, transferrin receptor concentrations ≥ 8.5 mg/L, and/or erythrocyte protoporphyrin concentrations > 1.42/μmol/L.[71,72] It is recommended that two or more abnormal values be used to diagnose iron deficiency for two reasons. First, on a population level, the prevalence of anemia was only elevated when at least two iron indices were abnormal; there was not a higher prevalence of anemia with one abnormal value compared to no abnormal values. Secondly, ferritin is an acute phase reactant and if used alone, it may be challenging to diagnose iron deficiency in the presence of an infection or inflammation.[71,72] Other markers used to assess iron status, though not as widely used in clinical practice, are reticulocyte hemoglobin content (CHr) and the ratio, log(sTfR concentrations/ferritin) concentrations or the ratio, sTfR/log(ferritin).[73,74] These markers may be useful for distinguishing anemia of iron deficiency from anemia of inflammation, and help to identify true deficits in iron storage (sTfR/ferritin ratio measures) as well as the iron incorporated during erythropoiesis (CHr). A sTfR/log(ferritin) value ≥ 1.03 mg/L is predictive of iron deficiency anemia.[73,75]

Iron deficiency is a gradual process, progressing from depletion of iron stores (reduced blood ferritin concentrations) to iron deficient erythropoiesis (reduced

circulating iron and transferrin saturation, and increased transferrin receptor), and finally iron deficiency anemia or reduced functional iron (decreased hemoglobin concentrations).[46,72] A specific type of iron deficiency anemia called iron-refractory iron deficiency anemia because it is resistant to oral iron therapy, may also manifest due to a mutation in the TMPRSS6 gene such that hepcidin is overexpressed. [51] These elevations in hepcidin result in reduced iron absorption and iron sequestration within macrophages.

Clinical symptoms of iron deficiency include glossitis, pallor, and koilonychia (spooning of nails).[72] Behaviors related to pica (consumption of non-food items such as dirt and ice) may also be symptomatic of iron deficiency.[72] Other health consequences associated with iron deficiency and iron deficiency anemia are immune compromise or increased susceptibility to infection, adverse effects on growth and development, reduced energy and work capacity, adverse effects on mental capacity and IQ, adverse effects on mother-child interactions, increased risk of labor and delivery complications, increased risk of low birth weight and preterm birth, maternal and neonatal mortality, and increased risk of cardiovascular morbidity and mortality.[46,76,77] Groups at risk for iron deficiency are pregnant women (due to blood volume expansion and increased demands from the placenta and fetus), infants and toddlers (due to the demands of rapid growth), women with heavy menstrual bleeding (due to increased losses), cancer patients (due to chemotherapy, blood loss, and inadequate diets or anorexia), those with gastrointestinal disorders such as inflammatory bowel disease or celiac disease (due to iron malabsorption, blood loss, or inadequate iron intake), and bariatric surgery patients (due to malabsorption, dietary restriction, or blood loss).[46] Treatment options for iron

deficiency include oral or intravenous iron therapy, but treatment may depend on the severity and etiology of the deficiency.[46]

Iron toxicity is rare, but may occur due to excessive supplement intakes. Outcomes related to iron toxicity include gastrointestinal distress, oxidative damage, and organ damage.[69] In addition to excessive supplement intake, iron overload can result due to a disease called hereditary hemochromatosis. Hereditary hemochromatosis occurs most often as a result of a mutation in the HFE gene, but can be due to mutations in genes encoding transferrin receptor 2 (TfR2) and hemojuvulin (HJV) as well.[60,67,78] Mutations in these genes coding for proteins involved in the regulation of hepcidin expression, lead to inappropriately low hepcidin concentrations.[60,67,78,] Hereditary hemochromatosis is most common in males and those of European descent.[67,79] There are no clear cut-offs for diagnosing iron toxicity, but some methods include blood biomarkers such as transferrin saturation > 45% or elevated blood ferritin concentrations (200-400 ug/L), or more invasive methods such as liver biopsy or quantitative iron assessment by MRI.[78,79] Symptoms of iron overload are fatigue, bronzing of the skin, organ damage such as cirrhosis of the liver, and increased susceptibility for chronic diseases including neurodegenerative disease, cardiovascular disease, and cancer.[79] Treatment options for iron overload include phlebotomy and chelation therapy.[78,79]

Anemia

Anemia is defined based on hemoglobin concentrations: < 11 g/dL for children 6 months to < 5 years; < 11.5 g/dL for children 5 to < 12 years of age; < 12 g/dL for children 12 to <15 years of age; < 12 g/dL for non-pregnant women aged 15 years and

older; < 11 g/dL for pregnant women; < 13 g/dL for men aged 15 years or older.[80] These criteria do not differentiate between iron deficiency anemia and anemia of inflammation.

Approximately 1/3 of the global population is anemic, and as noted above, it is estimated that half of all anemia cases are iron deficiency anemia.[81] Globally, the groups with the highest prevalence of anemia are preschool-age children, pregnant women, and non-pregnant women of reproductive age, and the regions with highest burden of anemia are sub-Saharan Africa and Southeast Asia.[80] There is a relatively low prevalence of anemia in the general United States population compared to other regions. However, there are certain groups with a higher prevalence than others. For example, blacks tend to have a higher prevalence of anemia than other race groups.[82-84] Certain diseases and conditions carry an elevated risk of anemia including chronic kidney disease, critical illness, cardiovascular disease, cystic fibrosis, and inflammatory bowel disease.[85-89] While each disease has its own specific risk factors for anemia, some common contributors are nutrient deficiencies (iron, folate, vitamin B_{12}, copper, etc.), blood loss, reduced erythropoietin or erythropoietin resistance, and inflammation.[46,47] Given that several of these diseases are characterized by chronic inflammation, anemia of inflammation is likely to account for at least part of the burden of anemia in these populations.[46,90]

Anemia of inflammation, also known as anemia of chronic disease, is estimated to be the second most common type of anemia after iron deficiency anemia.[90] In states of chronic inflammation, such as with chronic disease, the persistent inflammatory stimulus and consequent elevation in hepcidin may lead to prolonged iron sequestration within cells of the reticuloendothelial system, limiting iron availability for erythropoiesis and

hemoglobin synthesis, and ultimately resulting in anemia of inflammation.[66] Anemia of inflammation is a normochromic normocytic, mild-moderate anemia with hemoglobin concentrations typically in the 8-9.5 g/dL range.[90] It is often challenging to diagnose and differentiate from other types of anemia, but it is characterized by low concentrations of circulating iron despite adequate or slightly elevated ferritin concentrations.[66,90] As described in the previous section, ratio markers of sTfR/log(ferritin) have been suggested for use in differentiating between iron deficiency anemia and anemia of inflammation (sTfR/log(ferritin) $\geq$ 1.03 mg/L is predictive of iron deficiency anemia).[73] Use of this ratio helps to avoid misclassification of iron status in times of inflammation due to the acute phase reaction of ferritin, and performs better in differentiating between anemia subtypes than sTfR alone.[73] Additional markers that may be evaluated in clinical practice to identify and distinguish different types of anemia are red blood cell indices. As noted, anemia of inflammation is a normocytic normochromic anemia with normal values of mean cell volume (MCV), mean cell hemoglobin (MCH), and mean cell hemoglobin concentration (MCHC). This profile is distinct from iron deficiency anemia which is a microcytic hypochromic anemia, and anemia resulting from folate or vitamin B_{12} deficiency which is a macrocytic normochromic anemia.[72] Iron and red blood cell indices comparing iron deficiency anemia and anemia of inflammation are given in **Table 1.2**.

Correct classification of anemia subtype is important because while iron deficiency anemia may be treated through iron supplementation, therapeutic options for anemia of inflammation are less clear. Efforts to address the underlying disease or infection may improve the anemia, but this is challenging with chronic diseases. Red blood cell transfusions may be used if the anemia is sufficiently severe and erythropoiesis

stimulating agents may be used as necessary, but these therapies incur risks to the patient, such as thromboembolism, increased oxidative stress, increased length of hospital stay, and increased risk of mortality.[91,92] Other proposed treatment options include cytokine inhibitors and hepcidin binders or inhibitors, but these are not yet used in general clinical practice.[66] Therefore, additional treatment options should be investigated.

Table 1.2: Iron and Red Blood Cell Indices in Iron Deficiency Anemia and Anemia of Inflammation

Iron index	**Iron Deficiency Anemia**	**Anemia of Inflammation**
Serum iron	Reduced	Reduced
Transferrin saturation	Reduced	Reduced
Ferritin	Reduced	Normal-elevated
sTfR	Elevated	Normal
sTfR:log(ferritin)	≥1.03 mg/L	<1.03 mg/L
Red blood cell index		
MCV	Low	Normal
MCH	Low	Normal
MCHC	Low	Normal

Note: Changes listed are relative to normal values

Purpose of research

The purpose of this dissertation research is to explore the novel role of vitamin D in the regulation of iron metabolism, and provide preliminary data for future trials examining vitamin D as a therapeutic agent for anemia. The broad aims of this work are to **1)** review the existing literature regarding the link between vitamin D deficiency and anemia, and the mechanism underlying this association [chapter 2], **2)** examine and characterize the association between vitamin D status and anemia or hemoglobin concentrations in large cohorts of generally healthy adults [chapters 3 and 4], **3)** evaluate the effect of vitamin D supplementation on markers involved in the etiology of anemia in healthy adults [chapter 5], and **4)** test the effect of vitamin D supplementation on

hemoglobin concentrations in an ill population at risk for vitamin D deficiency and anemia [chapter 6].

The *central hypothesis* of this dissertation is that vitamin D deficiency, defined in this work as 25(OH)D concentrations < 20 ng/mL, will be associated with increased odds of anemia and/or reduced hemoglobin concentrations in generally healthy adults, and that supplementation with vitamin D will reduce inflammation and hepcidin concentrations and increase hemoglobin concentrations. Chapter 2 provides a review of the literature on the link between vitamin D status and anemia from epidemiologic studies, mechanistic studies, and clinical trials. The specific aims and hypotheses for the subsequent analyses (chapters 3-6) are as follows:

Specific Aim 1: To examine the association between vitamin D status and anemia in a generally healthy adult population. This was done by performing a cross-sectional analysis of N=638 adult employees of Emory University enrolled in the Emory/Georgia Tech Predictive Health Institute cohort, with vitamin D status as the predictor variable, and anemia as the outcome of interest. *For this aim the hypothesis was that lower vitamin D status would be associated with increased odds of anemia.*

Sub-aim 1: To examine the association between vitamin D status and specific sub-types of anemia. *Given that vitamin D has been shown to reduce cytokines implicated in the etiology of anemia of inflammation, the hypothesis for this sub-aim was that lower vitamin D status would be specifically associated with anemia of inflammation.*

Sub-aim 2: To examine the racial differences in the association between vitamin D and anemia. *Given that blacks have been reported to have a higher prevalence of both vitamin D deficiency and anemia compared to whites, the hypothesis for this sub-aim was that there would be significant effect modification by race in the association between vitamin D status and anemia.*

<u>Specific Aim 2:</u> To examine the association between dietary vitamin D intake and hemoglobin and anemia in women of reproductive age in rural Northern Vietnam. This was done by performing a cross-sectional analysis of 4,961 non-pregnant women enrolled in the PRECONCEPT Study, with dietary vitamin D intake as the predictor variable and hemoglobin and anemia as the outcomes of interest. *The hypothesis for this aim was that given the link between vitamin D status and anemia reported in other Asian populations, dietary vitamin D intake would follow a similar pattern and would be positively associated with hemoglobin concentration and inversely associated with odds of anemia.*

Sub-aim 1: To overcome concerns regarding the use of dietary vitamin D intake as a marker of vitamin D status, we examined the association between vitamin D status [25(OH)D concentrations] and hemoglobin and anemia in a randomly sampled subset of the population (n=88). *The hypothesis for this sub-aim was that lower vitamin D status would be associated with reduced hemoglobin concentrations and increased odds of anemia.*

<u>Specific Aim 3:</u> To examine the effect of high-dose vitamin D supplementation on plasma pro-inflammatory cytokine, hepcidin, and ferritin concentrations in healthy adults. This was done in the context of a pilot double-blind placebo-controlled trial in which N=28 healthy adults were randomized to receive a one-time oral bolus dose of 250,000 IU vitamin D_3 or matching placebo. Outcomes were measured at baseline and approximately 1 week later. *The hypothesis for this aim was that supplementation with high-dose vitamin D would significantly reduce circulating pro-inflammatory cytokine and hepcidin concentrations, and increase plasma ferritin concentrations relative to placebo.*

<u>Specific Aim 4:</u> To examine the effect of high-dose vitamin D supplementation on serum hepcidin concentrations and hemoglobin concentrations in critically ill adults. This was be done in the context of a pilot double-blind placebo-controlled trial in which N=30 critically ill adults requiring mechanical ventilation were randomized to receive placebo, a total of 250,000 IU vitamin D_3, or a total of 500,000 IU vitamin D_3, administered in five equal doses over the first 5 days after enrollment. *The hypothesis for this aim was that treatment with high-dose vitamin D_3 would reduce hepcidin concentrations and increase hemoglobin concentrations.*

CHAPTER 2

VITAMIN D AND ANEMIA: INSIGHTS INTO AN EMERGING ASSOCIATION

Ellen M. Smith[1] and Vin Tangpricha[1,2,3]

[1]Nutrition and Health Sciences Graduate Program, Laney Graduate School, Emory University, Atlanta, GA, USA

[2]Division of Endocrinology, Metabolism, and Lipids, Department of Medicine, Emory University School of Medicine, Atlanta, GA, USA

[3]Atlanta VA Medical Center, Decatur, GA, USA

Current Opinion in Endocrinology, Diabetes and Obesity.
December 2015;22(6):432-438.
doi: 10.1097/MED.0000000000000199.

Abstract

Purpose of review: This review highlights recent findings in the emerging association between vitamin D and anemia through discussion of mechanistic studies, epidemiologic studies, and clinical trials.

Recent findings: Vitamin D has previously been found to be associated with anemia in various healthy and diseased populations. Recent studies indicate that the association may differ between race and ethnic groups and is likely specific to anemia of inflammation. The mechanism underlying this association involves the reduction of pro-inflammatory cytokines by vitamin D as well as the direct suppression of hepcidin mRNA transcription. There is also evidence that vitamin D may be protective against anemia by supporting erythropoiesis. Other calciotropic hormones including fibroblast growth factor 23 and parathyroid hormone have also been found to be associated with iron homeostasis and erythropoiesis.

Summary: Recent advances in our understanding the association between vitamin D and anemia suggest that maintenance of sufficient vitamin D status may be important in preventing anemia, particularly in diseases characterized by inflammation. Early clinical trials have been promising, but further research is needed to define the efficacy of vitamin D as a future approach for the treatment of anemia.

Key words: vitamin D, anemia, hepcidin, inflammation

Introduction

As the role of vitamin D in health continues to be defined, particularly in terms of extra-skeletal functions, an association between vitamin D and anemia has emerged in recent years, indicating potential roles for vitamin D in iron homeostasis and erythropoiesis. This association has been described in several observational studies in various healthy and diseased populations,[6,8-12,93] and recent *in vitro* studies suggest that the mechanism underlying this association involves the action of vitamin D on inflammatory cytokines and the antimicrobial peptide, hepcidin (the hormone responsible for regulating systemic iron concentrations).[94-96]

Anemia is a common nutritional problem in public health, and may further complicate chronic diseases including kidney and heart disease, resulting in fatigue, shortness of breath, and decreased physical capacity, and if severe enough, cardiovascular morbidity and mortality. Several factors may contribute to anemia including nutrient deficiencies, namely iron, but also folate, vitamins B_{12} and B_6, as well as blood loss, infection, hemoglobinopathies, and inflammation. Given the multifactorial causes of anemia, it can be classified into different subtypes such as iron deficiency anemia or anemia of nutrient deficiency, and anemia of inflammation (also called anemia of chronic disease). Vitamin D, through its down-regulatory effects on inflammatory cytokines and hepcidin may favorably impact anemia, particularly anemia of inflammation. This review will highlight recent advances in our understanding of the vitamin D-anemia association though mechanistic studies, epidemiologic studies, and early clinical trials.

Mechanism of action in the vitamin D – anemia association

Studies have suggested that vitamin D, by down-regulating pro-inflammatory cytokines and hepcidin, may increase iron availability, and there is also evidence that vitamin D may support erythropoiesis (**Figure 2.1**). Through these potential mechanisms of action, vitamin D may therefore improve anemia, in particular, anemia of inflammation.[66]

Inflammation and hepcidin

Vitamin D has well-described anti-inflammatory functions,[28] and has recently been shown to act directly on the antimicrobial peptide hepcidin, which is responsible for the regulation of systemic iron concentrations. Hepcidin, which prevents further iron absorption and iron release from cells during times of iron sufficiency by binding to and inducing the degradation of the cellular iron exporter, ferroportin, is also up-regulated by pro-inflammatory cytokines, interleukin-6 (IL-6) and interleukin-1β (IL-1β).[60,97] This is a protective mechanism during times of acute infection to reduce bioavailable iron necessary for growth of invading microorganisms, in which iron absorption through enterocytes is decreased and iron release from macrophages in the process of iron recycling in diminished.[98] However, in chronic diseases which may carry a prolonged inflammatory stimulus, iron is pathologically sequestered within cells of the reticuloendothelial system and despite adequate iron stores, anemia may result due to impairments in iron recycling yielding insufficient iron available for erythropoiesis and hemoglobin synthesis.[99]

Findings by Zughaier *et al*. support the proposed anti-inflammatory mechanism of action of vitamin D on the hepcidin-ferroportin axis, demonstrating a dose-dependent decrease in release of IL-6 and IL-1β from a cultured human monocyte cell line in the

presence of increasing concentrations of hormonally active 1,25-dihydroxyvitamin D ($1,25(OH)_2D$), along with suppressed hepcidin mRNA expression and increased ferroportin mRNA expression.[96] Bacchetta *et al*. extended these findings, showing that treatment of human monocytes and hepatocytes with 25-hydroxyvitamin D (25(OH)D) or $1,25(OH)_2D$ resulted in significantly decreased expression of mRNA for the hepcidin antimicrobial peptide gene (HAMP).[95] Vitamin D response elements (VDREs) in the promoter region of the HAMP gene were subsequently identified, providing a strong mechanistic basis for the direct action of vitamin D on hepcidin. In healthy volunteers who received a bolus oral dose of 100,000 IU ergocalciferol, significant reductions in serum hepcidin were observed by 24 hours. Similar results were obtained in a second paper by Bacchetta *et al.* in which treatment of peritoneal macrophages obtained from non-infected chronic kidney disease (CKD) dialysis patients with 25(OH)D or $1,25(OH)_2D$ suppressed expression of HAMP. The HAMP expression was further suppressed in peripheral blood macrophages obtained from subjects with CKD and peritoneal infection. In translating these findings to patients who had received ergocalciferol as part of a pilot trial, HAMP mRNA expression in peritoneal macrophages was significantly reduced one month after supplementation.[94]

Based on the above studies, the association between vitamin D and anemia is likely through anemia of inflammation, in which the underlying mechanism involves the direct suppression of hepcidin mRNA expression by vitamin D as well as the reduction of hepcidin-stimulatory pro-inflammatory cytokines. In addition, these early *in vivo* pilot studies in humans indicate that vitamin D supplementation may be effective in suppressing hepcidin mRNA expression and lowering serum hepcidin concentrations.

The subsequent influence on markers of iron status needs to be elucidated in larger, longer-term studies.

Erythropoiesis

Another pathway contributing to anemia of inflammation is through depressed erythropoiesis and reduced red blood cell (RBC) lifespan.[66] This may occur through inflammation- and hepcidin-mediated disruptions in iron recycling as described above, leaving insufficient iron available to support erythropoiesis. Alternatively, inflammatory cytokines may impair erythropoiesis by inhibiting the production of erythropoietin and the differentiation and proliferation of erythroid progenitor cells.[90] In addition to decreasing pro-inflammatory cytokines, vitamin D has been shown to support erythropoiesis by increasing burst-forming unit-erythroid proliferation (BFU-E) and having a synergistic effect with erythropoietin to further enhance erythroid progenitor cell proliferation.[100,101]

In a recent pre-clinical study of ribavirin-induced anemia, Refaat *et al*. found that the addition of vitamin D_3 to chronic hepatitis C therapies, pegylated interferon-α and ribavirin, maintained RBC counts and hemoglobin concentrations and increased erythropoietin concentrations compared to rats who received ribavirin therapy alone.[102] Hemoglobin, RBC count, and erythropoietin concentrations were all positively correlated with serum 25(OH)D concentrations. These findings are suggestive of a protective role of vitamin D against drug-induced disturbances in erythropoiesis.

Studies in CKD patients have shown that vitamin D may reduce erythropoiesis stimulating agent (ESA) requirements.[103,104] In a study of children with CKD on dialysis, Rianthavorn *et al*., found that treatment with high-dose ergocalciferol resulted in

significant reductions in ESA dose at 12 weeks compared to baseline.[105] More recently, Afsar *et al*. found that patients receiving paricalcitol had the lowest ESA resistance compared to those on calcitriol, cincalcet, paricalcitol + cincalcet, or no treatment, and that paricalcitol was significantly inversely associated with ESA resistance.[106] Given these findings, vitamin D may support erythropoiesis, and shows promise as a potential adjunctive therapy for anemia.

Other calciotropic hormones and anemia

In addition to vitamin D, other hormones involved in the bone-mineral axis, including fibroblast growth factor 23 (FGF-23) and parathyroid hormone (PTH), have been shown to be involved in iron metabolism and erythropoiesis (**Table 2.1**).

Fibroblast Growth Factor-23 (FGF-23)

Coe *et al*. showed that FGF-23$^{-/-}$ mice had significantly greater RBC counts, a higher percentage of proerythroblast cells and erythroid cells, and increased BFU-E cells and serum erythropoietin concentrations compared with wild type (WT) mice.[107] The opposite was observed when WT mice were treated with a single dose of FGF-23 protein; serum erythropoietin, and percentage of proerythroblast and erythroid cells in the blood and bone marrow decreased significantly. Interestingly, genetic ablation of Klotho, a necessary cofactor for FGF-23, in a mouse model resulted in increased RBC counts and hemoglobin concentrations along with increased pro-erythroid cells, erythroid cells, and BFU-E cells in the bone marrow compared to heterozygous and WT mice, though hematopoietic stem cells in the bone marrow were reduced.[108] In humans, Scialla *et al*. found that among patients with stage 2-4 CKD, hemoglobin concentration decreased

significantly as FGF-23 quartile increased.[109] These results suggest that unlike vitamin D, FGF-23 is a negative regulator of erythropoiesis and iron metabolism. Elevations in FGF-23, which often accompany cardiovascular disease and renal disease, therefore, have the potential to increase risk for anemia.[110]

Parathyroid Hormone (PTH)

Previous studies have suggested that elevations in PTH may be associated with increased risk for anemia through alterations in erythropoiesis, including reductions in erythroid progenitor formation and erythropoietin synthesis, and PTH-induced fibrosis of the bone marrow.[111] Indeed, Russo *et al*. found that PTH concentrations were significantly inversely associated with hemoglobin concentrations among non-dialysis CKD patients.[112] Furthermore, a recent study assessing risk factors for hyporesponsiveness to ESAs found that elevated concentrations of PTH were associated with reduced odds of becoming responsive to ESAs.[113] While these findings suggest that elevations in PTH may impact iron metabolism and erythropoiesis, there remains uncertainty regarding the specific mechanism(s) and whether this association is independent of vitamin D.

Epidemiology of the vitamin D deficiency and anemia association

Vitamin D deficiency and anemia are important public health problems and are common in both acute and chronic illness. Past studies have demonstrated that low vitamin D status is associated with anemia risk in children, elderly adults, those with CKD, and those with heart failure.[8,9,12,93] Recent studies in patients scheduled for cardiac surgery and community-dwelling elderly men have also shown vitamin D status to be

inversely associated with odds of anemia and positively associated with hemoglobin concentrations, respectively[7,114]. New studies have also extended these findings to explore racial and ethnic differences in the association between vitamin D and anemia, and to further clarify the association with specific subtypes of anemia.

Association of vitamin D with subtypes of anemia and racial differences in the association

Previous population-based studies have indicated that the association between vitamin D and anemia may vary with respect to the etiology of anemia. Lee *et al*. found that among Korean children, the lowest quartile of 25(OH)D was associated with increased odds of anemia in females, but the effect was attenuated to non-significance after adjusting for iron deficiency.[115] Therefore, if iron deficiency is the primary contributor to anemia, improvements in vitamin D status may not confer added benefit.

These results are consistent with a study by Smith *et al*. of generally healthy adults in which serum 25(OH)D concentrations < 20 ng/mL were associated with increased odds of anemia in blacks but not whites. When the cohort was categorized by subtypes of anemia, vitamin D status was associated with anemia of inflammation but there was no association with anemia without inflammation.[116] This is in line with the mechanism of action of vitamin D on pro-inflammatory cytokines and hepcidin described above, and would suggest that when other factors such as iron deficiency, are the predominant contributors to the anemia, the association between vitamin D and anemia may be attenuated.

These results also point to effect modification by race in the vitamin D and anemia association. Vitamin D deficiency and anemia are more common in blacks than

in whites,[84,117] though whether lower 25(OH)D levels commonly found in blacks contribute to the higher prevalence of anemia is not clear. Atkinson *et al*. explored these racial differences in children, and found that hemoglobin increased significantly with increasing quartile of 25(OH)D in the entire study population and among the sub-group of whites, but not in blacks.[5] However, serum 25(OH)D concentrations were significantly lower among blacks compared to whites. When quartiles were determined based on 25(OH)D concentrations in black children only, hemoglobin increased significantly with increasing quartile of 25(OH)D.

Taken together, these epidemiologic studies provide strong evidence for the link between vitamin D deficiency and anemia, particularly anemia of inflammation, and indicate that the association may differ by race. However, several of these studies are limited by their cross-sectional nature. Additional longitudinal and interventional studies are required to determine whether the association between vitamin D deficiency and anemia is indeed causal.

Clinical trials

Data from clinical trials exploring the therapeutic effect of vitamin D on anemia are sparse, but early clinical trials have suggested that treatment with vitamin D may reduce ESA requirements in patients with chronic kidney disease and increase hemoglobin concentrations.[118-120] In a recent trial by Riccio *et al.*, patients with stage 3b-5 CKD and anemia were randomized to receive either paricalcitol (a vitamin D analogue) or calcitriol (the hormonally active form of vitamin D) over 6 months.[121] Subjects who received paricalcitol experienced a significant increase in hemoglobin over time, but interestingly, hemoglobin decreased in the group that received calcitriol.

In a placebo-controlled trial, Sooragonda *et al*. tested the efficacy of high-dose vitamin D in improving hemoglobin concentrations in subjects with iron deficiency anemia.[122] All subjects received iron supplementation. Those randomized to the intervention arm received a one-time intramuscular injection of 600,000 IU of vitamin D_3. After 12 weeks, hemoglobin concentrations did not differ between the vitamin D and placebo group, further confirming that among subjects with iron deficiency anemia, vitamin D is unlikely to offer additional improvements in hemoglobin after correction of iron deficiency.

While these trials add to the vitamin D and anemia literature, addressing which forms of vitamin D may be effective in raising hemoglobin levels and which type of anemia vitamin D may (or may not) improve, there remains a paucity of clinical trials specifically addressing the efficacy of vitamin D in improving anemia.

Implications for clinical practice

Given the mechanistic and epidemiologic evidence for an association specifically with anemia of inflammation, vitamin D may be especially important in preventing anemia in groups with chronic elevations in inflammation status. Patients with chronic kidney disease represent an especially vulnerable group given the characteristic reductions in erythropoietin production, erythropoietin resistance, and reduced ability to convert 25(OH)D to the active hormonal form due to reductions in functional renal mass, along with increased FGF-23 concentrations, and elevations in inflammatory cytokines that promote hepcidin release. In decreasing pro-inflammatory cytokines and directly suppressing hepcidin expression, vitamin D may be effective in mobilizing iron stores and promoting erythropoiesis and hemoglobin synthesis.

Hepcidin concentrations have been shown to be inversely associated with hemoglobin concentrations and positively associated with anemia risk, and therefore represent a potential therapeutic target for addressing anemia.[123] Furthermore, hepcidin concentrations have been used to distinguish iron deficiency anemia from anemia of inflammation, and this distinction may be important in targeting therapies to people with different types of anemia.[124] However, hepcidin is not yet available commercially to be measured in routine clinical practice. In the future, given its regulatory role on hepcidin mRNA expression, vitamin D may provide a promising therapy for anemia either alone or in conjunction with other pharmacotherapies; however, it is not currently FDA approved for this use.

Despite the recent advances in our understanding of the role of vitamin D in iron homeostasis, further clinical trials are needed confirm causality in the vitamin D and anemia association as well as determine optimal vitamin D dosing, the ideal population for therapy, and the preferred form of vitamin D to give.

Conclusions

Vitamin D is associated with anemia in various study populations and recent evidence suggests that the association may differ by race and is likely specific to anemia of inflammation. The link to anemia of inflammation is supported by recent investigations showing that vitamin D can reduce hepcidin-stimulatory pro-inflammatory cytokines, thereby reducing hepcidin, as well as act on hepcidin directly by down-regulating HAMP mRNA transcription. Recent studies have also suggested that vitamin D may support erythropoiesis, possibly through reduction of pro-inflammatory cytokines and increased erythroid progenitor cell proliferation. Other factors on the bone-mineral

axis, including FGF-23 and PTH, may have regulatory roles in iron homeostasis and erythropoiesis, and there is some evidence suggesting that the actions of FGF-23 may be independent of vitamin D.[107] The interplay between all three hormones in regulating iron metabolism will be an interesting area of future study.

In summary, there is strong evidence both epidemiologically and mechanistically to support a role for vitamin D in iron metabolism, but further clinical trials are need to clarify the therapeutic efficacy of vitamin D in improving anemia.

Key points

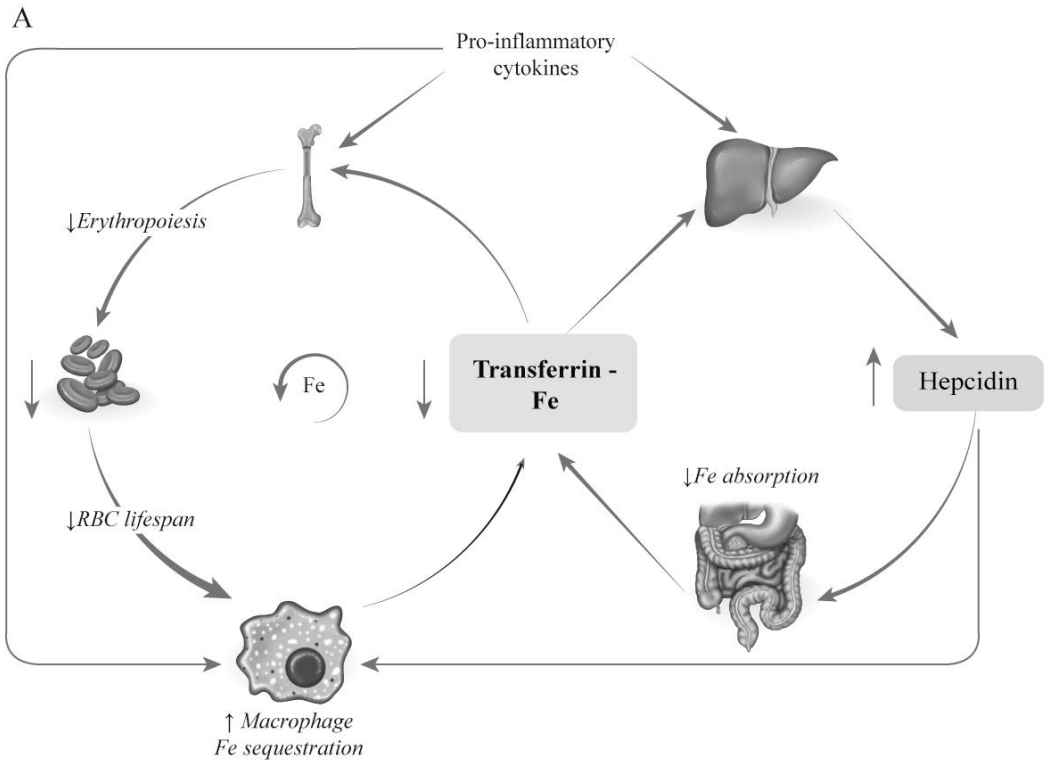
- Vitamin D status has been positively associated with hemoglobin concentrations and inversely associated with risk for anemia, particularly anemia of inflammation
- The mechanism underlying this association involves the reduction of pro-inflammatory cytokines and the direct suppression of hepcidin mRNA transcription by vitamin D.
- Treatment with vitamin D or its analogues has been shown to reduce ESA requirements and increase hemoglobin concentrations in patients with chronic kidney disease.
- Other calciotropic hormones, FGF-23 and PTH, may also be involved in the regulation of iron metabolism and erythropoiesis
- Vitamin D could be a future treatment option for anemia of inflammation, but additional trials are needed to further define its therapeutic efficacy and the interplay between vitamin D, FGF-23 and PTH.

Financial support and sponsorship

Supported by grants from the National Institutes of Health (T32 DK007734 (EMS)) and by the National Center for Advancing Translational Sciences of the National Institutes of Health under award number UL1TR000454 (VT). The content is solely the views of the authors and does not necessarily represent the official views of the National Institutes of Health.

Conflicts of interest

None


A
Pro-inflammatory cytokines
↓Erythropoiesis
Fe
Transferrin - Fe
Hepcidin
↓Fe absorption
↓RBC lifespan
↑ Macrophage Fe sequestration

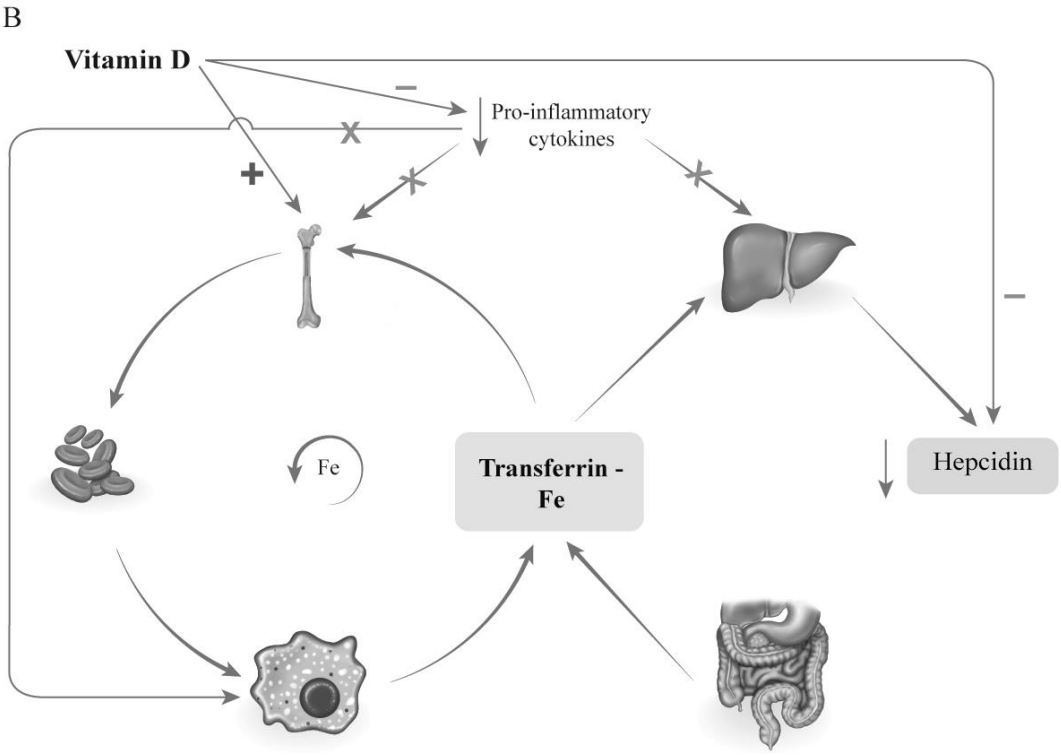

B
Vitamin D
Pro-inflammatory cytokines
Fe
Transferrin - Fe
Hepcidin

Figure 2.1: Alterations in Iron Recycling in Anemia of Inflammation and Proposed Role of Vitamin D. **A.** Alterations in Iron Recycling in Anemia of Inflammation: Iron recycling, under non-pathologic conditions, involves transferrin-bound iron in circulation traveling to the bone marrow to support erythropoiesis. Upon senescence, red blood cells (RBCs) are engulfed by macrophages and iron is recycled back into circulation to support further erythropoiesis. Dietary iron may also enter the circulating pool from absorption in the duodenum based on the body's needs. In anemia of inflammation, elevations in pro-inflammatory cytokines suppress erythropoiesis in the bone marrow and shorten RBC lifespan because of increased macrophage activation and erythrophagocytosis. Cytokines IL-6 and IL-1β stimulate the liver to up-regulate expression of hepcidin antimicrobial peptide (HAMP). Hepcidin inhibits iron egress from cells of the reticuloendothelial system, including enterocytes and macrophages, by binding to and inducing degradation of the cellular iron exporter, ferroportin, resulting in decreased iron absorption and increased iron sequestration in the macrophage. Collectively, depressed erythropoiesis, shortened RBC lifespan, iron sequestration in the macrophage, and reduced iron absorption impairs iron recycling and results in insufficient iron available for erythropoiesis and hemoglobin synthesis, ultimately leading to anemia. **B.** Proposed Role of Vitamin D in Counteracting Anemia of Inflammation: Vitamin D has been shown to promote erythropoiesis by increasing erythroid progenitor proliferation and decreasing pro-inflammatory cytokines. In addition, by decreasing hepcidin-stimulatory pro-inflammatory cytokines, and through direct transcriptional regulation of the HAMP gene, vitamin D may suppress hepcidin expression. Decreases in pro-inflammatory cytokines and hepcidin may increase iron bioavailability for erythropoiesis and

hemoglobin synthesis by restoring iron recycling, preventing iron sequestration in macrophages, and removing impairments on iron absorption, thus protecting against anemia.

Table 2.1: Associations of biomarkers in anemia pathophysiology with calciotropic hormones

Vitamin D	**FGF-23**	**PTH**
↓ Pro-inflammatory cytokines	↓ Hemoglobin	↓ Hemoglobin
↓ Hepcidin	↓ Erythropoietin	↓ Erythropoietin
↑ Serum iron	↓ % pro-erythroblasts	↓ Erythroid progenitor formation
↑ Hemoglobin	↓ Erythroid cells	↑ Fibrosis of bone marrow
↑ Erythroid progenitor proliferation	↓ RBC count	↑ Erythropoietin resistance
↑ RBC count		
↑ Erythropoietin		
↓ Erythropoietin resistance		

CHAPTER 3

VITAMIN D DEFICIENCY IS ASSOCIATED WITH ANAEMIA AMONG AFRICAN AMERICANS IN A U.S. COHORT

Ellen M. Smith[1], Jessica A. Alvarez[2], Greg S. Martin[3], Susu M. Zughaier[4], Thomas R. Ziegler[1,2], Vin Tangpricha[1,2,5]

[1]Nutrition and Health Sciences Program, Graduate Division of Biological and Biomedical Sciences, Laney Graduate School, Emory University, 1462 Clifton Rd Suite 314 Atlanta, GA 30322, USA

[2]Division of Endocrinology, Metabolism and Lipids, Department of Medicine, Emory University School of Medicine, Mailstop 1930-001-1AA Atlanta, GA 30322, USA

[3]Division of Pulmonary, Allergy and Critical Care, Department of Medicine, Emory University School of Medicine, Mailstop 1490-001-1AA, Atlanta, GA 30322, USA

[4]Department of Microbiology and Immunology, Department of Medicine, Emory University School of Medicine, Mailstop 4900-001-1AA, Atlanta, GA 30322, USA

[5]Atlanta VA Medical Center, Decatur, GA USA

British Journal of Nutrition. 2015;113(11):1732-1740.

doi: 10.1017/S0007114515000999

Abbreviations: 25(OH)D, 25-hydroxyvitamin D; CKD, chronic kidney disease; CRP, C-reactive protein; eGFR, estimated glomerular filtration rate; IFN-γ, interferon-γ; IL-6, interleukin-6; IL-8, interleukin-8; TNF-α, tumor necrosis factor-α

Abstract

Vitamin D deficiency is highly prevalent in the U.S. population and is associated with numerous diseases, including those characterized by inflammatory processes. We aimed to investigate the link between vitamin D status and anaemia, hypothesizing that lower vitamin D status would be associated with increased odds of anaemia, particularly anaemia with inflammation. A secondary aim was to examine the effects of race in the association between vitamin D status and anaemia. We conducted a cross-sectional analysis in a cohort of generally healthy adults in Atlanta, GA (*N* 638). Logistic regression was used to evaluate the association between vitamin D status and anaemia. Serum 25-hydroxyvitamin D (25(OH)D) < 50 nmol/l (compared to 25(OH)D ≥ 50 nmol/l) was associated with anaemia in bivariate analysis (OR 2·64; 95% CI 1·43, 4·86). There was significant effect modification by race (*P*=0.003), such that blacks with 25(OH)D < 50 nmol/l had increased odds of anaemia (OR 6·42; 95% CI 1·88, 21·99), versus blacks with 25(OH)D ≥ 50 nmol/l, controlling for potential confounders; this association was not apparent in whites. When categorized by subtype of anaemia, blacks with 25(OH)D < 50 nmol/l had significantly increased odds of anaemia with inflammation compared to blacks with serum 25(OH)D ≥ 50 nmol/l (OR 8·42; 95% CI 1·96, 36·23); there was no association with anaemia without inflammation. In conclusion, serum 25(OH)D < 50 nmol/l was significantly associated with anaemia, particularly anaemia with inflammation, among blacks in a generally healthy adult U.S. cohort.

Key words: Vitamin D, anaemia, inflammation, haemoglobin, hepcidin, African American

Introduction

Vitamin D deficiency has been well described in the U.S. population. Results from the National Health and Nutrition Examination Survey (NHANES) 2001-2006 suggest that vitamin D deficiency is highly prevalent in the U.S. population with approximately 32% of adults exhibiting vitamin D deficiency (defined as serum 25-hydroxyvitamin D (25(OH)D) concentrations < 50 nmol/l) and approximately 76% exhibiting vitamin D insufficiency (defined as serum 25(OH)D concentrations < 75 nmol/l). The prevalence is increased in blacks compared to other race and ethnic groups, where the prevalence of vitamin D deficiency and insufficiency have been reported to be 73% and 97%, respectively.[117] The high prevalence of vitamin D deficiency may have important implications for extra-skeletal health as vitamin D deficiency is associated with a number of disease processes including heart disease, cancer, and infections.[125-127] Recently, vitamin D deficiency has also been found to be associated with anaemia.[9,11]

Anaemia is characterized by a decrease in concentration of red blood cells or haemoglobin, resulting in impaired oxygen transport throughout the body. Furthermore, it is associated with a number of chronic conditions including kidney disease and cardiovascular disease.[85,87] The association between vitamin D status and anaemia has been shown in various populations including children, the elderly, chronic kidney disease patients, and those with heart failure.[5,8,9,12] However, the relationship between anaemia and vitamin D status in the generally healthy adult U.S. population has not been well described.

Previous studies have revealed that the strongest association between vitamin D status and anaemia may be with anaemia of inflammation.[9] The mechanism underlying

this relationship involves the antimicrobial peptide, hepcidin, a hormone involved in the regulation of iron recycling in the body that is induced by pro-inflammatory cytokines including interleukin-6 (IL-6).[57,58,65] Under chronic inflammatory conditions, iron can become sequestered within cells of the reticuloendothelial system and unavailable for erythropoiesis, which may ultimately lead to anaemia.[90,128] Recently, vitamin D has been reported to lower inflammatory cytokines implicated in the pathophysiology of anaemia of inflammation,[96] and suppress expression of hepcidin mRNA.[95] Thus, vitamin D may reduce the risk of anaemia through its anti-inflammatory effects.

The aim of the current paper is to examine the association between vitamin D status and anaemia in a generally healthy adult population. Based on the putative mechanism that vitamin D lowers pro-inflammatory cytokines, we hypothesized that lower vitamin D status would be associated with increased odds of anaemia, particularly anaemia with inflammation. Additionally, given that the prevalence of vitamin D deficiency is higher in blacks compared to whites, and lower haemoglobin concentrations have been reported in blacks compared to whites,[83,84] we hypothesized that there would be significant effect modification by race in the association between vitamin D and anaemia.

Materials and Methods

Study population

Participants were recruited from the Emory University/Georgia Institute of Technology (Georgia Tech) Predictive Health Initiative cohort within the Center for Health Discovery and Well Being.[129] This is a cohort of generally healthy adults (age ≥

18 years) living in the Atlanta area and working in a university setting. Recruitment into the cohort was based on invitation to a random list of Emory employees and members of the Emory and Georgia Tech communities. Exclusion was based on hospitalization for acute or chronic disease within the previous year; severe psychosocial disorder within the previous year; addition of new prescription medications to treat a chronic condition within the previous year (with the exception of changes in anti-hypertensive or anti-diabetic agents); history of substance/drug abuse or alcoholism; current active malignant neoplasm; history of malignancy other than localized basal cell cancer of the skin during the previous 5 years; uncontrolled or poorly controlled autoimmune, cardiovascular, endocrine, gastrointestinal, hematologic, infectious, inflammatory, musculoskeletal, neurologic, psychiatric or respiratory disease; and any acute illness in the twelve weeks before baseline visits. Participants enrolled between January 2008 and February 2013 with available serum 25(OH)D and haemoglobin concentrations were included in the current analysis. This study was conducted according to the guidelines laid down in the Declaration of Helsinki and all procedures involving human subjects were approved by the Emory University Institutional Review Board. Written informed consent was obtained from all participants.

Data collection

Upon enrolment, participants completed questionnaires on demographic information, personal and family health history, current health status, and medication and supplement use. Physical activity was assessed via the Cross-Cultural Activity Participation Study (CAPS) Physical Activity Questionnaire,[130] and glomerular filtration

rate was estimated, as a marker of kidney function, using the Modification of Diet in Renal Disease (MDRD) equation.[131] Venepuncture for biochemical measurements was performed after an overnight fast. Serum 25(OH)D concentrations were measured commercially via liquid chromatography/tandem mass spectrometry in a laboratory that participates in the Vitamin D External Quality Assessment Scheme (Quest Diagnostics, Tucker, GA, USA). Markers of iron status were measured as follows: serum ferritin via immunoassay, total serum iron and iron binding capacity via spectrophotometry, and haemoglobin and haematocrit via high performance liquid chromatography (Quest Diagnostics, Tucker, GA, USA). Serum high-sensitivity C-reactive protein (CRP) was measured using nephelometry (Quest Diagnostics, Tucker, GA, USA). Serum IL-6, IL-8, tumor necrosis factor- α (TNF-α), and interferon-gamma (IFN-γ) concentrations were measured using a Fluorokine® MultiAnalyte Profiling multiplex kit (R&D Systems, Minneapolis, MN, USA) with a Bioplex analyzer (Bio-Rad, Hercules, CA, USA).

Definitions

Anaemia was defined based on the World Health Organization criteria as haemoglobin concentration < 130 g/l for men, and < 120 g/l for women.[47] Anaemia was further categorized into subtypes based on inflammation status. Participants with anaemia who had CRP concentrations > 3 mg/l or were in the upper quartile of IL-6 concentration (≥ 1.76 pg/ml) were classified as having anaemia with inflammation. Those with CRP concentrations ≤ 3 mg/l or who were in the lower three quartiles of IL-6 concentrations (< 1.76 pg/ml) were classified as having anaemia without inflammation. The CRP cut-point used was based on the American Heart Association determination of

an increased risk of heart disease with CRP concentrations > 3 mg/l.[132] Given its role in upregulating hepcidin expression,[65] IL-6 was incorporated into our definition of inflammation; however in the absence of a standard clinical cut-point, inflammation was defined based on the upper quartile of IL-6 in our dataset. Participants were determined to be nutrient deficient if they had evidence of iron deficiency (serum ferritin < 12 μg/l and transferrin saturation < 15%)[71] or low serum vitamin B_{12} (levels < 147·6 pmol/l).[9] Other nutritional measures related to anaemia of nutrient deficiency, such as folate, were not available for this cohort.

Statistical Analysis

Descriptive statistics were examined for all variables. Continuous variables were reported as means and standard deviations (SD) for normally distributed variables or medians and interquartile ranges (IQR) for non-normally distributed variables; categorical variables were presented as numbers of subjects and percentages. Continuous variables not following a normal distribution were logarithmically transformed (serum ferritin, and all inflammatory markers) for modelling. For variables requiring log transformation with values of zero (IL-6, TNF-α, and IFN-γ), a constant of 1 was added to all non-missing values. Differences in demographic and biochemical variables by serum 25(OH)D status (dichotomized as serum 25(OH)D < 50 nmol/l compared to serum 25(OH)D concentrations ≥ 50 nmol/l, based on the Institute of Medicine (IOM) guidelines,[133] and by race (whites compared to blacks) were examined using two sample t-tests for normally distributed continuous variables, Wilcoxon-Mann-Whitney tests for non-normally distributed continuous variables, and Chi-Square or Fisher's exact test for

categorical variables. Pearson correlations and simple linear regression analyses were performed to examine bivariate associations of vitamin D status with biomarkers related the anaemia, haemoglobin and serum iron. To further explore these associations, multivariable linear regression analyses were performed with haemoglobin and serum iron as dependent variables and vitamin D status was the independent variable, controlling for age, sex, race, BMI, IL-6, and CRP as *a priori* covariates.

Simple logistic regression was used to examine demographic, health history, and biochemical variables associated with anaemia. Multivariable logistic regression was used to assess the association between vitamin D status (independent variable) and anaemia (dependent variable). Variables that were significantly associated with anaemia in bivariate analysis were included as covariates in these models. We assessed for an interaction between vitamin D status and race using a likelihood ratio test, given that both vitamin D status and anaemia prevalence are known to differ by race group.[117,83,84]

To further explore the association of vitamin D status with anaemia with and without inflammation, multivariable logistic regression analyses were performed using anaemia with inflammation and anaemia without inflammation as dependent variables and vitamin D status as the independent variable. We assessed for interaction between race and vitamin D status using a likelihood ratio test, and included the same covariates used in the overall anaemia models (with the exception of inflammatory markers given their use in the definition of the anaemia with inflammation outcome). All analyses were performed using SAS v 9.3 (SAS Institute, Inc., Cary, NC), with a two-sided P value < 0.05 used to define statistical significance.

Results

Participant Characteristics

Of the 719 participants enrolled in the Emory/Georgia Tech Predictive Health Institute cohort as of February 2013, 638 had available serum 25(OH)D and haemoglobin levels and were included in the current analysis. Demographic characteristics of these participants, as a whole and by vitamin D status (25(OH)D < 50 nmol/l vs 25(OH)D ≥ 50 nmol/l), are shown in **Table 3.1**. Among the whole cohort, the mean age was 48·3 (SD 10·9) years, and approximately two thirds of the participants were female. Race and ethnicity was based on self-report and those in this cohort were primarily non-Hispanic or Latino; 72% were white, 23% were black/African American, 5% were Asian, and 1% identified as another race. For the regression analyses, participants were restricted to white and black/African American (*n* 602). This was a relatively highly educated and affluent population. The cohort was generally overweight, and while participants were healthy by self-report, some did report a history of stable chronic conditions including hypertension and diabetes. Characteristics which differed by vitamin D status included age, race, education, income, BMI, comorbidities, supplementation, and season of study visit. Among the participants with serum 25(OH)D < 50 nmol/l the mean age was younger ($P < 0{\cdot}001$), a greater proportion were black/African American ($P < 0{\cdot}001$), a greater proportion reported less education ($P = 0{\cdot}007$) and lower income ($P = 0{\cdot}007$), the mean BMI was greater ($P < 0{\cdot}001$), there was a higher prevalence of hypertension ($P < 0{\cdot}001$) and diabetes ($P = 0{\cdot}002$), and a lower proportion took any vitamin D ($P < 0{\cdot}001$) or multivitamin supplements ($P < 0{\cdot}001$), compared to those with serum 25(OH)D concentrations ≥ 50 nmol/l. Compared to whites, blacks in our cohort were younger ($P =$

0·002), a higher proportion were female ($P < 0·001$), had lower education and income levels ($P < 0·001$), had higher BMIs ($P < 0·001$), had a higher prevalence of hypertension ($P < 0·001$) and diabetes ($P = 0·04$), and a higher proportion reported taking vitamin D supplements ($P = 0·004$) (**Table S3.1**).

Among the entire cohort, approximately 50% of the participants had serum 25(OH)D concentrations < 75 nmol/l, 18% had serum 25(OH)D concentrations < 50 nmol/l, and 3% had serum 25(OH)D concentrations < 30 nmol/l. The mean serum 25(OH)D concentration was in the range considered sufficient (**Table 3.2**). Mean haemoglobin was above the threshold for anaemia. Mean and median measures of iron status were all within normal ranges.[134] Approximately, eight percent of the cohort was anaemic, and of these, 4·9 had anaemia with inflammation and 3·3 had anaemia without inflammation. There were 16 anaemic participants with evidence of nutrient deficiency (15 with iron deficiency and one with low serum vitamin B_{12}). Those with serum 25(OH)D concentrations < 50 nmol/l had lower haemoglobin ($P = 0·008$), haematocrit ($P = 0·03$), and serum iron concentrations ($P < 0·001$), and higher CRP ($P < 0·001$), and IL-6 concentrations ($P < 0·001$) compared to those with serum 25(OH)D ≥ 50 nmol/l. Furthermore, there was a higher prevalence of anaemia overall ($P = 0·001$) and specifically anaemia with inflammation among those with serum 25(OH)D concentrations < 50 nmol/l ($P < 0·001$). Compared to whites, blacks in our cohort had lower serum 25(OH)D concentrations ($P < 0·001$), lower haemoglobin concentrations ($P < 0·001$), haematocrit ($P < 0·001$), and serum iron concentrations ($P < 0·001$), and higher CRP ($P < 0·001$) and IL-6 concentrations ($P < 0·001$) (**Table S3.2**). Blacks also had a higher prevalence of vitamin D deficiency ($P < 0·001$), and anaemia ($P < 0·001$).

Associations of vitamin D status with markers of iron status

In simple linear regression analysis, serum 25(OH)D was positively associated with haemoglobin concentrations (β ± SE: 0·05 ± 0·02, *P*=0·004); however adjustment for age, sex, race, BMI, CRP, and IL-6, attenuated the significance (*P*=0·23). Serum 25(OH)D was positively associated with serum iron concentrations (β ± SE: 0·04 ± 0·01, *P*<0·001, **Figure 3.1**), and the association remained significant after adjusting for age, sex, race, BMI, CRP, and IL-6 (β ± SE: 0·02 ± 0·01, *P*=0·006).

Association of vitamin D status with anaemia

Serum 25(OH)D as a continuous variable was significantly associated with decreased odds of anaemia (OR 0·98; 95% CI 0·97, 0·99). Odds of anaemia were increased when participants were dichotomized by various serum 25(OH)D cut-points for vitamin D insufficiency/deficiency: < 75 nmol/l (OR 2·15; 95% CI 1·18, 3·92), < 50 nmol/l (OR 2·64; 95% CI 1·43, 4·86), < 30 nmol/l (OR 4·97; 95% CI 1·84, 13·41). These associations remained significant after adjustment for season ($OR_{25(OH)D <75 nmol/l}$ 2·10; 95% CI 1·15, 3·85; $OR_{25(OH)D < 50 nmol/l}$ 2·54; 95% CI 1·37, 4·72, $OR_{25(OH)D < 30 nmol/l}$ 5·02; 95% CI 1·82, 13·85). Additional variables associated with anaemia in bivariate analysis included inflammatory markers CRP, IL-6, and IL-8, black race, female gender, BMI, history of diabetes, and lower annual income, age, and iron supplement intake (**Table S3.3**). TNF-α and IFN-γ, waist circumference, physical activity, smoking status, history of hypertension, education, multivitamin use, and vitamin D supplementation were not significantly associated with anaemia.

There was a significant interaction between race and vitamin D status (P = 0·003), such that the association between vitamin D status and anaemia remained significant only among blacks (Table 3). After adjustment for significant anaemia covariates from the bivariate analyses (age, sex, BMI, CRP, IL-6, IL-8, use of iron supplements, income, and diabetes) the odds of anaemia were 6 times higher for blacks with serum 25(OH)D < 50 nmol/l compared to blacks with serum 25(OH)D ≥ 50 nmol/l (OR 6·42; 95% CI 1·88, 21·99) (**Table 3.3**).

The magnitude of effect in blacks increased when vitamin D status was defined by 25(OH)D < 30 nmol/l and 25(OH)D ≥ 30 nmol/l (fully adjusted OR 17·3; 95% CI 2·27, 132·0). There was no significant association between anaemia and vitamin D status defined by 25(OH)D < 75 nmol/l and ≥ 75 nmol/l after adjustment for the same covariates.

Association of vitamin D status with subtypes of anaemia

The crude association between serum 25(OH)D < 50 nmol/l and anaemia with inflammation was statistically significant (OR 4·51; 95% CI 2·13, 9·55). However, there was significant effect modification by race (P = 0·03) such that in stratified analyses, controlling for age, sex, BMI, use of iron supplements, income, and history of diabetes, blacks with serum 25(OH)D concentrations < 50 nmol/l had 8 times higher odds of having anaemia with inflammation compared to blacks with serum 25(OH)D ≥ 50 nmol/l (OR 8·64; 95% CI 2·01, 37·23) (**Table 3.4**). The association between vitamin D status and anaemia with inflammation was not statistically significant in whites. When we excluded those with evidence of nutrient deficiency from the anaemia with

inflammation outcome, the crude association between vitamin D status and anaemia with inflammation remained statistically significant (OR 4·29 , 95% CI 1·80, 10·23); however, the limited sample size with this sub-analysis precluded adjustment for the anaemia covariates controlled for above. The crude association between vitamin D status and anaemia without inflammation was not statistically significant (OR 0·74; 95% CI 0·22, 2·57).

Discussion

This study reports an association between vitamin D status and anaemia in a generally healthy, working adult population. There were several notable findings: 1) we found a significant positive association between serum 25(OH)D concentrations and serum iron; 2) 25(OH)D < 50 nmol/l was associated with increased odds of anaemia in blacks, but not in whites, and 3) the association between vitamin D status and anaemia among blacks was especially prominent in anaemia with inflammation, consistent with our hypothesis that vitamin D that would be associated particularly with anaemia with inflammation.

Our results are supported by other epidemiologic studies which have demonstrated inverse associations between vitamin D status and odds of anaemia in patients with chronic kidney disease, and heart failure.[8,12] These studies indicate a consistent inverse association between vitamin D status and odds of anaemia. Our analysis adds to the literature by suggesting that the association may pertain particularly to anaemia with inflammation. However, there have been few trials examining the impact of vitamin D supplementation on anaemia. Lin, et al, showed in patients

undergoing hemodialysis, treatment with the active form of vitamin D, calcitriol, was effective in improving anaemia of chronic kidney disease.[120] In a study of patients with myelodysplastic syndromes, Mellibovsky, et al, demonstrated that treatment with calcitriol resulted in increases in hematologic markers including haemoglobin.[135] Further investigation is needed to better understand the therapeutic effects of vitamin D supplementation on anaemia, especially in generally health persons.

Our findings are consistent with the hypothesized mechanisms underlying the vitamin D-anaemia relationship. Anaemia resulting from chronic inflammation is characterized by disturbances in iron regulation such that iron becomes sequestered in cells of the reticuloendothelial system as a result of the action of pro-inflammatory markers, such as IL-6, on hepcidin, the global regulator of iron metabolism.[65,90] Hepcidin acts on ferroportin, the iron exporter on the surface of enterocytes, macrophages, and hepatocytes, resulting in its internalization and degradation, preventing iron efflux from the cell.[61] This leads to a decrease in iron available in circulation for erythropoiesis and heme synthesis (despite adequate iron stores and total body iron), ultimately leading to anaemia.[90,128] Vitamin D is thought to temper the effect of inflammation-induced anaemia by decreasing the secretion of pro-inflammatory cytokines. Our group recently demonstrated through a series of *in vitro* studies, that vitamin D can decrease the release of cytokines IL-6 and IL-1β from macrophages, and down-regulate hepcidin and up-regulate ferroportin expression in human monocytes.[96] These findings are consistent with those of Bacchetta, et al, showing that treatment of hepatocytes and monocytes with vitamin D, resulted in decreased expression of hepcidin mRNA.[95] In support of this putative mechanism for the role of vitamin D in iron metabolism, our findings showed

that serum 25(OH)D concentrations were positively associated with serum iron concentrations, suggesting that increases in vitamin D status may lead to increases in circulating iron available for use in erythropoiesis and heme synthesis. Furthermore, in our cohort, vitamin D deficiency was associated with anaemia with inflammation but not with anaemia without inflammation, supporting a potential role for vitamin D in iron recycling in the context of inflammation.

In our cohort, the association between vitamin D status and odds of anaemia was significant only among blacks. The odds of anaemia were approximately 6 times higher for blacks with serum 25(OH)D < 50 nmol/l compared to blacks with serum 25(OH)D ≥ 50 nmol/l, though the confidence intervals around the estimate were relatively wide. The magnitude of effect was even greater for blacks with serum 25(OH)D < 30 nmol/l compared to those with 25(OH)D≥30 nmol/l (OR 17·3; 95% CI 2·27, 132·0), though the estimate remained imprecise. A potential explanation for the racial differences in the association between vitamin D status and anaemia is the racial difference in circulating inflammatory markers. Increased IL-6 expression in African Americans compared to Caucasians has been demonstrated in human umbilical vein endothelial cells.[136] Further, a systematic review of 32 population-based studies found higher CRP concentrations in non-whites compared to whites.[137] Similarly, in our population, IL-6 and CRP were significantly increased in blacks compared to whites. Moreover, the magnitude of effect for the association of serum 25(OH)D < 50 nmol/l with odds of anaemia with inflammation increased above that of anaemia overall. Thus, higher inflammation in blacks may be augmenting the association between vitamin D status and anaemia in this racial group compared to whites.

There are currently no race-specific cut-offs for anaemia. However, it is known that blacks have a higher prevalence of vitamin D deficiency and lower haemoglobin concentrations compared to whites.[117,84,85] The clinical significance of this is not well understood. Vitamin D deficiency may provide one potential explanation for the differences observed in haemoglobin concentration and anaemia prevalence between blacks and whites.

Strengths of this study were a large sample size and a well-characterized cohort. However, this was a cross-sectional analysis, leaving us unable to conclude causality in the vitamin D-anaemia associations observed, and reverse causality bias cannot be excluded. We were also unable to measure hepcidin concentrations and, therefore, could not directly examine the putative mechanism underlying the vitamin D and anaemia association observed. Health status and socioeconomic variables were collected via self-administered questionnaires; thus recall error may be a limiting factor. Our population was self-selected from individuals invited to participate in the Emory-Georgia Tech Predictive Health Initiative. In addition, the majority of participants reported high income and education level and are therefore not representative of the general population. However, one may expect such a population to have regular access to health resources; thus the persistence of the vitamin D-anaemia association is noteworthy. Additional prospective studies exploring the relationship between vitamin D status and anaemia, including those in low-income populations, are warranted.

In conclusion, our results suggest that lower vitamin D status is associated with anaemia, particularly anaemia with inflammation, among blacks in a generally healthy and high socioeconomic cohort residing in Atlanta, GA. Given the duel burden of

vitamin D deficiency and anaemia prevalence among blacks, our findings have important public health implications. Clinical trials in racially diverse populations are necessary to elucidate the therapeutic effect of vitamin D supplementation on anaemia.

Financial Support: Information upon which this work is based is from the Emory/Georgia Tech Predictive Health Participant Database, and is supported by the National Center for Advancing Translational Sciences of the National Institutes of Health (UL1 TR000454). Other sources of support for this study include grants from the National Institutes of Health (E.M.S., grant number T32 DK007734), (V.T., grant number K23 AR054334), (J.A.A., grant numbers T32 DK007298, K01 DK102851), (T.R.Z., grant number K24 DK096574) and the Emory-Egleston Children's Research Center (S.M.Z.). The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health. The funders had no role in the design, analysis or writing of this article.

Conflict of Interest: None

Authorship: EMS, JAA, and VT formulated the research question; GSM and TRZ had leading roles in the cohort study design, implementation, and data collection; EMS, JAA, and VT analysed data; EMS, JAA, GSM, SMZ, TRZ, and VT wrote the article; EMS and VT had primary responsibility for final content. All authors read and approved the final manuscript.

Table 3.1: Demographic, socioeconomic, and health status characteristics of Emory-Georgia Tech Predictive Health Initiative cohort (2008-2013)[*], by serum 25(OH)D status (number and percentage of subjects, mean and standard deviation)

	All (*n* 638)		25(OH)D < 50 nmol/l (*n* 116)		25(OH)D ≥ 50 nmol/l (*n* 522)		
Characteristic	*n*	%	*n*	%	*n*	%	*P*[†]
Age[‡], years							< 0·001
Mean	48·3		45·3		49·0		
SD	10·9		11·3		10·7		
Sex							0.10
Male	206	32·3	30	25·9	176	33·7	
Female	432	67·7	86	74·1	346	66·3	
Ethnicity[‡]							0·21
Hispanic or Latino	9	1·4	3	2·6	6	1·2	
Non-Hispanic or Latino	628	98·6	113	97·4	515	98·9	
Race[‡]							< 0·001
White	457	71·7	50	43·1	407	78·1	
Black/African American	145	22·8	57	49·1	88	16·9	
Asian	29	4·6	8	6·9	21	4·0	
Other	6	0·9	1	0·9	5	1·0	
Education[§,‡]							0·007
Less than high school	2	0·3	0	0	2	0·4	
Completed high school	17	2·7	4	3·5	13	2·5	
Some college	99	15·5	31	26·7	68	13·1	
Four years of college	151	23·7	25	21·6	126	24·2	
Any graduate school	368	57·8	56	48·3	312	59·9	
Annual household income[‡]							0·007
≤ $50,000/yr	68	11·3	20	18·4	48	9·7	
>$50,000-$100,000/yr	117	29·4	38	34·9	139	28·2	
>$100,000 - $200,000/yr	217	36·1	35	32·1	182	36·9	
>$200,000/yr	140	23·3	16	14·7	124	25·2	
Physical activity[‖,‡]	154	24·3	29	25·0	125	24·1	0·84
BMI[‡], kg/m^2							<0·001
Mean	28·0		32·6		27·0		
SD	6·5		8·7		5·3		
Current smoker[‡]	35	5·5	10	8·7	25	4·8	0·10
Comorbidities							
History of hypertension[‡]	126	19·8	36	31·3	90	17·2	<0·001

History of diabetes‡	34	5·3	13	11·3	21	4·0	0·002
eGFR ≥ 60 ml/min/1.73m^{2}‡	621	97·8	115	99·1	506	97·5	0·48
Any vitamin D supplementation¶	262	41·1	20	17·2	242	46·4	<0·001
Multivitamin use	211	30·1	16	13·8	195	37·4	<0·001
Iron supplement use‡	10	1·9	3	3·5	7	1·6	0·22
Season of visit‡							0·03
Winter	137	21·5	24	20·7	113	21·7	
Spring	103	16·2	29	25·0	74	14·2	
Summer	187	29·4	33	28·5	154	29·6	
Fall	210	33·0	30	25·9	180	34·6	

*Restricted to participants with available vitamin D and haemoglobin values
†Two sample t-test for continuous variables, Chi-sq or Fisher's exact test for categorical variables, comparing 25(OH)D < 50 nmol/l and 25(OH)D ≥ 50 nmol/l
§Education refers to highest educational achievement; less than high school defined as less than 12th grade, completed high school defined as completion of 12th grade, some college defined as less than 4 years of college, and any graduate school includes both graduate and post-graduate education
‖Meet CAPS guidelines for moderate physical activity
¶Vitamin D supplementation from any source (alone, in combined supplement, or in multivitamin)
‡age: *n* 116 and *n* 521 for 25(OH)D < 50 nmol/l and 25(OH)D ≥ 50 nmol/l, respectively; ethnicity: *n* 116 and *n* 521 for 25(OH)D < 50 nmol/l and 25(OH)D ≥ 50 nmol/l, respectively; race: *n* 116 and *n* 521 for 25(OH)D < 50 nmol/l and 25(OH)D ≥ 50 nmol/l, respectively; education: *n* 116 and *n* 521 for 25(OH)D < 50 nmol/l and 25(OH)D ≥ 50 nmol/l, respectively; income: *n* 109 and *n* 493 for 25(OH)D < 50 nmol/l and 25(OH)D ≥ 50 nmol/l, respectively; physical activity: *n* 116 and *n* 518 for 25(OH)D < 50 nmol/l and 25(OH)D ≥ 50 nmol/l, respectively; BMI: *n* 115 and *n* 521 for 25(OH)D < 50 nmol/l and 25(OH)D ≥ 50 nmol/l, respectively; smoking: *n* 115 and *n* 521 for 25(OH)D < 50 nmol/l and 25(OH)D ≥ 50 nmol/l, respectively; hypertension: *n* 115 and *n* 522 for 25(OH)D < 50 nmol/l and 25(OH)D ≥ 50 nmol/l, respectively; diabetes: *n* 115 and *n* 522 for 25(OH)D < 50 nmol/l and 25(OH)D ≥ 50 nmol/l, respectively; eGFR: *n* 116 and *n* 519 for 25(OH)D < 50 nmol/l and 25(OH)D ≥ 50 nmol/l, respectively; iron supplementation: *n* 86 and *n* 431 for 25(OH)D < 50 nmol/l and 25(OH)D ≥ 50 nmol/l, respectively; season: *n* 116 and *n* 521 for 25(OH)D < 50 nmol/l and 25(OH)D ≥ 50 nmol/l, respectively

Table 3.2: Iron status and inflammatory markers of Emory-Georgia Tech Predictive Health Initiative cohort (2008-2013), by serum 25(OH)D status
(Mean values with their standard deviations, median values with interquartile range (IQR), number of subjects and percentages)

	All (*n* 638)		25(OH)D < 50 nmol/l (*n 116*)		25(OH)D ≥ 50 nmol/l (*n 522*)		
	Mean	SD	Mean	SD	Mean	SD	*P**
Serum 25(OH)D (nmol/l)	76·1	30·5	37·4	8·7	84·9	26·7	<0·001
Whites	82·4	30·2	40·2	8·0	87·7	27·9	
Blacks	57·9	23·7	35·3	8·6	72·8	18·2	
Haemoglobin (g/l)	137·9	14·8	134·8	15·9	138·6	13·2	0·008
Whites	140·7	12·9	139·9	17·4	140·8	12·3	
Blacks	129·2	12·5	128·9	11·2	129·4	13·3	
Haematocrit (%)	40·7	3·8	40·0	4·3	40·8	3·6	0·03
Serum ferritin (µg/l)†							0·34
Median	62·5		55·0		63·0		
IQR	91·0		77·0		92·0		
Serum iron (µmol/l)	16·8	6·3	14·9	5·6	17·3	6·4	<0·001
Iron binding capacity (µmol/l)	64·0	9·8	64·2	9·9	65·0	9·6	0·41
Serum CRP (mg/l)†,‡							<0·001
Median	1·5		3·2		1·4		
IQR	2·7		4·4		2·2		
Serum IL-6 (pg/ml)†,‡							<0·001
Median	1·0		1·6		1·0		
IQR	1·4		2·1		1·23		
Serum IL-8 (pg/ml)†,‡							0·28
Median	8·2		8·7		8·2		
IQR	5·5		7·3		5·2		
Serum TNF-α (pg/ml)†,‡							0·41
Median	3·7		4·0		3·7		
IQR	2·5		2·7		2·4		
Serum IFN-γ (pg/ml)†,‡							0·09
Median	0·2		0·1		0·2		
IQR	0·3		0·3		0·4		
	n	%	*n*	%	*n*	%	*P*
Anaemia	52	8·2	18	15·5	34	6·5	0·001
Anaemia with inflammation§	31	4·9	15	12·9	16	3·1	<0·001
Anaemia without inflammation‡	21	3·3	3	2·6	18	3·4	0·78

*Two sample t-tests for normally distributed continuous variables, Wilcoxon-Mann-Whitney test for non-normally distributed continuous variables, Chi-Sq or Fisher's exact test for categorical variables
†Median and IQR given for non-normally distributed variables
§Anaemia with inflammation defined as anaemia with serum CRP > 3 mg/l or upper quartile of IL-6 (≥ 1.76 pg/ml); anaemia without inflammation defined as anaemia with CRP ≤ 3mg/l or quartiles 1-3 of IL-6 (<1.76 pg/ml)
‡CRP: *n* 116 and *n* 521 for 25(OH)D < 50 nmol/l and 25(OH)D ≥ 50 nmol/l, respectively; IL-6: *n* 114 and *n* 509 for 25(OH)D < 50 nmol/l and 25(OH)D ≥ 50 nmol/l, respectively; IL-8: *n* 114 and *n* 510 for 25(OH)D < 50 nmol/l and 25(OH)D ≥ 50 nmol/l, respectively; TNF-α: *n* 114 and *n* 510 for 25(OH)D < 50 nmol/l and 25(OH)D ≥ 50 nmol/l, respectively; IFN-γ: *n* 114 and *n* 510 for 25(OH)D < 50 nmol/l and 25(OH)D ≥ 50 nmol/l, respectively

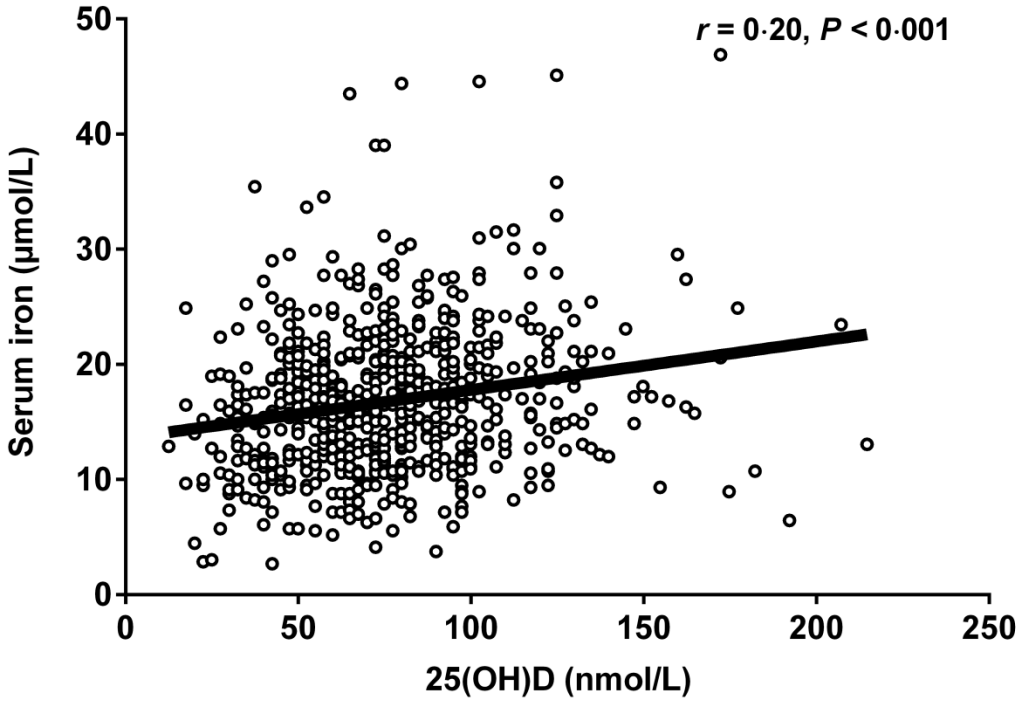


Figure 3.1: Correlation between vitamin D status (serum 25-hydroxyvitamin D (25(OH)D)) and total circulating iron concentrations in participants of the Emory/Georgia Tech Predictive Health Initiative cohort (2008 – 2013), *n* 638. Total serum iron was positively correlated with serum 25(OH)D concentration (Pearson's r=0.2, P<0.001), and this association remained statistically significant after adjusting for age, sex, race, BMI, CRP, and IL-6 ($\beta \pm$ SE: 0·02 ± 0·01, P=0·006).

Table 3.3: Association of serum 25(OH)D < 50 nmol/l and anaemia, stratified by race (Odds ratios and their 95% confidence intervals)

	All		White		Black	
	OR[*, †]	95% CI	OR[*]	95% CI	OR[*]	95% CI
Unadjusted[‡]	2·64	1·43, 4·86	0·77	0·33, 1·81	5·36	2·00, 14·33
Model 1[§]	2·02	0·99, 4·14	0·69	0·28, 1·68	5·36	1·88, 15·28
Model 2[‖]	1·75	0·84, 3·63	0·55	0·22, 1·40	5·08	1·74, 14·85
Model 3[¶]	2·08	0·92, 4·73	0·62	0·22, 1·75	6·42	1·88, 21·99

*Odds ratio comparing 25(OH)D < 50 nmol/l vs. 25(OH)D ≥ 50 nmol/l
†Race not included as covariate due to evidence of interaction (*P*=0.003)
‡Crude association between 25(OH)D < 50 nmol/l and anaemia, *n* 602
§Adjusted for age, sex, BMI, *n* 599
‖Model 1 + adjustment for CRP, IL-6, IL-8, *n* 586
¶Model 2 + adjustment for use of iron supplements, income, and history of diabetes, *n* 465

Table 3.4: Association of serum 25(OH)D < 50 nmol/l and anaemia with inflammation, stratified by race

(Odds ratios and their 95% confidence intervals)

	All		White		Black	
	OR[*, †]	95% CI	OR[*]	95% CI	OR[*]	95% CI
Unadjusted[‡]	4·51	2·13, 9·55	1·31	0·51, 3·40	8·93	2·49, 32·04
Model 1[§]	2·61	1·10, 6·17	0·98	0·36, 2·72	7·44	1·93, 28·75
Model 2[‖]	3·14	1·23, 7·99	1·20	0·39, 3·68	8·64	2·01, 37·23

*Odds ratio comparing 25(OH)D < 50 nmol/l vs. 25(OH)D ≥ 50 nmol/l

†Race not included as covariate due to evidence of interaction (P=0·03)

‡Crude association between 25(OH)D < 50 nmol/l and anaemia, n 602

§Adjusted for age, sex, BMI, n 599

‖Model 1 + adjustment for use of iron supplements, income, and history of diabetes, n 474

Table S3.1: Demographic, socioeconomic, and health status characteristics of Emory-Georgia Tech Predictive Health Initiative cohort (2008-2013)[*], by race

(number of subjects and percentages and mean and standard deviation)

	White (*n* 457)		Black (*n* 145)		
Characteristic	*n*	%	*n*	%	*P*[†]
Age[‡], years					0·002
Mean	49·4		46·1		
SD	11·0		9·5		
Sex					<0·001
Male	176	38·5	16	11·0	
Female	281	61·5	129	89·0	
Ethnicity					0·69
Hispanic or Latino	8	1·8	1	0·7	
Non-Hispanic or Latino	449	98·3	144	99·3	
Education[§]					< 0·001
Less than high school	1	0·22	1	0·7	
Completed high school	5	1·1	12	8·3	
Some college	44	9·6	51	35·2	
Four years of college	109	23·9	35	24·1	
Any graduate school	298	65·2	46	31·7	
Annual household income[‡]					< 0·001
≤ $50,000/yr	22	5·03	42	30·7	
>$50,000-$100,000/yr	109	24·9	59	43·1	
>$100,000 - $200,000/yr	179	41·0	33	22·6	
>$200,000/yr	127	29·1	5	3·7	
Physical activity[‖,‡]	112	24·7	37	25·7	0·8
BMI[‡], kg/m^2					<0·001
Mean	27·2		31·7		
SD	5·7		7·7		
Current smoker[‡]	20	4·4	12	8·3	0·07
Comorbidities					
History of hypertension[‡]	77	16·9	46	31·7	<0·001
History of diabetes[‡]	20	4·4	13	9·0	0·04
eGFR ≥ 60 ml/min[‡]	443	97·4	144	99·3	0·2
Any vitamin D supplementation[¶]	203	44·4	45	31·0	0·004
Multivitamin use	160	35·0	39	26·9	0·07
Iron supplement use[‡]	5	1·3	4	3·5	0·22
Season of visit[‡]					0·07

Winter	99	21·7	29	20·0
Spring	69	15·1	30	20·7
Summer	126	27·6	49	33·8
Fall	162	35·5	37	25·5

[*]Restricted to participants with available vitamin D and hemoglobin values and of black or white race

[†]Two sample t-test for continuous variables, Chi-sq or Fisher's exact test for categorical variables

[§]Education refers to highest educational achievement; less than high school defined as less than 12th grade, completed high school defined as completion of 12th grade, some college defined as less than 4 years of college, and any graduate school includes both graduate and post-graduate education

[‖]Meet CAPS guidelines for moderate physical activity

[¶]Vitamin D supplementation from any source (alone, in combined supplement, or in multivitamin)

‡age: *n* 456 and *n* 145 for whites and blacks, respectively; income: *n* 437 and *n* 137 for whites and blacks, respectively; physical activity: *n* 454 and *n* 144 for whites and blacks, respectively; BMI: *n* 456 and *n* 144 for whites and blacks, respectively; smoking: *n* 456 and *n* 144 for whites and blacks, respectively; hypertension: *n* 456 and *n* 145 for whites and blacks, respectively; diabetes: *n* 456 and *n* 145 for whites and blacks, respectively; eGFR: *n* 455 and *n* 145 for whites and blacks, respectively; iron supplementation: *n* 382 and *n* 116 for whites and blacks, respectively; season: *n* 456 and *n* 145 for whites and blacks, respectively

Table S3.2: Biochemical measurements and vitamin D and anaemia status, by race (Mean values with their standard deviations, median values with interquartile range (IQR), number of subjects and percentages)

	White (*n* 457)		Black (*n* 145)		
	Mean	SD	Mean	SD	*P*[*]
Serum 25(OH)D (nmol/l)	82·4	30·2	57·9	23·7	<0·001
Haemoglobin (g/l)	140·7	12·9	129·2	12·5	<0·001
Haematocrit (%)	41·3	3·6	38·6	3·5	<0·001
Serum ferritin (µg/l)†					0·06
Median	64·0		48·0		
IQR	92·0		87·0		
Serum iron (µmol/l)	17·5	6·3	14·4	5·0	<0·001
Iron binding capacity (µmol/l)	63·7	10·0	64·8	8·8	0·21
Serum CRP (mg/l)†,‡					<0·001
Median	1·4		3·0		
IQR	2·1		5·2		
Serum IL-6 (pg/ml)†,‡					<0·001
Median	0·97		1·56		
IQR	1·23		2·46		
Serum IL-8 (pg/ml)†,‡					0·49
Median	8·3		8·1		
IQR	5·8		5·2		
Serum TNF-α (pg/ml)†,‡					0·09
Median	3·8		3·5		
IQR	2·3		2·9		
Serum IFN-γ (pg/ml)†,‡					0·05
Median	0·2		0·1		
IQR	0·35		0·31		
	n	%	*n*	%	*P*
Serum 25(OH)D < 30 nmol/l	5	1·1	15	10·3	<0·001
Serum 25(OH)D < 50 nmol/l	50	10·9	57	39·3	<0·001
Serum 25(OH)D < 75 nmol/l	191	41·8	110	75·9	<0·001
Anaemia	19	4·2	29	20·0	<0·001
Anaemia with inflammation[§]	10	2·2	20	13·8	<0·001
Anaemia without inflammation‡	9	2·0	9	6·2	0·02

[*]Two sample t-tests for normally distributed continuous variables, Wilcoxon-Mann-Whitney test for non-normally distributed continuous variables, Chi-Sq or Fisher's exact for categorical variables

[†]Median and IQR given for non-normally distributed variables

[§]Anaemia with inflammation defined as anaemia with serum CRP > 3 mg/l or upper quartile of IL-6 (≥ 1.76 pg/ml); anaemia without inflammation defined as anaemia with CRP ≤ 3mg/l or quartiles 1-3 of IL-6 (<1.76 pg/ml)
‡CRP: *n* 456 and *n* 145 for whites and blacks, respectively; IL-6: *n* 444 and *n* 144 for whites and blacks, respectively; IL-8: *n* 445 and *n* 144 for whites and blacks, respectively; TNF-α: *n* 445 and *n* 144 for whites and blacks, respectively; IFN-γ: *n* 445 and *n* 144 for whites and blacks, respectively

Table S3.3: Bivariate associations of biochemical, demographic, socioeconomic, and health status variables with anemia
(Odds ratios and their 95% confidence intervals)

Variables	OR	95% CI
Biochemical		
25(OH)D (nmol/l), *n* 638	0·98	0·97, 0·99
25(OH)D < 30 nmol/l (Y vs. N)	4·97	1·84, 13·41
25(OH)D < 50 nmol/l (Y vs. N)	2·64	1·43, 4·86
25(OH)D < 75 nmol/l (Y vs. N)	2·15	1·18, 3·92
Log(CRP) (mg/l), *n* 637	3·19	1·62, 6·29
Log(IL-6) (pg/ml), *n* 623	4·91	1·83, 13·15
Log(IL-8) (pg/ml), *n* 624	5·23	1·63, 16·83
Log(TNF-α) (pg/ml), *n* 624	2·54	0·73, 8·82
Log(IFN-γ) (pg/ml), *n* 624	2·37	0·17, 32·75
Demographic		
Age (yrs), *n* 637	0·97	0·95, 0·996
Sex (F vs. M), *n* 638	6·.31	2·24, 17·75
Race (black vs. white), *n* 602	5.76	3·12, 10·65
Supplement use		
Multivitamin (Y vs. N), *n* 638	0·89	0·48, 1·65
Iron (Y vs. N), *n* 517	7·79	2·11, 28·73
Any vitamin D (Y vs. N), *n* 638	0·97	0·54, 1·73
Socioencomic status		
Education, *n* 637		
Completed high school	2·10	0·45, 9·75
Some college	3·74	1·93, 7·23
Completed college	1·00	0·45, 2·22
Any graduate school	Ref	
Annual household income, *n* 602		
≤ $50,000/yr	7·91	1·60, 39·18
>$50,000-$100,000/yr	9·79	2·26, 42·36
>$100,000 - $200,000/year	5·86	1·33, 25·77
>$200,000/yr	Ref	
Health status		
BMI (kg/m^2), *n* 636	1·05	1·01, 1·09
Waist circumference, *n* 626	1·12	0·60, 2·07
Physical activity (meeting CAPS guidelines vs. not), *n* 634	1·29	0·69. 2·43
Hypertension (Y vs. N), *n* 637	1·56	0·82, 2·97
Diabetes (Y vs. N), *n* 637	3·22	1·33, 7·79
Current smoking (Y vs. N), *n* 636	1·06	0·31, 3·57

eGFR ≥ 60 ml/min/1.73 m^2 (N vs. Y), *n* 635	0·86	0·11, 6·71
Season of visit, *n* 637		
Fall	0·71	0·34, 1·49
Winter	0·79	0·35, 1·78
Spring	1·20	0·54, 2·66
Summer	Ref	

CHAPTER 4

SERUM 25-HYDROXYVITAMIN D BUT NOT DIETARY VITAMIN D INTAKE IS ASSOCIATED WITH HEMOGLOBIN IN WOMEN OF REPRODUCTIVE AGE IN RURAL NORTHERN VIETNAM

Ellen M. Smith[a], Phuong H. Nguyen[b,c], Ines Gonzalez-Casanova[d], Son Nguyen[b], Reynaldo Martorell[a,d], Vin Tangpricha[a,e,f], Usha Ramakrishnan[a,d]

[a]Nutrition and Health Sciences Program, Laney Graduate School, Emory University, Atlanta, GA 30322, USA

[b]Thai Nguyen University of Pharmacy and Medicine, Thai Nguyen, Vietnam

[c]International Food Policy Research Institute, Hanoi, Vietnam

[d]Hubert Department of Global Health, Rollins School of Public Health, Atlanta GA 30322, USA

[e]Division of Endocrinology, Metabolism & Lipids, Emory University School of Medicine, Atlanta, GA 30322, USA

[f]Atlanta VA Medical Center, Atlanta, GA, 30033, USA

Submitted to *Nutrition Research* July 2016

Abbreviations:

25(OH)D; 25-hydroxyvitamin D

AGP; α_1-acid glycoprotein

AMDR; acceptable macronutrient distribution range

BMI; body mass index

CI; confidence interval

CRP; C-reactive protein

EAR; estimated average requirement

FFQ; food frequency questionnaire

IOM; Institute of Medicine

IQR; interquartile range

OR; odds ratio

RBP; retinol binding protein

RDA; recommended dietary allowance

SD; standard deviation

SE; standard error

SES; socioeconomic status

sTfR; soluble transferrin receptor

WHO; World Health Organization

WRA; women of reproductive age

Abstract

Hypovitaminosis D and anemia are both prevalent in Vietnam, and low vitamin D status may be a risk factor for anemia. This study aimed to examine the association between vitamin D intake and anemia in women of reproductive age (WRA) in Thai Nguyen, Vietnam (N=4,961). Associations between serum 25-hydroyxvitamin D [25(OH)D] and hemoglobin and anemia were also assessed in a subset of this population (n=88). We hypothesized that vitamin D intake and serum 25(OH)D concentrations would be positively associated with hemoglobin and inversely associated with odds of anemia. For this cross-sectional analysis, vitamin D intake was estimated using a semi-quantitative food frequency questionnaire, and multivariable regression models were used to assess determinants of vitamin D intake, and associations of vitamin D intake and serum 25(OH)D with hemoglobin and anemia. Median vitamin D intake was 0.2 µg/d [8.0 IU] (IQR: 0.4). Vitamin D intake was not associated with hemoglobin concentration or anemia after adjusting for age, body mass index, total energy intake, transferrin receptor, C-reactive protein, α_1-acid glycoprotein, socioeconomic status, occupation, education, ethnicity, and food insecurity (P=0.56 and P=0.65 for hemoglobin and anemia, respectively). Controlling for the same covariates, 25(OH)D <50 nmol/L (vs. ≥50 nmol/L) was associated with decreased hemoglobin concentrations (β=-0.91 (SE:0.42), P=0.03), but not with anemia (P=0.11). These findings suggest that low vitamin D status may be linked to reduced hemoglobin concentrations, but the role of diet in this association was not evident in this population of WRA in Vietnam where dietary vitamin D intake was very low.

Keywords: vitamin D; anemia; hemoglobin; Vietnam; women; dietary intake

Introduction

Anemia is a substantial public health problem throughout the world, present in nearly a third of the global population.[81] The burden is higher in certain population groups and regions such as in Southeast Asia where the prevalence of anemia among non-pregnant women is estimated to be over 40%.[138] In women of reproductive age (WRA) anemia is of particular concern as it has been associated with maternal and perinatal mortality[77] and adverse birth outcomes.[139] Furthermore, anemia is associated with decreased work capacity in adults, potentially resulting in economic consequences.[76,140]

Iron deficiency is a major cause of anemia but other factors including infection, inflammation, and other nutrient deficiencies have been recognized as important contributors to its etiology as well.[76,141,142] In a previous analysis from our group, we reported that nearly 20% of the non-pregnant WRA in this Vietnamese population were anemic, but iron deficiency anemia was relatively low, occurring in only 1.9% of women.[143] Similarly, Siridamrongvattana et al,[142] found that among pregnant women in the Thua Thien Hue province in the north central coast of Vietnam, the prevalence of anemia was nearly 20%, but only 6% had iron deficiency anemia. Given the burden of anemia but the low prevalence of iron deficiency in these studies of Vietnamese women, investigation of other factors contributing to anemia etiology is warranted.

One such factor that has recently been described is vitamin D deficiency. Epidemiologic studies in other Asian populations have linked vitamin D deficiency to anemia,[10,144] and studies in the United States have suggested that the association between vitamin D status and anemia may be particularly relevant to anemia of inflammation.[9,116]

The biochemical mechanism underlying this relationship may be explained by vitamin D's role in anti-inflammatory responses and gene expression.[145] Vitamin D has been reported to lower pro-inflammatory cytokines known to stimulate hepcidin expression[96] and directly suppress transcription of hepcidin, the major iron-regulatory hormone.[95]

In a recent analysis, Laillou et al[146] found that 90% of women in Vietnam had 25-hydroxyvitamin D [25(OH)D)] concentrations < 75 nmol/L and 40% had 25(OH)D concentrations < 50 nmol/L, indicating a fairly high prevalence of vitamin D insufficiency among Vietnamese women. Sources of vitamin D include sun exposure and dietary vitamin D intake. In the latter study, the authors found that the mean vitamin D intake among women was 0.15 μg/d based on a household level food consumption survey. This estimated intake is much lower than the Recommended Dietary Allowances (RDA) for vitamin D intake for WRA of 5 µg and 15 µg in Vietnam and the United States, respectively.[32,147] Therefore, given the burden of anemia in WRA in Vietnam, the low rate of iron deficiency anemia, the very low estimated vitamin D intake, and the link between vitamin D status and anemia in other populations, we hypothesized that vitamin D intake and serum 25(OH)D concentrations would be positively associated with hemoglobin concentrations, and inversely association with odds of anemia. The objectives of this cross-sectional population-based analysis were to 1) describe vitamin D intake and its determinants, and 2) examine the association between dietary vitamin D intake and hemoglobin concentration and anemia in WRA in the Thai Nguyen region of Vietnam. To overcome the concerns that dietary vitamin D intake may not be a good proxy for vitamin D status, we also examined the association of serum 25(OH)D concentrations with hemoglobin concentration and anemia in a subset of women (n=88)

in this population. To accomplish this, multivariable regression analyses were performed to evaluate determinants of dietary vitamin D intake and to model the associations of vitamin D intake and serum 25(OH)D concentrations with hemoglobin concentrations and anemia. Given that vitamin D deficiency and anemia are both highly prevalent nutrition-related public health concerns, this study contributes to the literature by evaluating the link between low vitamin D status and anemia in a population at risk for both conditions, and by exploring the role of diet in this association to potentially inform public health interventions.

Methods and materials

Study population

The data for this analysis came from the baseline survey of the PRECONCEPT study, a large double-blind randomized controlled pre-conceptual micronutrient supplementation trial in the Thai Nguyen region of northern Vietnam (NCT01665378). This trial aimed to improve maternal and infant health and a detailed description of the methodology has been previously published.[148] Briefly, women of reproductive age (18-40 years) who were planning to become pregnant within a year of enrollment were recruited from 20 communes located in four of the nine districts of the Thai Nguyen province between November 2011 and April 2012 (Winter to early Spring in Vietnam). Women were excluded if they were pregnant, had recently used or were currently using iron and folic acid or multiple micronutrient supplements, were severely anemic (hemoglobin < 7 g/dL), had a history of a high risk pregnancy, or had a chronic hematologic disease. The study was approved by the Vietnam Institute of Social and

Medical Studies and the Emory University Institutional Review Boards. Written informed consent was obtained from all participants at enrollment.

Data collection and processing

Dietary intake

Dietary vitamin D intake was estimated using a semi-quantitative food frequency questionnaire (FFQ) which included a list of 107 common foods and beverages consumed in Vietnam. This FFQ was previously validated by Vietnam's National Institute of Nutrition.[149] Trained interviewers asked participants to recall the frequency and portion size of listed foods and beverages consumed over the three months prior to the interview. Nutrient intake was estimated using the FFQ data and Vietnamese food composition tables.[150] Complex dishes not included in the food table were broken down into ingredients based on a Vietnamese recipe book and nutrient contents were calculated.[151]

Biochemical and anthropometric measurements

Hemoglobin concentration was measured in capillary blood using a portable hemoglobin analyzer, HemoCue® Hb 301. Venous blood samples (5 mL) were collected by trained nurses, stored in an icebox, and transported within 4 hours to Thai Nguyen University of Pharmacy and Medicine (TUMP) Hematology Department where they were centrifuged at 1500 x g. Plasma ferritin, soluble transferrin receptor (sTfR), retinol binding protein (RBP), C-reactive protein (CRP) and α_1-acid glycoprotein (AGP) were measured via sandwich ELISA.[152] The intra assay coefficient of variation (CV) was < 3.0%. Due to budget constraints, 25(OH)D concentrations were measured in only a

subset (n=88) of participants. The subset of women for 25(OH)D assessment was selected based on reported dietary vitamin D intake. Women were categorized into deciles based on their intake and 8-10 women from each decile were randomly selected for the analysis. Serum 25(OH)D concentrations were measured using an automated chemiluminescent technique (IDS-iSYS automated machine, Immunodiagnostic Systems, Inc., Fountain Hills, AZ) in a laboratory which participates in the Vitamin D External Quality Assessment Scheme (DEQAS, site #606) and the National Institute of Standards and Technology/NIH Vitamin D Metabolites Quality Assurance Program to ensure the accuracy of 25(OH)D measurements. Stool samples were analyzed using the Kato-Katz method for evaluation of intestinal helminth infection.[153] Height and weight were measured twice via standard methods.[154]

Demographic data

Demographic data were obtained using an interviewer-administered structured questionnaire. Socioeconomic status (SES) was assessed using the World Bank asset questionnaire for developing countries (and adapted for the local context in Vietnam), which includes questions about home and land ownership, house construction materials, access to services such as water and electricity, and household assets such as livestock.[155] Socioeconomic status was categorized into quintiles for this analysis. Household food insecurity was measured using the FANTA/USAID Household Food Insecurity Access Scale (HFIAS), and categorized by level of food security: food secure, mildly food insecure, moderately food insecure, and severely food insecure.[156] Other demographic variables used in this analysis included education, ethnicity, and occupation. Education

was categorized based on highest grade completed (0-5th grade, 6-9th grade, 10-12th grade, or greater than 12th grade). Ethnicity was dichotomized into majority and minority groups, with majority defined as those of Kinh ethnicity. Finally, occupation was dichotomized as farmer or other occupation.

Definitions

Anemia was defined based on the World Health Organization (WHO) criteria for non-pregnant WRA, as hemoglobin concentration < 12 g/dL.[80] Vitamin D status was categorized as 25(OH)D < 50 nmol/L and 25(OH)D < 75 nmol/L for descriptive and modelling purposes based on commonly accepted cut-offs for vitamin D insufficiency.[32,33] To further characterize the population by potential contributors to anemia etiology, namely inflammation and nutrient deficiency, we categorized inflammation as CRP concentrations > 5 mg/L or AGP concentrations > 1g/L,[157] and nutrient deficiency was defined as iron deficiency (plasma ferritin < 12 μg/L) or vitamin A deficiency (RBP < 1.05 mmol/L).[148]

Statistical analyses

Descriptive statistics were examined for all variables. Continuous variables were reported as means ± standard deviation (SD) for normally distributed variables or medians and interquartile range (IQR) for non-normally distributed variables; categorical variables were presented as percentages. Non-normally distributed variables were transformed to the natural logarithmic scale or categorized into tertiles for regression analyses. Because some values were zero, a constant of 0.01 was added to all non-

missing values prior to log transformation. Multivariable linear regression with step-wise elimination was used to evaluate significant determinants of vitamin D intake and 25(OH)D concentration.

Spearman correlation was used to assess the simple correlation between vitamin D intake and hemoglobin concentrations. Multivariable linear regression was then used to examine the association between vitamin D intake (log-transformed) and hemoglobin concentration. Multivariable logistic regression was used to assess the association between vitamin D intake, categorized into tertiles (independent variable), and anemia (dependent variable). All models were controlled for potential confounders, age, BMI, total energy intake, iron status (sTfR) and inflammatory variables (CRP, AGP) in model 1, with socio-economic variables (food insecurity, education, ethnicity, occupation, and SES) added in model 2. A two-stage least squares analysis was applied to assess indirect relationships among independent variables with hemoglobin concentration and anemia.

The relationship between dietary vitamin D intake and serum 25(OH)D concentrations was assessed via linear regression. Multivariable regression was used to assess the association between 25(OH)D (defined both as a continuous variable and categorized as 25(OH)D < 50 nmol/L vs. 25(OH)D ≥ 50 nmol/L) and hemoglobin concentration as well as anemia controlling for the potential confounders noted above.

Results were presented as β-coefficients and standard errors (SE) for linear regression models or odds ratio (OR) with 95% confidence intervals (CI) for logistic regression models. All analyses were performed using SAS v 9.4 (SAS Institute, Inc., Cary, NC), with a two-sided significance level of 0.05. This secondary analysis used all available data from the parent study for examining the associations between vitamin D

intakes and hemoglobin/anemia. The subsample for 25(OH)D assays was determined by available resources.

Results

Population characteristics

Of the 5,011 women recruited for the PRECONCEPT Study, 4,961 who had hemoglobin and dietary intake data were included in the present analysis. The socio-demographic, biochemical, and dietary intake characteristics of this sample of Vietnamese WRA are presented in **Table 4.1**. The mean age was approximately 26 years, and mean BMI was in the normal range at 19.6 ± 2.0 kg/m^2. The mean hemoglobin concentration was 13.0 ± 1.4 g/dL, and 19.6% of the women were anemic. Approximately 7% of women had low ferritin and/or low RBP, and a similar proportion had evidence of inflammation (elevated CRP and/or elevated AGP). The majority of women were farmers and approximately half reported being of an ethnic minority. Twelve percent of the women reported completing greater than a 12th grade education and about 20% were moderately or severely food insecure.

Distribution and determinants of dietary vitamin D intake

The median estimated dietary vitamin D intake was 0.2 μg/d [8.0 IU] (IQR: 0.4), **Table 4.1**. The distribution of dietary vitamin D intake is shown in **Figure 4.1**. The majority of women consumed less than 1 μg/d on average of dietary vitamin D, less than 1% of women met the Vietnamese RDA of 5 µg/d, and none met the United States' RDA of 15 μg/d.[32] Dietary sources of vitamin D included milk, eggs, and pork ribs, which contained

only 1.0, 0.88, and 0.69 μg (40, 35.2, and 27.6 IU) of vitamin D per serving, respectively as determined by the Vietnamese food composition table.[150] A third of the women reported consuming any milk in the three months prior to interview, and about 20% reported consuming a glass of milk at least once per week. Approximately 90% of women reported consuming eggs over the three months prior to interview, and nearly 75% reported consuming them at a frequency of at least once per week. Approximately 46% of women reported eating pork at all, and about 30% consumed it at least once per week. Other potential dietary sources of vitamin D including mushrooms and fish did not contribute to dietary vitamin D intake in this population. Mushrooms were consumed in negligible amounts, and the fish in this region is not the fatty fish known to be a source of dietary vitamin D.

Significant determinants of vitamin D intake are presented in **Table 4.2**. In multivariable linear regression analysis, older age, farming as an occupation, increased food insecurity, and higher BMI were statistically significantly associated with lower vitamin D intake (P<0.001 for all), while higher socioeconomic status, higher energy intake, and higher educational attainment were associated with higher vitamin D intake (P<0.001 for all). Ethnic minority, hookworm infection, and gravidity were not significantly associated with dietary vitamin D intake.

Associations of vitamin D intake with hemoglobin and anemia

Vitamin D intakes were significantly correlated with hemoglobin concentration in bivariate analysis (Spearman r = 0.03, P = 0.02). However, dietary vitamin D intake was no longer significantly associated with hemoglobin concentration (β = -0.01 (SE: 0.02), P

= 0.56) in the multivariable linear regression model that adjusted for age, BMI, energy intake, sTfR, CRP, AGP, ethnicity, occupation, food insecurity, education level and socioeconomic status (**Table 4.3**).

Vitamin D intakes were also significantly associated with anemia in bivariate analysis. Women in the highest tertile of vitamin D intake were 20% less likely to be anemic compared to those in lowest tertile (OR: 0.80, 95% CI: 0.67, 0.95; *P* = 0.01), and these associations remained significant after controlling for age, total energy intake, sTfR (or ferritin), CRP, and AGP (Model 1: OR: 0.78, 95% CI: 0.64, 0.94; *P* = 0.01) (**Table 4.3**). However, the association between vitamin D intake and anemia was attenuated and no longer statistically significant when ethnic minority, occupation, education, food insecurity, and SES quintile, were added to the model (Model 2: OR: 0.95, 95% CI: 0.78, 1.17; *P* = 0.65). The results of the two stage least squares analysis also showed that there was no residual association between vitamin D intake and hemoglobin concentration (*P* = 0.97) or anemia (*P* = 0.19), after accounting for the association of vitamin D intake with SES (results not shown).

Association of dietary vitamin D intake with 25(OH)D status

The mean 25(OH)D concentrations in the subset of women (n=88) was 57.4 ± 10.7 nmol/L (**Table 4.4**). Approximately 20% of this sample had 25(OH)D concentrations < 50 nmol/L and 93% had 25(OH)D concentrations < 75 nmol/L. This subset of women was similar to the larger study population in terms of socio-demographic, health status, and biochemical characteristics (**Table 4.4**). Dietary vitamin

D intake was not significantly associated with 25(OH)D concentrations in linear regression analysis controlling for total energy intake (β = 1.21 (SE: 0.88), P = 0.17).

Determinants of 25(OH)D concentration and association of 25(OH)D with hemoglobin and anemia

None of the predictors of dietary vitamin D intake, including age, BMI, total energy intake, occupation, food insecurity, education and socioeconomic status, were significantly associated with 25(OH)D concentrations ($P > 0.05$ for all).

After full adjustment for all covariates as mentioned above, women with 25(OH)D < 50 nmol/L had significantly lower hemoglobin concentration compared to women with 25(OH)D ≥ 50 nmol/L (β = -0.91 (SE: 0.42), P = 0.03) (**Table 4.5**). In the models with anemia as the outcome, neither 25(OH)D as a continuous variable nor 25(OH)D < 50 nmol/L were significantly associated with anemia in unadjusted or adjusted models (P=0.08 and P=0.11, respectively) (**Table 4.5**). Similar non-significant associations were observed with hemoglobin or anemia when 25(OH)D was categorized as < 75 nmol/L vs. ≥ 75 nmol/L (P = 0.62 and P = 0.72 for hemoglobin and anemia, respectively). Serum 25(OH)D modeled either continuously or dichotomized as 25(OH)D < 50 nmol/L vs. 25(OH)D ≥ 50 nmol/L was not statistically significantly associated with other markers of iron status, plasma sTfR (P=0.23 and P=0.18, for serum 25(OH)D continuously and dichotomized, respectively) or plasma ferritin (P=0.053 and P=0.12, for serum 25(OH)D continuously and dichotomized, respectively), controlling for age, total energy intake, BMI, AGP, CRP, ethnicity, food insecurity, occupation, education, and SES.

Discussion

This paper reports the dietary vitamin D intake among WRA in the largely rural and mountainous Thai Nguyen province of northern Vietnam, and its association with hemoglobin concentration and anemia. We found that reported dietary vitamin D intake was profoundly low in this population, with less than 1% of women reporting intakes of vitamin D meeting the Vietnamese RDA of 5 μg/d, and none of the women reporting intakes of vitamin D meeting even the estimated average requirement (EAR) of 10 μg/d (400 IU/day), as recommended by the IOM in the United States.[32] Dietary vitamin D intake was not significantly associated with hemoglobin concentration or anemia after adjustment for sociodemographic variables; therefore, our hypothesis regarding the link between vitamin D intake and hemoglobin and anemia is rejected However, in the subset of women with available 25(OH)D concentrations, 25(OH)D concentrations < 50 nmol/L were significantly associated with a 0.91 g/dL reduction in hemoglobin concentration compared to 25(OH)D concentrations ≥ 50 nmol/L, consistent with our hypothesis.

Low dietary vitamin D intake in this population is likely due to the dearth of food sources of vitamin D consumed by women in this region; the only food sources of vitamin D consumed were eggs, milk, and pork ribs. As the food supply in Vietnam is not fortified with vitamin D, the content of vitamin D in these foods is quite low. Though overall intake of vitamin D was extremely low, we found that higher SES (higher quintile of SES, higher educational attainment, better food security, and non-farming occupation) was associated with increased dietary vitamin D intake. These findings are consistent with an earlier nationwide study that assessed vitamin D intake among WRA in Vietnam

using a national household food intake survey.[146] Studies of food consumption patterns in Vietnam indicate that rural households were more likely to consume diets higher in carbohydrates, and lower in animal proteins and fats compared to those living in urban areas.[158,159] Previous findings from our study population have shown that nearly 55% of our study population consumed carbohydrates in excess of the Acceptable Macronutrient Distribution Range (AMDR) as recommended by the IOM,[160] and a similar proportion did not meet the recommended intakes from fats.[161] The high carbohydrate-low fat composition of the diet may explain why vitamin D intakes are low, since the primary natural dietary sources of vitamin D are foods rich in fats and proteins such as fatty fish and cheese.

Dietary vitamin D intake was not associated with hemoglobin or anemia after controlling for socio-demographic variables. One potential explanation is that vitamin D intake and anemia appeared to be largely determined by SES. This is supported by our two-stage least squares analysis in which we found that after controlling for the association between quintile of SES and vitamin D intake, residual vitamin D intake was not significantly associated with hemoglobin concentration or anemia. The association of vitamin D intake with SES is consistent with findings of other micronutrient intakes in this population,[162] suggesting that those with higher SES may have more diverse diets, potentially translating to improved health outcomes, such as in the case of anemia.

Another explanation for why vitamin D intake was not associated with hemoglobin or anemia is that dietary vitamin D intake may not be reflective of an individual's vitamin D status, as measured by 25(OH)D concentrations. Approximately 90% of an individual's vitamin D requirement comes from sun exposure,[15] and given the

extremely low dietary vitamin D intake in our population, it is likely that dietary vitamin D intake was not a primary contributor to 25(OH)D status. Indeed, dietary vitamin D intake was not significantly associated with 25(OH)D concentrations in our study population among the group in which 25(OH)D was available.

Although we did not observe significant associations of dietary vitamin D intake with hemoglobin or anemia, we did find a significant inverse association between 25(OH)D concentrations < 50 nmol/L and hemoglobin concentrations in a smaller subset of women. In our fully adjusted model, 25(OH)D concentrations < 50 nmol/L were associated with a 0.91 g/dL reduction in hemoglobin concentrations, compared to 25(OH)D concentrations ≥ 50 nmol/L. This association is not inconsequential, as a meta-analysis by Stoltzfus, *et al*., found that a 1 g/dL increase in hemoglobin was associated with a 25% reduction in maternal mortality.[77] Therefore, improvements in 25(OH)D concentrations to maintain a level of 50 nmol/L or greater (the level designated by the IOM as sufficient to maintain bone and overall health),[32] may have beneficial implications in terms of hemoglobin concentrations.

The potential mechanism underlying the association between vitamin D and hemoglobin likely involves hepcidin, the major iron-regulatory hormone.[145] When hepcidin concentrations are elevated, such as in response to an inflammatory stimulus, this prevents iron egress from cells of the reticuloendothelial system, limiting iron absorption and sequestering iron within macrophages.[66] Vitamin D has been shown to lower hepcidin-stimulatory pro-inflammatory cytokines and act directly on the hepcidin antimicrobial peptide gene to suppress hepcidin expression in hepatocytes and

macrophages.[95,96] Thus, vitamin D, in lowering pro-inflammatory cytokines and hepcidin, may increase iron bioavailability for hemoglobin synthesis and erythropoiesis.

We did not observe a significant association between 25(OH)D concentrations with anemia in the population. It is possible that while 25(OH)D concentrations < 50 nmol/L were associated with reductions in hemoglobin, this was not severe enough to affect anemia status. In our small subset, we were unable to determine whether the relationship between 25(OH)D and anemia differed by inflammation or nutrient status. Previous studies have reported vitamin D status to be associated particularly with anemia of inflammation, and this is in line with the potential mechanism described above.[9,116] Furthermore, the lack of an association between either dietary vitamin D intake or serum 25(OH)D concentrations with anemia may suggest that anemia is largely determined by SES in this population. Indeed, in a previous analysis by our group exploring the multi-causal etiology of anemia in this study population, it was found that minority ethnicity, lower education, and lower SES quintile were all significant predictors of anemia.[143]

Strengths of this analysis included a large sample size and use of a validated semi-quantitative FFQ to estimate nutrient intake at an individual level. However, there are important limitations of our study. First, the cross-sectional nature of our analysis precludes us from drawing causal inferences from the associations we observed. Second, FFQs rely on recall, which may be inaccurate and especially difficult in foods eaten infrequently (which may be the case for food sources of vitamin D). However, given that relatively few foods are good natural sources of vitamin D, and that the majority of women in our population eat a diet high in carbohydrates and low in fats, errors in recall are unlikely to affect our vitamin D intake estimates. Another limitation is

that we were unable to measure serum 25(OH)D concentrations in the entire study population due to limited resources. Although this sample had sufficient power to detect the observed association between vitamin D status and hemoglobin concentrations, we may have been under powered to detect an association between vitamin D status and anemia. Moreover, we cannot conclude that the associations observed with hemoglobin in the smaller subset, holds for the entire study population. However, the socio-demographic, health status, and biochemical characteristics of the subset were similar to those of the entire population, and the 25(OH)D levels observed in our subset were similar to those reported in other studies of vitamin D status among Vietnamese women.[146,163,164] It is also possible that there may be residual confounding in our associations due to variables that we were unable to control for such as outdoor physical activity, so we cannot exclude the possibility that our results may be biased. Additionally, we were unable to examine the associations of other vitamin D metabolites including the active form of vitamin D (1,25-dihydroxyvitamin D) and free 25(OH)D concentrations, with our outcomes which may have allowed us to more comprehensively evaluate the link between vitamin D and iron metabolism. Finally, this was a study in a primarily rural and mountainous region of northern Vietnam, where the majority of women worked as farmers; our findings may not be generalizable to the entire country of Vietnam, especially urban or southern areas.

In conclusion, we found that dietary vitamin D intake was very low in this population, and inversely associated with SES, but was not associated with hemoglobin concentrations or anemia. Serum 25(OH)D concentrations < 50 nmol/L were significantly inversely associated with hemoglobin concentrations, suggesting that

achieving a vitamin D status ≥ 50 nmol/L may result in improvements in hemoglobin concentrations. Further research, including experimental studies, is warranted to fully evaluate the implications of this association, and understand the role of vitamin D in iron metabolism.

Acknowledgment: The authors would like to acknowledge Dr. Hoa Pham, Dr. Truong V. Truong, and Dr. Hieu Nguyen for their work in field supervision and data collection, data management, and field work organization, respectively. This work was supported by the Mathile Institute for the Advancement of Human Nutrition; the Micronutrient Initiative; and the National Institute of Health [grants T32 DK007734 (EMS) and UL1TR000454 (Atlanta Clinical and Translational Science Institute)]. The funders had no role in the design, analysis or writing of this article. The authors declare no conflict of interest.

Table 4.1
Baseline descriptive characteristics for women in PRECONCEPT Study[a]

Sociodemographic and health status characteristics	**Mean ± SD or n (%)**
Age (y)	26.2 ± 4.6
Education (completed 12th grade)[*]	592 (12.0)
Occupation (farmer)[*]	3,991 (80.6)
Ethnicity (minority)[*]	2,448 (49.5)
Food insecurity (moderately or severely food insecure)[*]	936 (18.9)
BMI (kg/m^2)[*]	19.6 ± 2.0
Gravidity[*]	1.3 ± 0.8
Dietary intake	
Vitamin D intake (µg/d)	0.2 (0.4)[b]
Iron intake (mg/d)	15.7 (8.3)[b]
Total energy intake (kcal/d)	2,104.1 (842.9)[b]
Intake of foods containing vitamin D in last 3 months	
Any milk[*]	1,676 (33.8)
A glass of milk at least once per week	1,035 (20.9)
Any eggs[*]	4,464 (90.0)
An egg at least once per week	3,639 (73.4)
Any pork[*]	2,285 (46.1)
A small piece at least once per week	1,466 (29.6)
Biochemical markers	
Hemoglobin (g/dL)	13.0 ± 1.4
Anemia[c]	974 (19.6)
Plasma Ferritin (µg/L)[*]	68.2 (66.3)[b]
Plasma sTfR (mg/L)[*]	4.5 (1.5)[b]
Plasma RBP (mmol/L)[*]	1.6 (0.5)[b]
Nutrient deficiency[d]	337 (6.8)
Plasma AGP (g/L)[*]	0.7 ± 0.2
Plasma CRP (mg/L)[*]	0.3 (0.7)[b]
Inflammation[e]	337 (6.8)
Hookworm (proportion of women with any eggs)[*]	953 (21.5)

Values are Means ± SD or n (%) unless otherwise noted
Abbreviations: AGP, α_1-acid glycoprotein; BMI, body mass index; CRP, C-reactive protein; RBP, retinol binding protein; sTfR, soluble transferrin receptor
[a]n = 4,961 women with hemoglobin measurements and dietary intake data included in this analysis
[b]median (interquartile range) for non-normally distributed variables
[c]anemia defined as hemoglobin < 12 g/dL
[d]ferritin < 12 µg/L or RBP < 1.05 mmol/L
[e]AGP > 1 g/L or CRP > 5 mg/L
[*]n = 4960 for AGP, CRP, ferritin, RBP, sTfR; n = 4959 for intake of milk, eggs, pork; n = 4958 for BMI; n = 4948 for gravidity; n = 4951 for occupation, education, food insecurity; n = 4949 for ethnicity; n = 4432 for hookworm

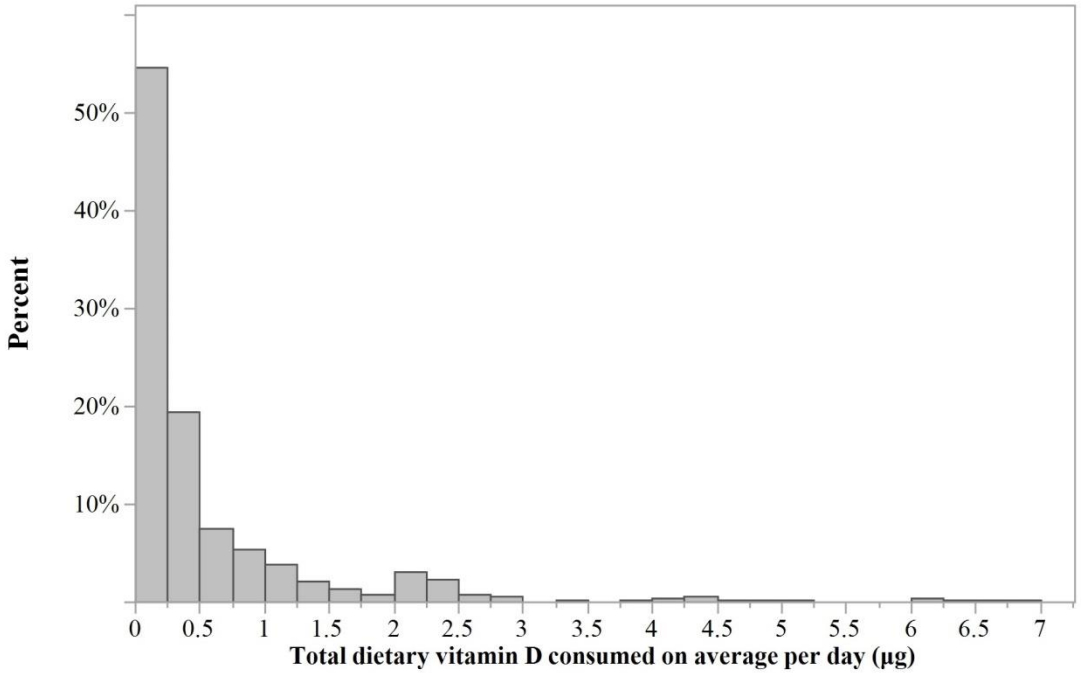


Figure 4.1.

Distribution of dietary vitamin D intake among women of reproductive age in northern Vietnam (n = 4,961).

Table 4.2.
Determinants of dietary vitamin D intake[a] in women of reproductive age in northern Vietnam[b]

	***β* (SE)**	***P***
Age (years)	-0.01 (0.004)	<0.001
BMI (kg/m^2)	-0.05 (0.01)	<0.001
Total energy intake (Kcal/d)[a]	1.50 (0.05)	<0.001
Occupation (farmer)	-0.39 (0.05)	<0.001
SES quintile	0.15 (0.01)	<0.001
Lowest	Ref	
Low	0.13 (0.05)	0.01
Middle	0.24 (0.05)	<0.001
High	0.40 (0.06)	<0.001
Highest	0.60 (0.06)	<0.001
Food insecurity category[c]	-0.15 (0.02)	<0.001
Food secure	Ref	
Mildly food insecure	-0.22 (0.05)	<0.001
Moderately food insecure	-0.31 (0.05)	<0.001
Severely food insecure	-0.43 (0.10)	<0.001
Level of education[d]	0.15 (0.03)	<0.001
0-5th grade	Ref	
6-9th grade	0.19 (0.06)	0.004
10-12th grade	0.40 (0.07)	<0.001
Greater than 12th grade	0.37 (0.09)	<0.001

Values are β coefficients and SE, n = 4,961
Abbreviations: BMI, body mass index; SES, socioeconomic status
[a]Transformed to natural logarithmic scale
[b]Results from multivariable linear regression analysis with step-wise elimination
[c]Categories of food insecurity: food secure, mildly food insecure, moderately food insecure, severely food insecure
[d]Levels of education: 0-5th grade, 6-9th grade, 10-12th grade, or greater than 12th grade

Table 4.3.
Multivariable regression analysis of vitamin D intake with hemoglobin and anemia

	Model 1[a]		**Model 2**[b]	
Hemoglobin as outcome[c]	***β* (SE)**	***P***	***β* (SE)**	***P***
Vitamin D intake[d]	0.03 (0.02)	0.04	-0.01 (0.02)	0.56
Tertile of dietary vitamin D intake				
Low	Ref		Ref	
Middle	0.001 (0.05)	0.99	-0.03 (0.05)	0.55
Higher	0.11 (0.05)	0.04	-0.01 (0.05)	0.87
Anemia as outcome[e]	**OR (95% CI)**	***P***	**OR (95% CI)**	***P***
Tertile of dietary vitamin D intake				
Low	Ref		Ref	
Middle	1.00 (0.84, 1.19)	1.00	1.07 (0.90, 1.27)	0.45
Higher	0.78 (0.64, 0.94)	0.01	0.95 (0.78, 1.17)	0.65

Values are β coefficients and SE for linear regression analyses and OR and 95% CI for logistic regression analyses, n = 4,961

[a]Model 1: Association of vitamin D intake with hemoglobin or anemia, adjusted for age, BMI, total energy intake, and transferrin receptor, C-reactive protein, and α_1-acid glycoprotein

[b]Model 2: Model 1 + adjustment for ethnicity, occupation, education level, food insecurity, and socioeconomic quintile.

[c]Results from multivariable linear regression analysis

[d]Transformed to natural logarithmic scale

[e]Results from multivariable logistic regression analysis, BMI dropped from these models due to collinearity

Table 4.4
Biochemical and sociodemographic characteristics of subset with available 25(OH)D[a]

Sociodemographic and health status characteristics	**Mean ± SD or n (%)**
Age (y)	26.6 ± 5.1
Education (completed 12th grade)	13 (14.8)
Occupation (farmer)	69 (78.4)
Ethnicity (minority)	44 (50.0)
Food insecurity (moderately or severely food insecure)	13 (14.8)
BMI (kg/m^2)	19.1 ± 2.0
Gravidity	1.2 ± 0.8
Dietary intake	
Vitamin D intake (µg/d)	0.2 (0.4)[b]
Iron intake (mg/d)	15.9 (9.8)[b]
Total energy intake (kcal/d)	2149.9 (895.6)[b]
Biochemical markers	
Serum 25(OH)D (nmol/L)	57.4 ± 10.7
25(OH)D < 50 nmol/L	18 (20.5)
25(OH)D < 75 nmol/L	82 (93.2)
Hemoglobin (g/dL)	12.9 ± 1.5
Anemia[c]	21 (23.9)
Plasma Ferritin (µg/L)	76.5 (58.5)[b]
Plasma sTfR (mg/L)	4.7 ± 1.0
Plasma RBP (mmol/L)	1.7 (0.4)[b]
Nutrient deficiency[d]	5 (5.7)
Plasma AGP (g/L)	0.7 ± 0.2
Plasma CRP (mg/L)	0.4 (0.6)[b]
Inflammation[e]	3 (3.4)
Hookworm (proportion of women with any eggs)[*]	18 (22.5)

Values are Means ± SD or n (%) unless otherwise noted
Abbreviations: 25(OH)D, 25-hydroxyvitamin D; AGP, α_1-acid glycoprotein; BMI, body mass index; CRP, C-reactive protein; RBP, retinol binding protein; sTfR, soluble transferrin receptor
[a]n=88
[b]median (IQR) for non-normally distributed variables
[c]anemia defined as hemoglobin < 12 g/dL
[d]ferritin < 12 µg/L or RBP < 1.05 mmol/L
[e]AGP > 1 g/L or CRP > 5 mg/L
*n=80

Table 4.5
Multivariable regression analysis of 25(OH)D with hemoglobin and anemia

	Model 1[a]		**Model 2**[b]	
Hemoglobin as outcome[c]	***β* (SE)**	***P***	***β* (SE)**	***P***
25(OH)D (continuous)	0.02 (0.02)	0.25	0.02 (0.02)	0.24
25(OH)D (categorical)				
25(OH)D ≥ 50 nmol/L	Ref		Ref	
25(OH)D < 50 nmol/L	-0.68 (0.42)	0.11	-0.91 (0.42)	0.03
Anemia as outcome[d]	**OR (95% CI)**	***P***	**OR (95% CI)**	***P***
25(OH)D (continuous)	0.97 (0.92, 1.03)	0.34	0.92 (0.85, 1.01)	0.08
25(OH)D (categorical)				
25(OH)D ≥ 50 nmol/L	Ref		Ref	
25(OH)D < 50 nmol/L	1.28 (0.36, 4.54)	0.70	5.44 (0.67, 43.98)	0.11

Values are β coefficients and SE for linear regression analyses and OR and 95% CI for logistic regression analyses, n = 88
Abbreviations: 25(OH)D, 25-hydroxyvitamin D
[a]Model 1: Association of 25(OH)D with hemoglobin or anemia, adjusted for age, BMI, total energy intake, and transferrin receptor, C-reactive protein, and α_1-acid glycoprotein
[b]Model 2: Model 1 + adjustment for ethnicity, occupation, education level, food insecurity, and socioeconomic quintile.
[c]Results from multivariable linear regression analysis
[d]Results from multivariable logistic regression analysis, BMI dropped from these models due to collinearity

CHAPTER 5

HIGH-DOSE VITAMIN D_3 REDUCES CIRCULATING HEPCIDIN CONCENTRATIONS: A PILOT, RANDOMIZED, DOUBLE-BLIND, PLACEBO-CONTROLLED TRIAL IN HEALTHY ADULTS

Ellen M. Smith[a], Jessica A. Alvarez[a,b], Malcolm D. Kearns[b], Li Hao[b], John H. Sloan[c], Robert J. Konrad[c], Thomas R. Ziegler[a,b], Susu M. Zughaier[d,e], Vin Tangpricha[a,b,e,*]

[a]Nutrition and Health Sciences Program, Laney Graduate School, Emory University, Atlanta, GA, 30322 USA;

[b]Division of Endocrinology, Metabolism and Lipids, Emory University School of Medicine, Atlanta, GA, 30322 USA;

[c]Lilly Research Laboratories, Eli Lilly and Company, Indianapolis, IN, 46285 USA;

[d]Department of Microbiology and Immunology, Emory University School of Medicine, Atlanta, GA, 30322 USA;

[e]Atlanta Veterans Administration Medical Center (VAMC), Decatur, GA, 30033 USA.

Clinical Nutrition (2016), http://dx.doi.org/10.1016/j.clnu.2016.06.015

Abbreviations: $1,25(OH)_2D$: 1,25-dihydroxyvitamin D; 25(OH)D: 25-hydroxyvitamin D; BMI: body mass index; CKD: chronic kidney disease; GMR: geometric mean ratio; IL-1β: interleukin-1β; IL-6: interleukin-6; IL-8: interleukin-8; MCP-1: monocyte chemoattractant protein-1

Abstract

Background & Aims: *In vitro* studies suggest that vitamin D may reduce hepcidin expression and pro-inflammatory cytokine release from monocytes. However, data assessing the vitamin D-mediated effects on iron recycling in healthy individuals are lacking. We aimed to examine the effect of high-dose vitamin D_3 on plasma hepcidin, inflammatory cytokine, and ferritin concentrations in healthy adults.

Methods: This was a pilot, double-blind, placebo-controlled trial in healthy adults (N=28) randomized to receive a one-time oral dose of 250,000 IU of vitamin D_3 or placebo. Between- and within-group differences in plasma hepcidin, pro-inflammatory cytokine [interleukin (IL)-1β, IL-6, IL-8, monocyte chemoattractant protein-1 (MCP-1)], and ferritin concentrations at baseline and 1 week were determined using two-sample and paired *t*-tests, respectively.

Results: At baseline, plasma 25-hydroxyvitamin D [25(OH)D], hepcidin, pro-inflammatory cytokine, and ferritin concentrations did not differ between the two groups, and greater than 70% of subjects in both groups were vitamin D deficient (25(OH)D < 20 ng/mL). After 1 week, plasma hepcidin concentrations decreased by 73% from baseline in those who received vitamin D_3 (geometric mean ratio [GMR] = 0.27 (95% CI: 0.11-0.62); $P = 0.005$); there was no significant change in the placebo group (GMR = 0.73 (95% CI: 0.49-1.09); $P = 0.11$). Plasma cytokine and ferritin concentrations did not change significantly in either group.

Conclusions: High-dose vitamin D_3 significantly reduced plasma hepcidin concentrations in healthy adults 1 week post-dosing, without a change in plasma pro-inflammatory cytokine or ferritin concentrations. These data suggest that vitamin D may

have a role in regulating iron recycling by acting independently of changes in pro-inflammatory markers.

Keywords: vitamin D, hepcidin, inflammation, anemia, iron

Introduction

Vitamin D deficiency and anemia are both prominent nutrition-related public health concerns. In the United States, it has been reported that 32% of adults have vitamin D deficiency as defined by 25-hydroxyvitamin D [25(OH)D] concentrations < 20 ng/mL.[117] In 2010, it was estimated that nearly one third of the global population had anemia.[81] Co-existence of vitamin D deficiency and anemia is not uncommon, as poor diets and illness are contributing factors to both conditions,[1,46] and chronic diseases, including chronic kidney disease (CKD) and cardiovascular disease, incur high rates of both.[164-168] Recently, vitamin D deficiency was identified as a potential risk factor for anemia, particularly anemia of inflammation, in the general population.[9,116]

Anemia of inflammation may develop due to disturbances in iron recycling secondary to pro-inflammatory cytokine-induced increases in the hepatic production of hepcidin, the major iron-regulatory hormone.[66] Elevations in hepcidin promote iron sequestration within cells of the reticuloendothelial system, thus limiting iron availability for erythropoiesis and hemoglobin synthesis.[51] Recent *in vitro* studies suggest a role for vitamin D in down-regulating both pro-inflammatory cytokines and hepcidin.[145] Treatment of cultured human monocytes with vitamin D has been shown to decrease the release of pro-inflammatory cytokines interleukin-6 (IL-6) and interleukin-1β (IL-1β) and down-regulate the expression of hepcidin mRNA.[95,96] Furthermore, the hepcidin antimicrobial peptide (HAMP) gene has been found to contain a vitamin D response element, suggesting a mechanism for transcriptional regulation of hepcidin by vitamin D.[95]

Despite the strong biological plausibility for the association between vitamin D status and anemia, rigorous randomized controlled trials examining the effect of high-dose vitamin D supplementation on specific biomarkers involved in the pathophysiology of anemia of inflammation, namely pro-inflammatory cytokines and hepcidin, are lacking. It remains unclear whether vitamin D-mediated effects on iron recycling occur due to reductions in inflammation or through direct action on hepcidin expression. Moreover, the down-stream effects of vitamin D on makers of iron status have not yet been elucidated. Therefore, the purpose of this study was to examine the acute effect of high-dose vitamin D_3 supplementation on plasma hepcidin, inflammatory cytokine, and ferritin concentrations in healthy adults to better understand the mechanism by which vitamin D may influence iron recycling. We hypothesized that treatment with vitamin D_3 would reduce circulating hepcidin and plasma inflammatory cytokine concentrations, and increase plasma ferritin concentrations.

Materials and Methods:

Subjects and Protocol

Subjects were participants in a double-blind, randomized, placebo-controlled trial designed to evaluate the impact of a large bolus dose of vitamin D_3 given prior to winter, on 25(OH)D concentrations year round in healthy adults.[169] Briefly, adults between the ages of 18 and 65 who were healthy by self-report were recruited from the Emory University campus in Atlanta, GA between August and December 2012. Participants were excluded if they were currently pregnant or breastfeeding, had granulomatous conditions, a history of kidney or liver disease, diabetes, a history of malignancy,

thyrotoxicosis, a history of calcium or bone abnormalities including hyperparathyroidism, osteoporosis, and Paget's disease, an inability to ambulate, an intake of greater than 1000 mg/day of calcium, and/or used medications including antihypertensives, barbituates, anticonvulsants, or steroids. Sex, race, height, weight, time spent outdoors, and vitamin D supplement use were collected via participant self-report. Participants were asked to refrain from taking any additional vitamin D supplementation during the course of the study. A total of 28 participants were randomized to receive a one-time oral bolus dose of 250,000 IU of vitamin D_3 (Biotech Pharmacal, Fayetteville, AR) or matching placebo (Biotech Pharmacal, Fayetteville, AR). This study was approved by the Emory University Institutional Review Board, and is registered at clinicaltrials.gov (NCT01924910). All participants provided written informed consent upon enrollment. The current study uses samples drawn from participants at baseline (n=14 in the vitamin D group; n=14 in the placebo group) and approximately 1 week (5-10 days) later (n=13 in the vitamin D group; n=11 in the placebo group).

Analytical procedures

Plasma 25(OH)D concentrations were determined using an automated chemiluminescent technique (IDS-iSYS automated machine, Immunodiagnostic Systems, Inc., Fountain Hills, AZ), as previously described.[169] Plasma pro-inflammatory cytokines, interleukin (IL)-1β, IL-6, and IL-8, were measured using a high-sensitivity magnetic bead-based Luminex Performance Assay multiplex kit (R&D Systems, Minneapolis, MN) with a Bioplex analyzer (Bio-Rad, Hercules, CA). Plasma monocyte chemoattractant protein-1 (MCP-1) concentrations were assayed using a bead-based

Luminex Performance assay kit (R&D Systems, Minneapolis, MN) on a Bioplex analyzer (Bio-Rad, Hercules, CA). Plasma ferritin concentrations were determined via ELISA (ab108698 – Ferritin Human ELISA kit, Abcam Inc., Cambridge, MA) following manufacturer instructions; the intra-assay CV was 2.48%. A ferritin cut-off value of < 12 ng/mL was used to define low iron stores.[71]

Plasma hepcidin concentrations were determined using an electrochemiluminescence immunoassay as previously described.[96,170,171] Briefly, streptavidin-coated and blocked 96-well plates were incubated with 25 μL of biotin-labeled capture antibody (4 μg/mL) for 1 hour. Plasma samples were diluted 1:50 in assay buffer, added to their respective washed wells, and incubated at room temperature for 1 hour. Captured plasma hepcidin was detected with 25 μL of 0.1 μg/mL ruthenium-labeled conjugate hepcidin-specific detection antibody, and hepcidin concentrations were interpolated against a standard curve of reference standard hepcidin (Eli Lilly and Company, Indianapolis, IN, USA).[170,171]

Statistical Analyses

Descriptive statistics were performed for all variables and reported as mean ± SD or geometric mean (95% confidence interval (CI)) for continuous variables, and number (%) for categorical variables. Variables which were not normally distributed were transformed to the natural logarithmic scale; in the case of variables with values of zero (IL-1β and IL-6), a constant of 0.01 was added to all non-missing values prior to log-transformation. Baseline comparisons between the vitamin D and placebo groups were examined using two sample *t*-tests for continuous variables, and χ^2 or Fisher's exact test

for categorical variables. Between- and within-group differences in plasma 25(OH)D, cytokine, ferritin, and hepcidin concentrations from baseline to 1 week were evaluated using two sample independent *t*-tests, and paired *t*-tests, respectively. For variables requiring log-transformation, the results were back-transformed so as to be expressed in the original unit of measurement, as geometric means and their corresponding 95% CI. The mean differences between groups and between time points of the log-transformed data were exponentiated (back-transformed) to generate geometric mean ratios (GMR). A GMR of 1 indicates no treatment effect. All analyses were performed in SAS version 9.4 (SAS Institute Inc, Cary, NC) using a two-sided *P*-value of 0.05 to define statistical significance.

Results

Participant characteristics

Baseline demographic and biochemical characteristics for this study population are shown in **Table 5.1**. This was a young and predominantly female cohort. The mean body mass index (BMI) was within the normal range for both groups. Most participants were Caucasian and reported spending less than 10 hours outdoors per week. Very few participants (n=5 total) reported regular intake of vitamin D supplements prior to the start of the study. Baseline demographic and health status characteristics did not differ significantly between vitamin D and placebo groups. As previously reported,[169] geometric mean baseline plasma 25(OH)D concentrations for the vitamin D and placebo groups were in the vitamin D deficient range with greater than 70% of participants in either group having a baseline 25(OH)D concentration < 20 ng/mL. Plasma 25(OH)D

concentrations increased 150% relative to baseline after 1 week in the group that received high-dose vitamin D_3, compared to no significant change in the placebo group [GMR = 2.5 (95% CI: 2.0-3.0), $P < 0.001$ for the vitamin D group vs. GMR = 1.1 (95% CI: 0.9-1.1), $P = 0.38$ for the placebo group]. Baseline geometric mean ferritin concentrations in both groups were greater than the cut-off value of 12 ng/mL used to define low iron stores. Plasma pro-inflammatory cytokine, hepcidin, and ferritin concentrations did not differ significantly between vitamin D and placebo groups at baseline.

Effect of high-dose vitamin D on pro-inflammatory cytokine concentrations

Geometric means of plasma IL-6, IL-1β, IL-8, and MCP-1 concentrations with their corresponding 95% confidence intervals at baseline and approximately 1 week later are shown in **Figure 5.1**. For any of the plasma cytokines, geometric means did not differ significantly between vitamin D and placebo groups 1 week after dosing ($P = 0.23$-0.87). Likewise, there were no significant differences in plasma cytokines within groups from baseline to 1 week ($P = 0.14$-0.99).

Effect of high-dose vitamin D on plasma hepcidin concentrations

Plasma hepcidin concentrations are shown in **Figure 5.2**. There were no significant differences between groups at baseline [GMR = 0.72 (95% CI: 0.29, 1.80), $P = 0.47$]. By the 1 week time point plasma hepcidin concentrations were 73% lower in the vitamin D group relative to the placebo group [GMR = 0.27 (95% CI: 0.08-0.96), $P = 0.04$]. The within-group difference was also statistically significant in the vitamin D group such that hepcidin decreased by 73% from baseline to 1 week [GMR = 0.27 (95%

CI: 0.11-0.62); $P = 0.005$]. There was no significant change in hepcidin from baseline to 1 week in the placebo group [GMR = 0.73 (95% CI: 0.49-1.09), $P = 0.11$].

Effect of high-dose vitamin D on plasma ferritin concentrations

Plasma ferritin concentrations are shown in **Figure 5.3**. Baseline and 1 week ferritin concentrations did not differ significantly between the vitamin D and placebo groups ($P = 0.35$ and $P = 0.44$, respectively). Neither group had significant changes in plasma ferritin concentrations from baseline to 1 week ($P = 0.95$; $P = 0.55$, for vitamin D and placebo groups, respectively).

Discussion

In this cohort of healthy adults with a high prevalence of vitamin D deficiency, treatment with a single large dose of vitamin D_3 significantly reduced plasma hepcidin concentrations after 1 week, but did not have a statistically significant effect on plasma concentrations of pro-inflammatory cytokines or ferritin. To our knowledge, this study is the first to evaluate the effect of high-dose vitamin D supplementation on markers involved in the pathophysiology of anemia of inflammation in healthy adults in the context of a randomized controlled trial. Mechanisms by which vitamin D has been proposed to influence iron recycling and anemia are summarized in **Figure 5.4**. Our findings suggest that even in the absence of inflammatory conditions, vitamin D may act directly on hepcidin, independent of inflammatory cytokines.

While few studies have explored the association of vitamin D with hepcidin, our findings are consistent with the data of Bacchetta et al.[95] in which seven healthy

volunteers given 100,000 IU vitamin D_2 had a 33% reduction in hepcidin concentrations 72 hours post-dosing. Similarly, in patients with CKD, treatment with vitamin D has been associated with reductions in hepcidin mRNA expression in peritoneal macrophages[94] and circulating hepcidin concentrations.[96] Results from observational studies have been mixed. In a study of pregnant adolescents, circulating 25(OH)D concentrations were not associated with hepcidin concentrations,[172] while a study in CKD patients showed that lower levels of $1,25(OH)_2D$ were associated with increased hepcidin concentrations.[173] Taken together, our data and the previous reports provide evidence for hepcidin-lowering by vitamin D as a potential mechanism by which vitamin D may influence iron recycling. The recent discovery of a vitamin D response element on the HAMP gene also lends strong biological plausibility to our results.[95] Further long-term studies are needed to establish the clinical implications of inflammation-independent changes in circulating hepcidin concentrations.

Concentrations of pro-inflammatory cytokines did not change significantly in response to high-dose vitamin D supplementation in this healthy cohort. This is in contrast to epidemiologic studies, which have reported increased odds of anemia of inflammation with lower vitamin D status.[9,116] Vitamin D has also been shown to have anti-inflammatory effects in various *in vitro* studies, observational studies, and clinical trials.[28,96] However, these studies have largely been conducted in populations with chronic conditions or infections, where elevations in pro-inflammatory cytokines are more likely to be observed. In utilizing healthy volunteers for this study, we were able to demonstrate that vitamin D may affect hepcidin concentrations even without altering inflammatory cytokine concentrations.

Despite the significant reduction in hepcidin, circulating ferritin concentrations were unchanged in response to high-dose vitamin D_3 supplementation. However, given the physiological response to iron recycling in the presence of elevated hepcidin concentrations, this is not entirely unexpected. Any effects of hepcidin reduction on iron egress from cells and iron bioavailability may have been better captured as transport iron, using markers such as serum iron, total iron binding capacity, and transferrin saturation as opposed to ferritin, the storage form of iron.[45,51] In a previous observational study, we found that 25(OH)D status was significantly positively associated with hemoglobin and serum iron concentrations in a cohort of generally healthy adults, while serum ferritin concentrations did not differ between participants who were vitamin D deficient and those who were not.[116] Similarly, Thomas et al.[172] found that 25(OH)D status was significantly positively associated with hemoglobin concentrations and serum iron among pregnant adolescents, but did not observe a significant association between vitamin D status and ferritin concentrations.

Our findings may have potential implications for vitamin D as a therapy in combatting anemia. As hepcidin concentrations have been inversely associated with hemoglobin concentrations and positively associated with risk for anemia,[123] reducing hepcidin levels in the blood may be a target for anemia therapies. However, very few trials have directly evaluated the effect of vitamin D treatment on hemoglobin concentrations or anemia. Among those that have, the results have been mixed, likely due to the differences in dosage and form of vitamin D administered, and the population studied.[121,122,174] In studies of patients with CKD, vitamin D or its analogues have been shown to increase hemoglobin concentrations.[118-121] Larger studies examining the effect

of vitamin D administration on circulating hepcidin concentrations, and the subsequent impact on hemoglobin concentrations or anemia in both healthy and diseased populations at risk for anemia are necessary.

A major strength of this study was the double-blind, randomized, placebo-controlled clinical trial design. Also, the healthy, young study population allowed us to ascertain the effects of vitamin D on iron recycling in the absence of potentially confounding conditions. However, we were limited in that we were unable to measure hemoglobin concentrations in our study participants to determine the influence of vitamin D treatment on hemoglobin and anemia status. Given the high prevalence of vitamin D deficiency in this population, the generalizability of our results to other populations with a lower prevalence of vitamin D deficiency may be limited. However, as a pilot efficacy study, the high prevalence of vitamin D deficiency in this population may have been advantageous in allowing us to ascertain the impact of vitamin D therapy on our outcomes. Other limitations of this study include the lack of measures of iron status other than ferritin. With our relatively short duration of observation and the small sample size we were possibly underpowered to detect changes in inflammatory markers and ferritin, and the generalizability of our findings may be limited. However, the fact that we observed a statistically significant reduction in hepcidin concentrations in response to vitamin D supplementation in spite of the short duration and small sample size suggests a robust effect of vitamin D on hepcidin concentrations, and lends merit to our findings.

In summary, this pilot study addresses a gap in the literature related to the mechanism underlying the association between vitamin D status and anemia observed in several epidemiologic studies. Supplementation with high-dose vitamin D_3 significantly

reduced circulating hepcidin concentrations after 1 week among healthy adults without chronic or inflammatory disease and independent of circulating cytokine markers of inflammation. The down-stream effects of vitamin D on markers of iron status and anemia require further examination in larger studies of longer duration.

Statement of authorship

Contributions of authors to the manuscript: EMS, MDK, and VT designed the study; EMS, JAA, MDK, JHS, RJK, LH, SMZ, and VT collected the data and conducted the analytical procedures; EMS and VT analyzed the data; EMS, JAA, SMZ, TRZ, and VT interpreted the data; EMS wrote the draft of the manuscript; all authors critically reviewed the manuscript, contributed to revisions, and read and approved the final manuscript.

Conflict of interest

JHS and RJK are employed by Eli Lilly and Company and performed the hepcidin assay. Eli Lilly and Company played no role in the study design or decision to publish.

Funding sources

This work was supported, in part, by National Institutes of Health grants T32 DK007734 (EMS), K01 DK102851 (JAA), K24 DK096574 (TRZ), and UL1 TR000454 (Atlanta Clinical and Translational Science Institute). The content is solely the responsibility of the authors and does not necessarily represent the official views of the

National Institutes of Health. The funders had no role in the design, analysis or writing of this article.

Table 5.1

Baseline characteristics of study population by treatment group

	Vitamin D (n = 14)	Placebo (n = 14)	P-value[a]
Age (years), mean ± SD	28.2 ± 6.7	26.5 ± 5.2	0.44
Female, *n* (%)	12 (85.7)	10 (71.4)	0.65
Caucasian, *n* (%)	9 (64.3)	9 (64.3)	1.00
BMI (kg/m^2)[b], mean ± SD	23.7 ± 2.9	22.3 ± 2.2	0.17
Hours spent outdoors per week[b], mean ± SD	9.0 ± 5.2	7.0 ± 5.4	0.32
Vitamin D supplementation prior to trial[c] (yes), *n* (%)	4 (28.6)	1 (7.1)	0.33
Plasma 25-hydroxvitamin D [25(OH)D] (ng/mL)[d]	16.6 (13.6-20.2)	16.5 (13.4-20.4)	1.00
Plasma 25(OH)D < 20 ng/mL, *n* (%)	10 (71.4)	11 (78.6)	1.00
Plasma ferritin (ng/mL)[d,e]	24.5 (12.9-46.4)	36.1 (19.8-65.8)	0.35
Plasma IL-1β (pg/mL)[d]	0.70 (0.41-1.18)	1.05 (0.63-1.75)	0.24
Plasma IL-6 (pg/mL)[d]	1.93 (1.29-2.90)	1.87 (0.71-4.95)	0.95
Plasma IL-8 (pg/mL)[d]	1.96 (1.63-2.36)	2.01 (1.53-2.65)	0.88
Plasma MCP-1 (pg/mL)[d]	84.9 (72.2-99.9)	82.0 (69.1-97.5)	0.75
Plasma hepcidin (ng/mL)[d]	9.3 (4.1-20.8)	12.8 (7.8-21.1)	0.47

Abbreviations: IL-1β: interleukin-1β; IL-6: interleukin-6; IL-8: interleukin-8; MCP-1: monocyte chemoattractant protein-1

[a]Two-sample *t*-test for continuous variables, χ^2 or Fisher's exact test for categorical variables

[b]*n*=13 for vitamin D group

[c]reported dosages ranged from 400-1000 IU/day

[d]geometric mean (95% confidence interval)

[e]*n*=13 for vitamin D group, n=11 for placebo group

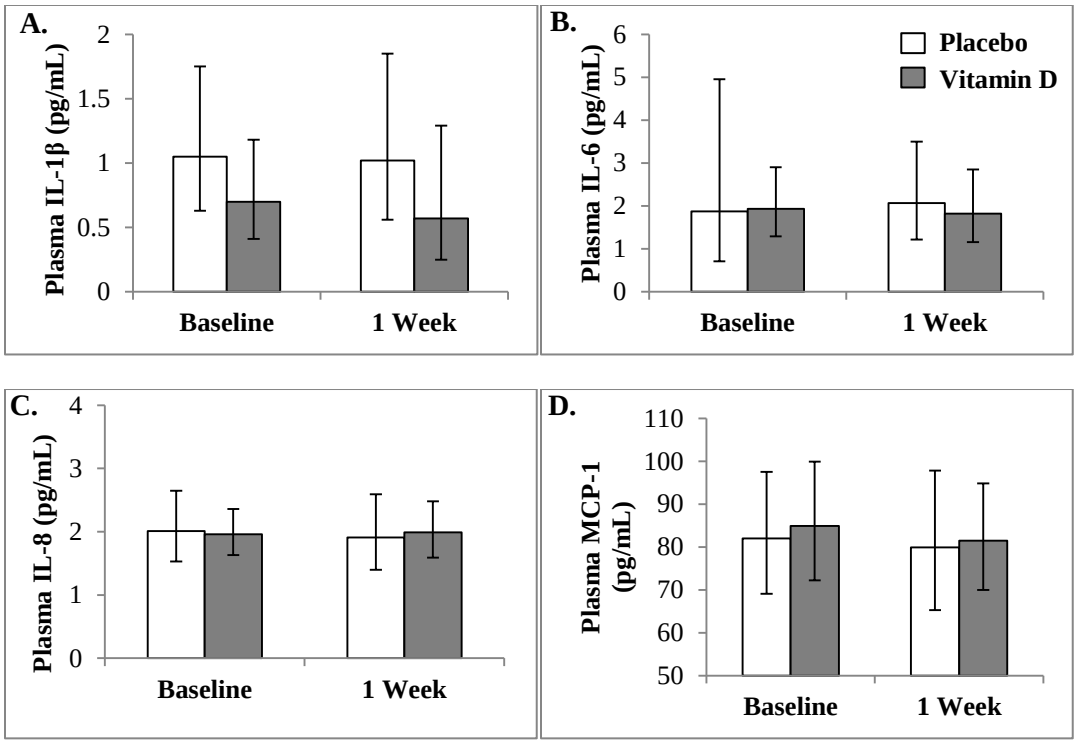


Figure 5.1. Geometric means and 95% confidence intervals for plasma IL-1β, IL-6, IL-8, and MCP-1 concentrations at baseline and 1 week in the placebo- and vitamin D-treated subjects. Concentrations of IL-1β (**A**), IL-6 (**B**), IL-8 (**C**), and MCP-1 (**D**) did not differ between vitamin D and placebo groups at baseline or 1 week, and there were no significant changes in concentrations of any of the cytokines from baseline to 1 week in either treatment group. Specific results for each cytokine are listed below. IL-1β **(**A), difference between groups at 1 week: GMR = 0.56 (95% CI: 0.21-1.50), *P* = 0.23; within group difference from baseline to 1 week: GMR = 0.86 (95% CI: 0.55-1.33), *P* = 0.46; GMR = 0.92 (95% CI: 0.69-1.23), *P* = 0.53, for the vitamin D and placebo groups, respectively. IL-6 **(B),** difference between groups at 1 week: GMR = 0.88 (95% CI: 0.46-1.68), *P* = 0.69; within group difference from baseline to 1 week: GMR = 0.93 (95% CI: 0.77-1.12), *P* = 0.40; GMR = 0.80 (95% CI: 0.58-1.09), *P* = 0.14, for vitamin D and placebo groups, respectively. IL-8 **(C),** difference between groups at 1 week: GMR

= 1.04 (95% CI: 0.74-1.48), *P* = 0.80; within group difference from baseline to 1 week: GMR = 1.05 (95% CI: 0.79-1.39), *P* = 0.73; GMR = 0.94 (95% CI: 0.75-1.17), *P* = 0.52, for vitamin D and placebo, respectively. MCP-1 **(D),** difference between groups at 1 week: GMR = 1.02 (95% CI: 0.81-1.29), *P* = 0.87; within group difference from baseline to 1 week: GMR = 1.00 (95% CI: 0.89-1.12), *P* = 0.99; GMR = 0.96 (95% CI: 0.89-1.05), *P* = 0.34, for vitamin D and placebo groups, respectively.

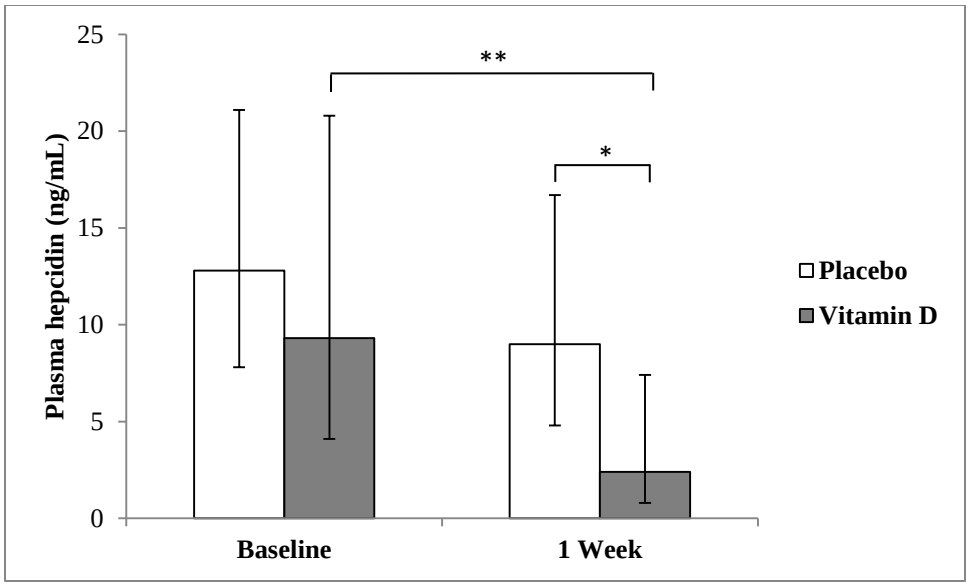


Figure 5.2. Geometric means and 95% confidence intervals of plasma hepcidin concentrations at baseline and 1 week in the placebo- and vitamin D-treated subjects. Plasma hepcidin concentrations did not differ between groups at baseline [GMR = 0.72 (95% CI: 0.29, 1.80), *P* = 0.47). Plasma concentrations of hepcidin in the vitamin D and placebo groups differed significantly at 1 week [2.4 ng/mL (95% CI: 0.8-7.4) for the vitamin D group and 9.0 ng/mL (95% CI: 4.8-16.7) for placebo; GMR =0.27 (95% CI: 0.08-0.96), *P* = 0.04]. Plasma hepcidin concentrations in the vitamin D group decreased significantly from baseline values after 1 week [GMR = 0.27 (95% CI: 0.11-0.62), *P* = 0.005]; there was no significant change from baseline in the placebo group [GMR = 0.73 (95% CI: 0.49-1.09), *P* = 0.11]. ***P* <0.01, **P* < 0.05.

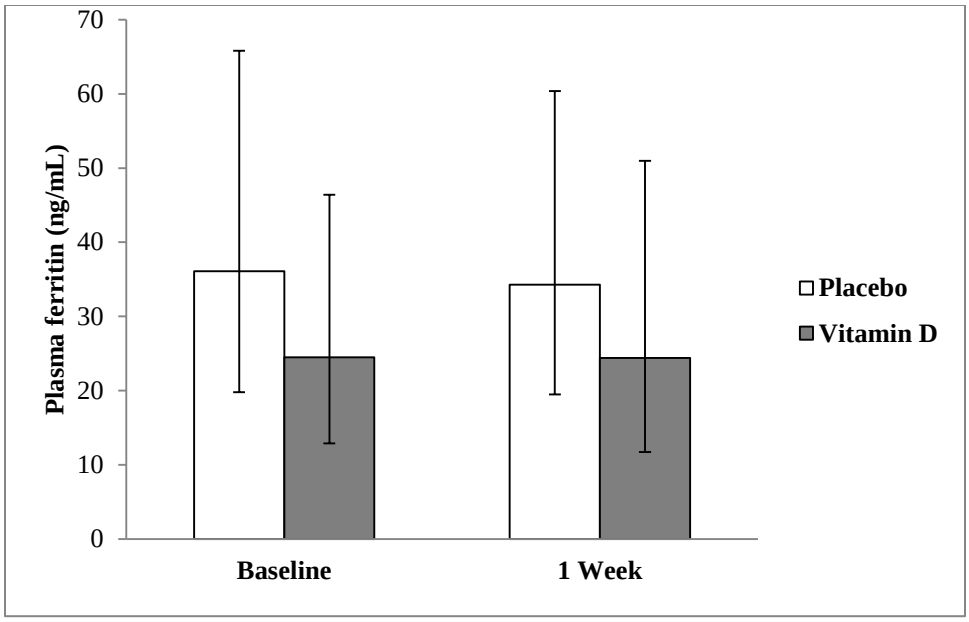


Figure 5.3. Geometric mean plasma ferritin concentrations with their 95% confidence intervals at baseline and 1 week in the placebo- and vitamin D-treated subjects. Vitamin D and placebo groups did not differ in plasma ferritin concentrations at baseline [GMR = 0.68 (95% CI: 0.29-1.57), *P* = 0.35] or 1 week post-dosing [GMR = 0.71 (95% CI: 0.29-1.75), *P* = 0.44]. Neither group had significant changes in plasma ferritin concentrations from baseline to 1 week [GMR = 0.99 (95% CI: 0.83-1.19), *P* = 0.95; GMR = 0.95 (95% CI: 0.80-1.14), *P* = 0.55, for vitamin D and placebo groups, respectively].

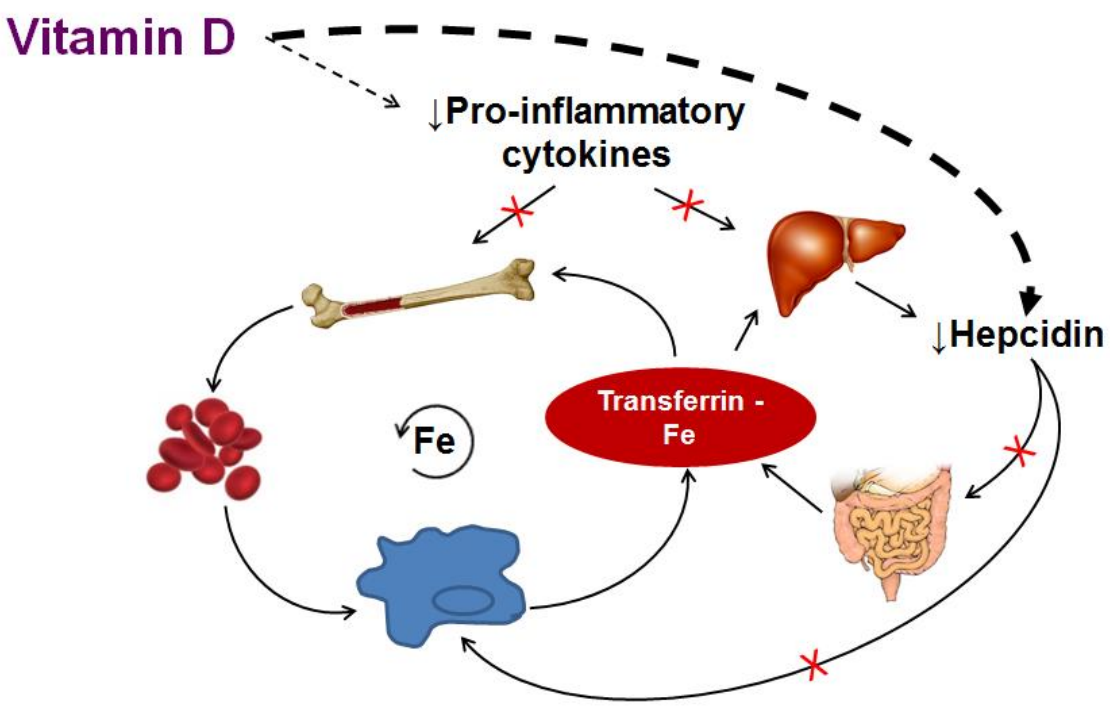


Figure 5.4. Proposed Role of Vitamin D in Enhancing Iron Recycling. Iron recycling, under non-pathologic conditions, involves transferrin-bound iron in circulation traveling to the bone marrow to support erythropoiesis. Upon senescence, red blood cells (RBCs) are engulfed by macrophages and iron is recycled back into circulation to support further erythropoiesis. Dietary iron may also enter the circulating pool from absorption in the duodenum based on the body's needs. Elevations in pro-inflammatory cytokines suppress erythropoiesis in the bone marrow and shorten RBC lifespan due to increased macrophage activation and erythrophagocytosis. Pro-inflammatory cytokines IL-6 and IL-1β also stimulate the liver to up-regulate the expression of hepcidin antimicrobial peptide (HAMP). Hepcidin inhibits iron egress from cells of the reticuloendothelial system, including enterocytes and macrophages, by binding to and inducing the degradation of the cellular iron exporter, ferroportin, resulting in decreased iron absorption from the small intestine, and increased iron sequestration within the

macrophage. Vitamin D has been shown to promote erythropoiesis and iron recycling by increasing erythroid progenitor proliferation, decreasing pro-inflammatory cytokines, and suppressing hepcidin expression. The results from the current study demonstrate that treatment with vitamin D may directly reduce circulating hepcidin concentrations independent of changes in inflammatory cytokines. Decreases in pro-inflammatory cytokines and hepcidin may increase iron bioavailability for erythropoiesis and hemoglobin synthesis by preventing iron sequestration in macrophages, and removing impairments on iron absorption, thus restoring iron recycling.

Figure adapted with permission from Smith EM and Tangpricha V. Vitamin D and anemia: insights into an emerging association. Curr Opin Endocrinol Diabetes Obes 2015, 22:432–38.

CHAPTER 6

HIGH-DOSE VITAMIN D_3 ADMINISTRATION IS ASSOCIATED WITH INCREASES IN HEMOGLOBIN CONCENTRATIONS IN MECHANICALLY VENTILATED CRITICALLY ILL ADULTS: A PILOT DOUBLE-BLIND RANDOMIZED PLACEBO-CONTROLLED TRIAL

Ellen M. Smith, BS[1]; Jennifer L. Jones, PhD, RD[2]; Jenny E. Han, MD, MSc[3]; Jessica A. Alvarez, PhD, RD[1,2]; John H. Sloan, PhD[4]; Robert J. Konrad, MD[4]; Susu M. Zughaier, PhD[5]; Greg S. Martin, MD, MSc[3]; Thomas R. Ziegler, MD[1,2]; Vin Tangpricha, MD, PhD[1,2,6.]

[1]Nutrition and Health Sciences Program, Laney Graduate School, Emory University, Atlanta, GA, USA;

[2]Division of Endocrinology, Metabolism and Lipids, Emory University School of Medicine, Atlanta, GA, USA;

[3]Division of Pulmonary, Allergy, Critical Care and Sleep Medicine, Emory University School of Medicine, Atlanta, GA, USA

[4]Lilly Research Laboratories, Eli Lilly and Company, Indianapolis, IN, USA;

[5]Department of Microbiology and Immunology, Emory University School of Medicine, Atlanta, GA, USA;

[6]Atlanta VA Medical Center, Decatur, GA, USA

Journal of Parenteral and Enteral Nutrition 2016; in press
DOI: 10.1177/0148607116678197

Abstract

Background: Anemia and vitamin D deficiency are highly prevalent in critical illness, and vitamin D status has been associated with hemoglobin concentrations in epidemiologic studies. We examined the effect of high-dose vitamin D therapy on hemoglobin and hepcidin concentrations in critically ill adults.

Materials and Methods: Mechanically ventilated critically ill adults (N=30) enrolled in a pilot double-blind, randomized, placebo-controlled trial of high-dose vitamin D_3 (D_3) were included in this analysis. Participants were randomized to receive placebo, 50,000 IU D_3, or 100,000 IU D_3 daily for 5 days (totaling 250,000 IU D_3, and 500,000 IU D_3, respectively). Blood was drawn weekly throughout hospitalization for up to 4 weeks. Linear mixed-effects models were used to assess change in hemoglobin and hepcidin concentrations by treatment group over time.

Results: At enrollment, >75% of participants in all groups had plasma 25-hydroxyvitamin D [25(OH)D] concentrations <30 ng/mL and >85% of participants across groups were anemic. In the 500,000 IU D_3 group, hemoglobin concentrations increased significantly over time ($P_{group*time}$=0.01) compared to placebo, but did not change in the 250,000 IU D_3 group ($P_{group*time}$=0.59). Hepcidin concentrations decreased acutely in the 500,000 IU D_3 group relative to placebo after 1 week (P=0.007). Hepcidin did not change significantly in the 250,000 IU D_3 group.

Conclusion: In these critically ill adults, treatment with 500,000 IU vitamin D_3 was associated with increased hemoglobin concentrations over time and acutely reduced serum hepcidin concentrations. These findings suggest that high-dose vitamin D_3 may improve iron metabolism in critical illness, and should be confirmed in larger studies.

Keywords: vitamin D, hemoglobin, hepcidin, anemia, critical illness

Clinical Relevancy Statement

Anemia is highly prevalent in critical illness and associated with adverse patient outcomes. Vitamin D deficiency, also common in critical illness, has been identified as a risk factor for anemia. Our finding that hemoglobin concentrations increased following treatment with high-dose vitamin D is clinically relevant for clinicians and researchers contemplating therapeutic options for anemia in critically ill adults. Pending confirmation in larger studies, vitamin D repletion may have implications as a safe, alternative or adjunct therapy to traditional therapies for anemia in critical illness, such as transfusions which may carry risks for the patient.

Introduction

Anemia is highly prevalent in critical illness. Nearly two thirds of adults have anemia on admission to the intensive care unit (ICU) and an even greater proportion develops anemia within the first week of admission.[175,176] The presence of anemia is associated with increased risk of mortality, cardiovascular morbidity, and decreased oxygen-carrying capacity, potentially prolonging the requirement for mechanical ventilation.[86] This may be particularly concerning in critically ill patients with pre-existing cardiopulmonary disease. Traditional therapies for anemia in critical illness include blood transfusions, erythropoiesis stimulating agents, and iron repletion, however, these are not without risks and there is controversy regarding their efficacy in improving patient survival.[91,177-179] In light of the high burden of anemia and associated adverse outcomes, investigation into safe and efficacious alternative or complementary therapies to improve hemoglobin concentrations in critically ill adults is warranted.

Anemia in critical illness may develop due to repeated blood sampling, hemorrhage, renal disease, inflammation, and nutrient deficiencies including iron, folate, and vitamin B_{12}.[86] Recently, vitamin D deficiency has also been identified as a risk factor for anemia, particularly anemia of inflammation.[9,116] Where iron deficiency anemia occurs due to absolute iron deficiency - depletion of iron stores, most often measured via ferritin concentrations in the blood, and reduced circulating iron - anemia of inflammation is characterized by reduced circulating iron in the context of normal or elevated ferritin concentrations due to sequestration of iron within cells of the reticuloendothelial system.[90] In critical illness, inflammation may contribute to anemia due to elevations in pro-inflammatory cytokines and hepcidin, the major iron-regulatory hormone.[180] Increases in cytokines and hepcidin result in shortened red blood cell

lifespan, decreased iron absorption, and iron sequestration within macrophages, limiting the amount of iron in circulation to support erythropoiesis and hemoglobin synthesis.[66] Vitamin D has been shown to reduce pro-inflammatory cytokines and suppress hepcidin transcription, thereby potentially improving iron egress from cells and increasing the amount of iron in circulation to support erythropoiesis. Furthermore, the hepcidin antimicrobial peptide gene (*HAMP*) has been found to contain a vitamin D response element, thus lending biological plausibility to the observed association between vitamin D deficiency and anemia.[95,96,181]

We have previously reported a high prevalence of vitamin D insufficiency and anemia in the ICUs at our centers,[182,183] and the VITdAL-ICU study found that serum hemoglobin concentrations were higher in the vitamin D-treated group than the placebo group 28 days after intervention.[184] However, few studies, particularly in the ICU population, have explored the therapeutic effect of vitamin D with hemoglobin as the primary outcome of interest. Therefore, we aimed to 1) examine the impact of high-dose vitamin D_3 (D_3**)** supplementation on hemoglobin concentrations in critically ill adults and 2) evaluate the effect of vitamin D on serum hepcidin concentrations to better understand the role of vitamin D in iron metabolism in this population. We hypothesized that treatment with high-dose vitamin D_3 would increase hemoglobin concentrations and reduce hepcidin concentrations in this population of critically ill adults.

Methods

Study design and participants

The study population for this analysis was derived from participants enrolled in a pilot (N=30) double-blind, randomized, placebo-controlled trial of high-dose vitamin D_3 regimens in mechanically ventilated critically ill adults (NCT01372995).[185] The parent study was designed to test the efficacy of high-dose vitamin D_3 regimens in improving plasma 25-hydroxyvitamin D [25(OH)D] concentrations to levels ≥ 30 ng/mL. Briefly, upon enrollment participants were stratified on Acute Physiology and Chronic Health Evaluation II (APACHE II) score (≤ 15 or > 15) and randomized to receive placebo, a total enteral dose of 250,000 IU (6,250 μg) of cholecalciferol (D_3), or a total enteral dose of 500,000 IU (12,500 μg) D_3. Participants were not included/excluded from the study on the basis of their 25(OH)D status at enrollment. The study drug was administered in 5 equal doses over 5 days (i.e. the 500,000 IU group received a dose of 100,000 IU daily for 5 days after enrollment). Pills were dissolved in sterile water and administered through an enteral feeding tube. The cholecalciferol was manufactured from Tischon Corp. (Westbury, NY) and BioTech Pharmacal (Fayetteville, AR). Inclusion criteria were age ≥ 18 years, receiving care in ICUs at Emory University Hospital, Emory University Hospital Midtown, or Grady Memorial Hospital, anticipated mechanical ventilation of ≥ 72 hours after study enrollment, anticipated survival and ICU stay of ≥ 96 hours, and enteral access and ability to tolerate enteral study drug administration. Potential participants were excluded due to pregnancy, shock, hypercalcemia, receipt of high-dose vitamin D therapy in the preceding 6 months, chronic renal dysfunction requiring dialysis, AIDS, cirrhosis, and receipt of any investigational drug in the 60 days prior to study entry. This study was approved by the Emory University Institutional

Review Board and written informed consent was obtained from the patient or legally authorized representative prior to enrollment.

Data collection

Data on demographics, medical history, and admitting diagnosis were collected on study entry.[185] Blood was drawn at study enrollment, and weekly throughout the hospitalization for up to 4 weeks for assessment of plasma 25(OH)D and serum hepcidin concentrations. Plasma 25(OH)D concentrations were measured using an automated chemiluminescent technique (IDS-iSYS automated machine, Immunodiagnostic Systems, Inc., Fountain Hills, AZ) in a laboratory which participates in the Vitamin D External Quality Assessment Scheme (DEQAS, site #606) and the National Institute of Standards and Technology/NIH Vitamin D Metabolites Quality Assurance Program to ensure the accuracy of 25(OH)D measurements. Hepcidin concentrations were measured using an electrochemiluminescence immunoassay (Eli Lilly and Company, Indianapolis, IN, USA) as previously described.[96,170,171,181] Hemoglobin measurements within 24 hours of the 25(OH)D measurement were abstracted from the electronic medical record, starting when the patient was enrolled in the study. Hemoglobin concentrations were determined via automated cell counting using standard hospital methods. Anemia was defined based on World Health Organization criteria as hemoglobin concentrations < 13 g/dL in men and < 12 g/dL in non-pregnant women.[47] Data on receipt and volume of red blood cell transfusion were extracted from participant electronic medical records. The transfusion trigger used at our sites is typically a hemoglobin concentration of 7 g/dL.

Statistical analysis

Descriptive statistics were performed for all variables and presented as mean ± standard deviation (SD) for normally distributed continuous variables, median (interquartile range[IQR]) for non-normally distributed continuous variables, or percentages for categorical variables. Comparisons of characteristics between treatment groups at enrollment were performed using one-way ANOVA for normally distributed continuous variables, Kruskal-Wallis tests for non-normally distributed continuous variables, and Fisher's exact tests for categorical variables. Non-normally distributed variables (hemoglobin and hepcidin) were transformed to the natural logarithmic scale for subsequent analyses. Such variables were subsequently back-transformed so as to be expressed in their original unit of measurement as geometric means (95% confidence interval [CI]).

Analysis of repeated measures was performed using linear mixed-effects models to ascertain mean differences in hemoglobin and hepcidin concentrations by time, treatment group, and time*treatment group interaction. Any differences between groups in outcomes over time are reflected in the time*treatment group term. Beta-coefficients from linear mixed-effects models with log-transformed outcome variables (hemoglobin and hepcidin) were back-transformed and expressed as geometric mean ratios (GMR) with their corresponding 95% CI. A GMR of 1 indicates no treatment effect. A one-way ANOVA with Tukey's multiple testing correction was used to determine differences in treatment groups at specific time points, and paired t-tests were used to examine within group changes in outcomes.

Sensitivity analyses were performed in which potentially confounding variables (age, sex, race, hemoglobin concentration at enrollment, hepcidin concentration at

enrollment, transfusion volume, and admission ICU) were added to the model one at a time to determine the influence of each of these variables on the hemoglobin and hepcidin outcomes. Additional sensitivity analyses were performed with the linear mixed-effects models in which models were restricted to those with 25(OH)D concentrations < 30 ng/mL at enrollment to determine if enrollment 25(OH)D status affected our outcomes. All analyses were two-sided with an alpha of 0.05, and performed using SAS v. 9.4 (SAS Institute Inc., Cary, NC).

Results

Demographic, biochemical, and health status characteristics at study enrollment were similar across treatment groups (**Table 6.1**). The median time from ICU admission to study enrollment was 4 days (IQR: 5), and did not differ between groups (*P*=0.53). The study population was largely male, overweight or obese, and approximately half were African American. The vast majority of participants had 25(OH)D concentrations < 30 ng/mL at study enrollment. Nearly all of the participants were anemic at study enrollment, and the mean APACHE II score indicated a high degree of illness severity among the study population. Several participants had underlying comorbidities at enrollment including diabetes and heart disease. In the 500,000 IU D_3 group, there was a higher proportion of a prior diagnosis of coronary artery disease compared to the other two groups. The groups did not differ in the proportion of participants who received blood transfusions, or in the total volume of blood received throughout the course of the study.

As previously reported,[185] plasma 25(OH)D concentrations increased significantly after 1 week in the groups that received 250,000 IU D_3 and 500,000 IU D_3, (to 45 ± 20

ng/mL and 55 ± 14 ng/mL, respectively), compared to no change in the placebo group (P<0.001). These effects were sustained through week 4.

Hemoglobin concentrations increased significantly over time in the group that received 500,000 IU D_3 (**Figure 6.1**). Compared to the placebo group, those who received 500,000 IU D_3 demonstrated a significant 8% increase in hemoglobin concentrations per week [GMR: 1.08 (95% CI: 1.02, 1.15), $P_{group*time}$=0.01]. Hemoglobin concentrations in the 250,000 IU D_3 group did not change significantly over time relative to placebo [GMR: 0.99 (95% CI: 0.94, 1.04), P=0.59]. By week 3, hemoglobin concentrations were significantly higher in the 500,000 IU D_3 group compared to placebo [11.30 g/dL (95% CI: 9.34, 13.68) vs. 8.19 g/dL (95% CI: 7.26, 9.24), P=0.03]. The prevalence of anemia remained high throughout the course of the study and did not differ significantly between groups at any time point (P=1.00 at week 1, and P=0.72 at week 2; all remaining participants were anemic at weeks 3 and 4).

Hepcidin concentrations were only obtained for up to 3 weeks (**Figure 6.2**). During that period, there was not a statistically significant group-by-time interaction with hepcidin concentrations in either vitamin D-treated group relative to the placebo group [$P_{group*time}$ (500,000 IU D_3 vs placebo) = 0.44; $P_{group*time}$ (250,000 IU D_3 vs placebo) = 0.60]. However, by 1 week, hepcidin concentrations did differ significantly between the 500,000 IU D_3 and placebo groups (P=0.007). The 250,000 IU D_3 group did not differ from either the 500,000 IU D_3 group (P=0.22) or the placebo group (P=0.38) at 1 week. Within group analyses showed that hepcidin concentrations decreased acutely by 66% after 1 week in the 500,000 IU D_3 group, relative to study enrollment [GMR: 0.34 (95%

CI: 0.20, 0.59), P=0.002]. No significant changes were observed within the 250,000 IU D_3 or placebo groups from enrollment to1 week (P=0.08 and P=0.48, respectively).

Sensitivity analyses

Controlling separately for potentially confounding variables age, sex, race, hemoglobin concentration at enrollment, hepcidin concentration at enrollment, transfusion volume, and admission ICU did not affect the results of the linear mixed-effects models. The group-by-time interaction for hemoglobin remained significant in the 500,000 IU D_3 group (P<0.03 for all) and non-significant in the 250,000 IU D_3 (P>0.45 for all). The group-by-time interaction for hepcidin remained non-significant for both the 500,000 IU D_3 (P>0.35 for all) and 250,000 IU D_3 (P>0.45 for all) relative to placebo with the addition of each control variable.

When linear mixed-effects models were restricted to those with 25(OH)D < 30 ng/mL at the time of study enrollment, hemoglobin and hepcidin results were largely unchanged. Compared to the placebo group, those who received 500,000 IU D_3 demonstrated a significant 10% increase in hemoglobin concentrations per week [GMR: 1.10 (95% CI: 1.04, 1.18), $P_{group*time}$=0.003]. Hemoglobin concentrations in the 250,000 IU D_3 group remained unchanged relative to placebo [GMR: 0.99 (95% CI: 0.93, 1.05), P=0.67]. There was not a statistically significant group-by-time interaction with hepcidin concentrations in either vitamin D-treated group relative to the placebo group [$P_{group*time}$ (500,000 IU D_3 vs placebo) = 0.34; $P_{group*time}$ (250,000 IU D_3 vs placebo) = 0.47].

Discussion

In this population of mechanically ventilated critically ill adults, we found that treatment with a total enteral dose of 500,000 IU D_3 was associated with a significant increase in hemoglobin concentrations over time. Although not sustained, there was an acute decrease in hepcidin concentrations by 1 week following treatment with 500,000 IU D_3 relative to the placebo group. No significant changes in hemoglobin or hepcidin concentrations were observed in the 250,000 IU D_3 group compared to the placebo group, suggesting a potential dose-related effect of vitamin D_3 on these outcomes.

Our findings are consistent with studies of patients with chronic kidney disease (CKD) in which treatment with vitamin D or its analogues increased hemoglobin concentrations.[120,121] However, studies in other patient populations have been mixed, but this is likely due to differences in the dose and form of vitamin D administered, as well as the prevalence of vitamin D deficiency and type of anemia in these studies.[122,174,186] Indeed, Sooragonda et al[122] found that among generally healthy adults with iron deficiency anemia, treatment with 600,000 IU of cholecalciferol did not result in further increases in hemoglobin concentrations following correction of iron deficiency. This would suggest that the effects of vitamin D are likely specific to anemia of inflammation, which is consistent with the proposed mechanism of action outlined below.

The acute reduction in hepcidin that we observed is consistent with another study from our group in which healthy volunteers who received high-dose vitamin D_3, experienced a significant reduction in hepcidin concentrations after one week, compared to the placebo group.[181] Given the physiology of iron recycling, the acute reduction in hepcidin observed in the 500,000 IU D_3 group likely potentiated the increase in hemoglobin, even though the change in hepcidin was not sustained over time.[45] During

anemia of inflammation, which is a component of anemia in critical illness,[180] hepcidin is elevated, blocking iron egress from cells, and sequestering iron within cells of the reticuloendothelial system.[66] This process limits the iron in circulation that can be used to support erythropoiesis and hemoglobin synthesis. Reductions in hepcidin may restore iron recycling by allowing iron to exit cells and be utilized in hemoglobin synthesis and erythropoiesis. Thus increases in hemoglobin concentrations are likely to be preceded by reductions in hepcidin in the context of anemia of inflammation. Our findings of an acute reduction in hepcidin concentration with an increase over time in hemoglobin concentrations in the 500,000 IU D_3 group are in line with this process. Further, the gene coding for hepcidin has been demonstrated to contain a vitamin D response element,[95] lending strong biological plausibility to our findings. Other potential mechanisms underlying our findings may involve a role for vitamin D in supporting erythropoiesis through induction of erythroid progenitor cell proliferation,[100,101,145] however this was not evaluated in the present study.

Unexpectedly, we did not see significant changes in either hemoglobin or hepcidin concentrations in response to 250,000 IU D_3, despite significant increases in 25(OH)D concentrations in this group. It is possible that there may be differences in characteristics of the individuals randomized to these two groups which were not measured in this study, or differences in unknown factors related to response to high-dose vitamin D_3. Another possibility is that there is a threshold level of 25(OH)D that may need to be reached to provide adequate substrate for the local production of the active form of vitamin D, 1,25-dihydroxyvitamin D [$1,25(OH)_2D$] by the macrophage to regulate hepcidin concentrations. While the groups were generally balanced at study

enrollment, they differed in the prevalence of coronary artery disease, with the 500,000 IU D_3 group having a higher proportion with this condition. It is possible that this group was more responsive to vitamin D therapy in terms of hemoglobin and hepcidin concentrations. Indeed, in observational studies in patients scheduled for cardiac surgery and coronary angiography, higher 25(OH)D concentrations were associated with higher hemoglobin concentrations, and protective against anemia.[13,187] Another potential explanation may be that even though 25(OH)D concentrations increased with both dosing regimens, it is possible that there was a differential between free 25-hydroxyvitamin D in these groups. Recent evidence has indicated that free 25-hydroxyvitamin D may be a better marker of vitamin D bioactivity than total 25(OH)D concentrations.[21] Thus if the higher dose of vitamin D resulted in greater concentrations of free 25-hydroxyvitamin D, this may explain the observed changes in hemoglobin and hepcidin.

Our findings have potential clinical implications for vitamin D repletion as a safe and efficacious adjunct therapy for anemia in critical illness. Even though anemia was not completely resolved in our study population, hemoglobin concentrations did improve to a clinically meaningful extent in the group which received 500,000 IU D_3. The improvement in hemoglobin may reduce the frequency and necessity of blood transfusions. Transfusions are widely used in the ICU, but there is controversy regarding their use and threshold for initiation, as studies have found transfusions to be associated with increased length of hospital stay, hospital costs, and mortality.[175,176,178,189] Studies indicate that the hemoglobin threshold for initiation of transfusions typically falls between 7-9 g/dL and may vary by patient characteristics.[175,190,191] If vitamin D can improve hemoglobin concentrations to a level above which transfusions are typically

initiated, as was demonstrated in our study, this may have positive effects on patient outcomes.

Strengths of our study included the rigorous clinical trial design, novel research question, and the well-characterized study population. However, there were important limitations. First, this trial was not specifically designed and powered with hemoglobin as the primary outcome, and the sample size of this pilot trial was relatively small. Nonetheless, the significant increase in hemoglobin that was observed, despite the small sample size, suggests that this was a fairly robust finding. We were also limited in that the randomization was unbalanced with respect to coronary artery disease. With only one person in the placebo group having the disease, we were unable to adjust for this variable in our analysis. We therefore cannot rule out the effect of this potential confounder on our outcomes, and our findings related to the 500,000 IU D_3 group may lack generalizability to populations with a different prevalence of underlying comorbidities. Another potential limitation of this study was that the median time from ICU admission to study enrollment was 4 days. Therefore, the plasma 25(OH)D, serum hepcidin, and hemoglobin concentrations obtained at study enrollment may not reflect the participants' true baseline levels as concentrations of these biochemical markers may decrease after ICU admission due to hemodilution or other unknown factors. Finally, we were unable to fully evaluate the effect of other anemia treatments that may have been given as part of the patients' medical care. We did, however, find that the number of patients who received transfusions during their hospitalization did not differ by treatment group, nor did the volume of packed red cells received, suggesting that the increase in hemoglobin

in the 500,000 IU D_3 group was unlikely due to differences in receipt of transfusions in this group.

In conclusion, we found that treatment with 500,000 IU of vitamin D_3 was associated with a significant increase in hemoglobin concentrations over time and an acute reduction in hepcidin concentrations in critically ill adults. Larger clinical trials of high-dose vitamin D_3 in critically ill adults with hemoglobin as the primary outcome are warranted to confirm these findings and further elucidate the therapeutic effect of vitamin D on anemia in critical illness.

Statement of authorship: Ellen M. Smith, Greg S. Martin, Thomas R. Ziegler, and Vin Tangpricha contributed to the conception and design of the work; Ellen M. Smith, Jennifer L. Jones, Jenny E. Han, Jessica A. Alvarez, Susu M. Zughaier, John H. Sloan, and Robert J. Konrad contributed to the acquisition and analysis of the data. Ellen M. Smith drafted the manuscript, and all authors contributed to the interpretation of the data and critically revised the manuscript. All authors read and approved the final manuscript and agree to be accountable for ensuring the accuracy and integrity of all aspects of the work.

Table 6.1. Characteristics by Treatment Group at Time of Study Enrollment.

Characteristic	Placebo (n = 10)	250,000 IU D_3 (n = 9)	500,000 IU D_3 (n = 11)	*P*[a]
Age (yrs)[b]	64.8 ± 17.5	56.4 ± 15.4	68.1 ± 18.6	0.33
Men [n (%)]	6 (60.0)	5 (55.6)	8 (72.7)	0.72
Race [n (%)]				0.09
African American	4 (40.0)	7 (77.8)	3 (27.3)	
Caucasian	5 (50.0)	2 (22.2)	8 (72.7)	
American Indian/Alaskan	1 (10.0)	0 (0.0)	0 (0.0)	
BMI (kg/m^2)[b,c]	28.2 ± 9.9	33.4 ± 6.3	30.2 ± 6.1	0.36
Plasma 25(OH)D (ng/mL)[b]	21.5 ± 12.2	23.2 ± 7.8	20.0 ± 7.3	0.75
25(OH)D<20 ng/mL [n (%)]	5 (50.0)	3 (33.3)	5 (45.5)	0.81
25(OH)D<30 ng/mL [n (%)]	8 (80.0)	7 (77.8)	10 (90.9)	0.70
Hemoglobin (g/dL)[d]	8.3 (2.0)	9.4 (3.2)	9.5 (1.8)	0.22
Anemia [n (%)][e]	10 (100.0)	8 (88.9)	10 (90.9)	0.75
Hepcidin (ng/mL)[d]	82.1 (135.7)	36.5 (67.7)	15.7 (25.1)	0.09
APACHE II Score[b]	23.2 ± 8.8	20 ± 10.1	19.0 ± 7.5	0.53
Admission ICU				0.16
Medical	7 (70.0)	4 (44.4)	3 (27.3)	
Surgical	3 (30.0)	5 (55.6)	8 (72.7)	
Infection on admission [n (%)]	6 (60.0)	4 (44.4)	3 (27.3)	0.38
Coronary artery disease [n (%)]	1 (10)	2 (22.2)	7 (63.6)	0.03
Congestive heart failure [n (%)]	1 (10)	2 (22.2)	5 (45.5)	0.20
COPD [n (%)]	2 (20)	1 (11.1)	4 (36.4)	0.49
Asthma [n (%)]	1 (10)	1 (11.1)	0 (0)	0.52
Diabetes [n (%)]	4 (40.0)	1 (11.1)	2 (18.2)	0.32
Received transfusion [n(%)][f]	4 (40.0)	2 (22.2)	3 (27.3)	0.78
Transfusion volume (mL)[b,g]	775 ± 505.8	700 ± 495.0	916.7 ± 700.6	0.91

25(OH)D, 25-hydroxyvitamin D; APACHE II, Acute Physiology and Chronic Health Evaluation II; BMI, body mass index; COPD, chronic obstructive pulmonary disease; ICU, intensive care unit

[a]ANOVA for normally distributed continuous variables, Kruskal-Wallis test for non-normally distributed continuous variables, Fisher's exact test for categorical variables

[b]mean ± SD

[c]n=8 for 250,000 IU D_3 group

[d]Median (IQR)

[e]Hemoglobin <13 g/dL for men, <12 g/dL for women

[f]Receipt of any blood transfusion during the course of the study

[g]Total volume of packed red cells received during the course of the study , among those who received a transfusion

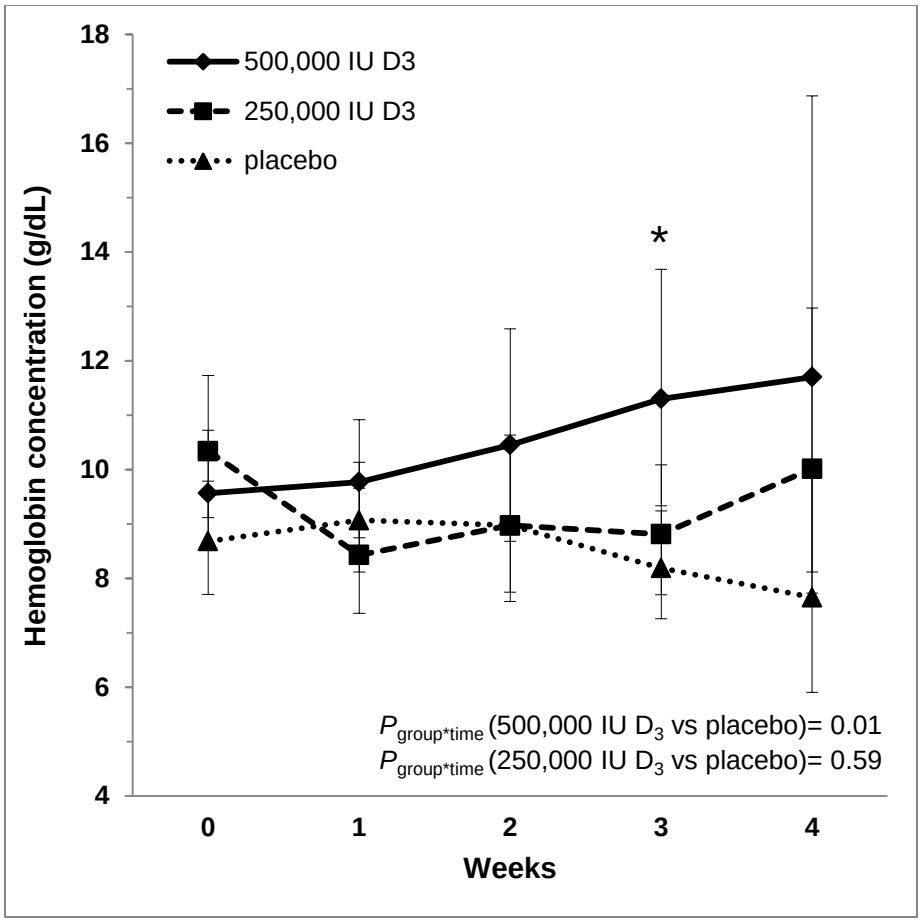


Figure 6.1. Geometric mean hemoglobin concentrations with corresponding 95% confidence intervals in critically ill adults. Hemoglobin concentrations are reported across time and by treatment group. Hemoglobin concentrations increased significantly over time in the group that received 500,000 IU D_3compared to the placebo group; there was no significant change in the 250,000 IU D_3 group. By three weeks, hemoglobin concentrations in the 500,000 IU D_3 group differed significantly from the placebo group; there were no statistically significant differences between groups at other time points. *P<0.05; group*time, group-by-time interaction. Sample sizes in the placebo, 250,000 IU D_3, and 500,000 IU D_3 groups, respectively: enrollment n = 10, 9, 11; 1 week n = 9, 9, 9; 2 weeks n = 8, 6, 5; 3 weeks n = 5, 4, 2; 4 weeks n = 2, 2, 1.

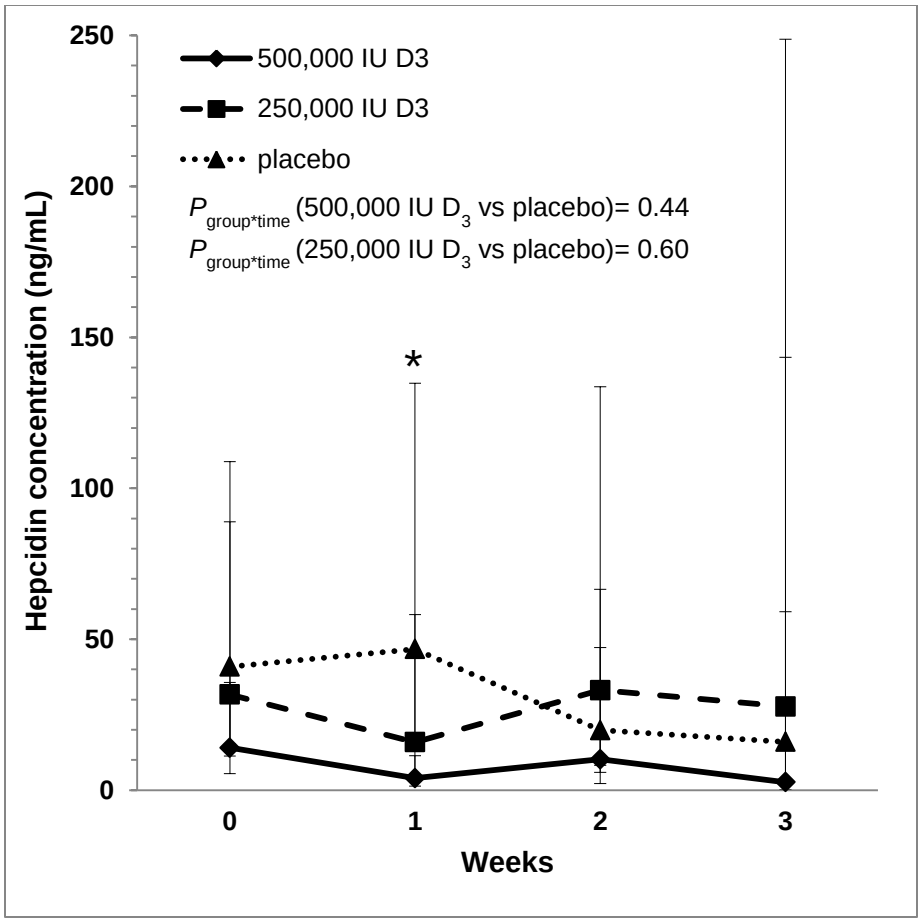


Figure 6.2. Geometric mean hepcidin concentrations with corresponding 95% confidence intervals in critically ill adults. Hepcidin concentrations are reported across time and by treatment group. There were no significant differences over time in hepcidin concentrations in either vitamin D-treated group relative to placebo. Hepcidin concentrations in the 500,000 IU D_3 group differed significantly from the placebo group one week after dosing; there were no statistically significant differences between groups at other time points. *P<0.05; group*time, group-by-time interaction. Sample sizes in the placebo, 250,000 IU D_3, and 500,000 IU D_3 groups, respectively: enrollment n = 10, 9, 11; 1 week n = 9, 6, 9; 2 weeks n = 8, 6, 5; 3 weeks n = 4, 4, 2.

CHAPTER 7

DISCUSSION AND CONCLUSIONS

Key Findings

The objectives of this dissertation were to examine and characterize the association between vitamin D status and hemoglobin/anemia, investigate the mechanism underlying this association, and evaluate the effect of vitamin D supplementation on markers of iron metabolism, in healthy and ill populations. There were several notable findings from this body of work. First, in a cohort of generally healthy adults working and living in the Atlanta area, serum 25(OH)D concentrations < 20 ng/mL were independently associated with increased odds of anemia among African Americans, but not among Caucasians (chapter 3). Further, when categorized by anemia subtype, serum 25(OH)D concentrations < 20 ng/mL were significantly associated with anemia of inflammation, but not with anemia without inflammation among African Americans; similar to anemia overall, no such associations were observed among whites.

Based on the results of chapter 3, we aimed to further characterize the link between vitamin D and anemia by exploring the association in a different population, that of generally healthy women of reproductive age in rural northern Vietnam, and determine whether dietary vitamin D intake was also associated with anemia. In chapter 4, we examined associations of both dietary vitamin D intake and serum 25(OH)D concentrations with hemoglobin and anemia. We found that dietary vitamin D intake was profoundly low in this population and largely determined by socioeconomic status. Dietary vitamin D intake, however, was not associated with hemoglobin concentrations or anemia. In this study population, dietary vitamin D intake was not associated with

serum 25(OH)D concentrations in the subset in which 25(OH)D was measured. The lack of association between dietary vitamin D intake and serum 25(OH)D concentrations indicated that dietary vitamin D intake was not a good proxy for vitamin D status and may explain why we did not observe associations between dietary vitamin D intake and hemoglobin or anemia. However, 25(OH)D concentrations < 20 ng/mL were independently associated with reduced hemoglobin concentrations, compared to those with 25(OH)D concentrations ≥ 20 ng/mL. We did not observe an association between serum 25(OH)D concentrations and anemia in this subset, though we were unable to examine the association by subtype of anemia, which based on the previous chapter, may have provided further insight into a vitamin D-anemia link in this population.

Taken together, these population-based studies suggest that there is a link between vitamin D status and anemia/hemoglobin in generally healthy adults in both the Atlanta area and in the Thai Nguyen region of Vietnam. The results from the analysis of women of reproductive age in Vietnam indicate that dietary vitamin D intake may not be a good proxy for vitamin D status in areas where vitamin D intake is very low. In addition, these population-based studies suggest that achieving serum 25(OH)D concentrations ≥ 20 ng/mL may lead to improvements in hemoglobin or anemia. However, this may vary by race/ethnicity and anemia etiology, with the anemia association most prominent in anemia of inflammation.

These findings are consistent with other population-based studies of generally adults in the U.S. and Asia, which have documented an association between vitamin D deficiency and anemia,[4,10,11] and studies in adults and children using NHANES data have

suggested that the association may be particularly prominent among African Americans, and specific to anemia of inflammation.[5,9]

Following our finding of a link between vitamin D status and hemoglobin and anemia, we sought to better understand the mechanism underlying this association. In chapter 5 we evaluated the effect of high-dose vitamin D_3 supplementation on markers thought to be involved in the pathophysiology of anemia of inflammation, including hepcidin and pro-inflammatory cytokines IL-1β, IL-6, IL-8, and MCP-1, where the association was most prominently observed in chapter 3. Previous studies assessing the effects of vitamin D on these markers were largely *in vitro.* A study from our group found that treatment of cultured human monocytes with $1,25(OH)_2D$ resulted in a reduction in IL-6 and IL-1β release, a reduction in hepcidin mRNA expression, and an increase in ferroportin mRNA expression.[96] Similarly, Bacchetta et al,[95] showed that treatment of cultured human monocytes or hepatocyes with 25(OH)D or $1,25(OH)_2D$ resulted in a reduction in hepcidin mRNA expression with associated increases in ferroportin mRNA expression and reductions in cellular ferritin mRNA expression. This group subsequently identified VDREs in the promoter region of the hepcidin antimicrobial peptide gene, *HAMP*, indicating that the observed reductions in hepcidin expression may be due to direct transcriptional suppression of *HAMP* by vitamin D.

Other observational and clinical studies have shown that vitamin D status is inversely associated with pro-inflammatory cytokine[28] and hepcidin concentrations.[173] However, these questions had not been previously assessed in the context of a randomized controlled trial. In our population of young, healthy adults with a high prevalence of vitamin D deficiency, we found that treatment with 250,000 IU of vitamin

D_3 significantly reduced circulating hepcidin concentrations after one week compared to placebo, but had no effect on circulating pro-inflammatory cytokine or ferritin concentrations. These findings suggest that even in the absence of chronic disease or inflammatory conditions, vitamin D may influence iron metabolism through effects on hepcidin. Reductions in hepcidin may have the potential to increase the circulating iron pool available for erythropoiesis and hemoglobin synthesis, thus increasing hemoglobin concentrations and improving anemia. However, the small sample size and short duration of this study, as well as the lack of iron biomarkers other than ferritin left us unable to conclude on the down-stream effects of vitamin D on markers of iron status.

Therefore, to further evaluate the effect of vitamin D on iron metabolism, we used a double-blind randomized controlled trial of vitamin D in a population with a documented high prevalence of both vitamin D deficiency and anemia – critically ill adults (chapter 6). In this pilot study we found that treatment with 500,000 IU vitamin D_3 resulted in an acute reduction in hepcidin after one week, consistent with our findings from chapter 5. While this reduction was not maintained over time, it likely potentiated an increase in hemoglobin that was observed. The group that received 500,000 IU vitamin D_3 experienced a significant increase in hemoglobin concentrations over time, compared to the placebo group. These results suggest that vitamin D may improve hemoglobin concentrations, potentially through down-regulatory actions on hepcidin.

The findings from this dissertation work are consistent with our central hypothesis that vitamin D deficiency would be associated with increased odds of anemia or reduced hemoglobin concentrations and that treatment with high-dose vitamin D would reduce circulating hepcidin concentrations and increase hemoglobin concentrations. However,

contrary to our hypothesis, we failed to observe an effect of high-dose vitamin D_3 on inflammatory markers in chapter 5 despite the significant association observed with anemia of inflammation in chapter 3. This may be a result of the healthy study population where changes in inflammatory markers may be unlikely to be observed. Unfortunately, inflammatory markers were not measured in the ICU study so whether the effects on hemoglobin and hepcidin observed in chapter 6 were independent of changes in inflammation status, is unknown. It is also possible that the small sample size and short duration of observation left us underpowered to detect effects on pro-inflammatory cytokines in chapter 5. However, this unexpected result may suggest that the associations between vitamin D status and hemoglobin/anemia observed in chapters 3 and 4, may be due to direct transcriptional action of vitamin D on the hepcidin antimicrobial peptide gene, *HAMP*, instead of through anti-inflammatory pathways. There is biological plausibility to support the direct action of vitamin D on *HAMP* given the VDREs found to be present in the promoter region of the gene.[95] The results from chapter 5 and 6 should be confirmed in larger randomized controlled trials of longer duration to fully evaluate the effectiveness of vitamin D in improving hemoglobin concentrations and reducing risk of anemia, and to better understand the specific mechanism by which it may do so.

Strengths and Limitations

A major strength of this dissertation work is the progressive and systematic approach used to address a novel research question. We first evaluated the association of vitamin D status with hemoglobin concentrations and anemia in two diverse and well-

characterized large population-based studies. Then, applying the results from the previous studies we aimed to evaluate the efficacy of vitamin D in modifying markers involved in iron regulation and the pathophysiology of anemia of inflammation. This efficacy study was performed in a healthy cohort and therefore free of potentially confounding factors such as disease processes and medications which may otherwise have influenced the results. Following successful demonstration of the efficacy of vitamin D_3 in reducing hepcidin concentrations, we sought to translate this finding and further test the effect of vitamin D_3 on hemoglobin concentrations in a population at high risk for vitamin D deficiency and anemia (i.e. one most likely to benefit from vitamin D supplementation should our hypotheses hold). Additional strengths of chapters 5 and 6 were the rigorous randomized controlled trial design which had not previously been applied to these research questions.

Despite these strengths, there were several limitations to this dissertation work to note. First, the population-based analyses were both cross-sectional, leaving us unable to conclude causality in the vitamin D and hemoglobin/anemia associations observed. Secondly, the clinical trials used in this work were pilot studies, both small in number and short in duration and not originally designed or powered for the outcomes of our analyses. While significant results were observed for some markers, we were unable to determine if the non-significant results in others were due to a null effect or because we were underpowered for those outcomes. Another potential limitation of chapters 3-5 is that study questionnaires including health history were collected via participant self-report and therefore subject to recall bias. Finally, while some markers were measured in multiple studies, there was a lack of consistency in markers of iron metabolism and

inflammation measured across all studies, precluding complete comparison of results across all studies. Furthermore, as some of these markers are subject to diurnal variation, the lack of consistent timing of blood draw across all studies limits our ability to compare results from one study to the next. Nonetheless, these pilot studies and the work of this dissertation as a whole provide compelling preliminary evidence for a role for vitamin D in the regulation of iron metabolism.

Implications of this research

The results of this dissertation have both public health and clinical implications. The prevalence of anemia is approximately 33% worldwide, and it was estimated that anemia accounted for 68.4 million years lived with disability from all causes globally in 2010.[81] Anemia of inflammation is estimated to be the second-most common anemia after iron deficiency anemia.[90] More than a third of U.S. adults are obese and approximately half of all Americans have one or more chronic disease,[192,193] putting a substantial proportion of the population at risk for anemia of inflammation, also referred to as anemia of chronic disease. With the concomitant high prevalence of vitamin D deficiency in the U.S., this body of work suggests that a large segment of the general population may benefit from achieving 25(OH)D concentrations $\geq$ 20 ng/mL, as these levels were found to be protective against anemia and associated with increased hemoglobin (chapters 3 and 4).

Pending confirmation of these results in larger studies, potential public health recommendations may be considered to increase vitamin D uptake at a population level. While supplementation may be appropriate for groups at elevated risk for both vitamin D

deficiency and anemia, such as African Americans, and those with chronic diseases characterized by chronic inflammation including CKD, cardiorenal syndrome, inflammatory bowel disease (IBD), and cystic fibrosis (CF), [111,116,194-198] this strategy may be best applied in a clinical setting, as supplement uptake typically does not exceed 40% on a population level, and is most often consumed in the form of a multivitamin.[199] In addition, supplementation recommendations on a population level may run the risk of vitamin D toxicity with excess supplement intake. Diet-based strategies are less likely to result in vitamin D toxicity as the amount of vitamin D from food sources is unlikely to cause toxicity. Therefore, given the duel burden of vitamin D insufficiency and anemia on a population-level, food fortification and education strategies may be more efficacious and safe in increasing vitamin D intake on the population level.

In the United States, the median dietary vitamin D intake was estimated to be approximately 1.75 μg/day from naturally occurring food sources of vitamin D, and 6 μg/day for all sources (natural, fortified, supplements), indicating that most people do not meet the recommended level of intake of vitamin D.[200] There are thus opportunities to improve dietary vitamin D intake through education and food fortification strategies. Food fortification has been shown to be effective in raising blood 25(OH)D concentrations in a dose-dependent manner, though increases in the amount of vitamin D typically added to fortified or enriched foods may be necessary to achieve these improvements in 25(OH)D concentrations.[201] Furthermore, additional vehicles may be considered to further expand the reach of this strategy. Milk and ready-to-eat cereals are the most widely consumed fortified sources of vitamin D,[202] but among people who do not consume these products or do so in limited amounts, the efficacy of these products in

improving 25(OH)D status is limited. To expand the reach of vitamin D fortified foods, some have proposed scaling-up fortification of grain productions to include corn meal, rice, and pasta,[203] and using biofortification to enhance the vitamin D content in foods such as eggs and baker's yeast.[204]

Education-based strategies may also be undertaken to increase the consumption of food sources of vitamin D. Milk and fatty fish are the two most commonly consumed sources of vitamin D,[202] but education-based strategies to inform the consumer of other sources of vitamin D including eggs and mushrooms may be considered to increase vitamin D intake as part of a healthy, balanced diet.

In addition to potential public health implications, this work has clinical relevance as well. Pending confirmation in larger, longer term clinical trials, the results of this dissertation research suggest that vitamin D may have efficacy as a potential therapeutic option for anemia, particularly anemia of inflammation in those with chronic diseases characterized by chronic inflammation including CKD, cardiorenal syndrome, IBD, and CF. Anemia of inflammation is challenging to treat as it is often tied to an underlying chronic condition. However, if vitamin D can lower hepcidin concentrations as demonstrated in chapters 5 and 6, this may lead to an increase in the circulating iron pool available for use in erythropoiesis and hemoglobin synthesis, thereby improving hemoglobin concentrations, as indicated in chapter 6, and potentially anemia.

While anemia of inflammation is typically a mild-moderate anemia, it is not without consequence. In addition to associations with fatigue and decreased physical capacity, anemia of inflammation is associated with increased disease severity and increased risk for cardiovascular disease, and is an independent risk factor for death is

some patient populations.[86,92,194,205-209] Therefore, additional therapeutic options are needed to address the burden of anemia. If, as the results from this work suggest, vitamin D can lower hepcidin concentrations and increase hemoglobin concentrations, vitamin D may provide a safe alternative or complementary therapeutic option for addressing anemia of inflammation. Even if complete resolution of anemia is not achieved, vitamin D may prove beneficial if it can increase hemoglobin levels to an extent such that riskier and more expensive therapies including transfusions and erythropoiesis stimulating agents need not be administered.[178,179,188,189,210-214]

Results from this work and others suggest that the effect of vitamin D on anemia may be limited to anemia of inflammation. While hepcidin-lowering by vitamin D may increase circulating iron by increasing iron egress from cells of the reticuloendothial system, this may only have positive benefits in the absence of absolute iron deficiency.[122] In other words, improvements are only likely if there is sufficient iron to be mobilized from cells. Despite the potential implications being specific to anemia of inflammation, as noted above, this would still be likely to benefit a substantial portion of the general and clinical populations.

In addition to the potential therapeutic implications of this work, these results have broad scientific implications in that they address the call from the Institute of Medicine (US) Committee to Review Dietary Reference Intakes for Vitamin D and Calcium in 2011 for further research exploring the causal relationships between vitamin D and non-skeletal health outcomes, including outcomes related to immune function and inflammation.[32] While more research is needed to fully evaluate the role of vitamin D in the regulation of iron metabolism, this dissertation research provides strong preliminary

data to inform future, large-scale trials testing the therapeutic effectiveness of vitamin D for improving anemia, exploring the role of vitamin D in the hepcidin-ferroportin axis, and more broadly continuing to define the role of vitamin D in human health.

Future Directions

One of the broad aims of this research was to provide preliminary data for larger, longer-term randomized controlled trials designed to test the effectiveness of vitamin D supplementation in increasing hemoglobin concentrations. To date, few studies have been specifically designed and powered for this aim. Some studies have examined the effect of vitamin D supplementation on ESA use, but the results of these have been mixed with some studies reporting lower ESA requirements with vitamin D therapy[104,105] and others report no effect.[215] The few studies that have assessed the effect of vitamin D supplementation on hemoglobin or anemia were largely secondary analyses of previously completed vitamin D trials. In addition, these studies have varied in the form and dose of vitamin D administered, as well as the population studied. Studies in patients with CKD have shown that vitamin D or its analogues may increase hemoglobin concentrations.[118-121] Studies in other populations have found no effect of vitamin D therapy on hemoglobin concentrations, however these may have been limited in that they included those with iron deficiency anemia or were in populations with a low prevalence of anemia and vitamin D deficiency.[122,174,186]

Therefore, while the results of this work do provide preliminary evidence, further questions remain regarding a role for vitamin D in the regulation of iron metabolism. To more completely examine the role of vitamin D in the regulation of iron homeostasis,

future work must consider the ideal population(s) to study, the dose and form of vitamin D to give, the duration of the study, and which iron and vitamin D biomarkers to evaluate. Other potential mechanisms of action, the interplay of vitamin D and other regulators of iron metabolism, the effect of lifestyle factors on the link between vitamin D and iron metabolism, and the influence of vitamin D on functional outcomes should also be considered to fully evaluate the role of vitamin D iron metabolism in the context of the system as a whole. These considerations for future work are discussed in more detail below.

Populations to study

Given the results of the dissertation work, studies aiming to evaluate the effect of vitamin D supplementation on anemia should consider racially diverse populations with a high prevalence of vitamin D deficiency and anemia, specifically anemia of inflammation. Studies in those with iron deficiency anemia are unlikely to show a benefit of vitamin D. Care should be taken to exclude those with iron deficiency as the sole contributor to anemia from studies testing the effectiveness of vitamin D in improving anemia. Furthermore, based on the results of chapter 3, investigators should stratify their randomization and analysis by race given that vitamin D deficiency and anemia are more prevalent in African Americans than Caucasians and there may be racial differences in response profiles to vitamin D.[9,34,83,84,116,215,216]

Several chronic diseases are characterized by high rates of vitamin D deficiency and anemia, as well as chronic inflammation, including CKD, CVD, CF, and IBD.[111,194-

[198] Studies in patients with these diseases may therefore be appropriate for evaluating the role of vitamin D in iron metabolism.

We are currently performing a large retrospective chart review of adult CF patients to examine the association between vitamin D deficiency and onset of anemia in this population. In patients with CF, a group prone to hypoxemia, reductions in iron availability for erythropoiesis secondary to elevations in pro-inflammatory cytokines and hepcidin may be particularly concerning.[218-220] To compensate for hypoxemia, the body responds by increasing hemoglobin concentration to improve oxygen-carrying capacity.[219,221] However, if inflammation is present as a competing signal, iron may not be available to support hemoglobin synthesis, thereby negatively affecting oxygen-carrying capacity in these patients, making this an important population to study.

Our group has also recently completed a study in children with IBD, in which we examined the link between vitamin D status and hepcidin, hemoglobin, and anemia. In this study we found that those with 25(OH)D concentrations < 30 ng/mL had significantly higher serum hepcidin concentrations [β(SE)=0.55(0.22), *P*=0.01] and significantly lower hemoglobin concentrations [β(SE)= -1.0 (0.5), *P*=0.03] controlling for age, sex, race, inflammation, insurance, BAZ score and disease duration, compared to those with 25(OH)D concentrations ≥ 30 ng/mL (Syed S, Smith EM, et al, unpublished observations).

Data from epidemiologic studies of CKD and CVD patients suggest a link between vitamin D status and anemia/hemoglobin in these populations.[8,12,13,222] Critically ill patients may also be considered for studying the effect of vitamin D on hemoglobin and anemia given the high rates of vitamin D deficiency and anemia in this

population.[175,176,182,183] However, the dynamic nature of their condition and difficulty in establishing true "baseline" status may make this a particularly challenging population to study and draw generalizable conclusions from.

Form and dose of vitamin D, duration of study

Future trials evaluating the role of vitamin D in iron metabolism should use vitamin D_3 as the study drug, as there is evidence that vitamin D_3 is more effective in raising blood 25(OH)D concentrations compared to vitamin D_2.[44,223] The native form of vitamin D_3 (cholecalciferol) should be used as opposed to the active form (calcitriol) because adequate substrate [25(OH)D] must be available at the tissue (in this case, the monocyte or hepatocyte) for local activation to $1,25(OH)_2D$ via CYP27B1 to then carry out local transcriptional regulation of hepcidin.[3]

In terms of the dosing regimen, it likely depends on the population being studied. In previous studies from our group, we have been successful in achieving blood 25(OH)D concentrations $\geq$ 20 ng/mL, the level found to be associated with either reduced odds of anemia or higher hemoglobin concentrations in chapters 3 and 4, using high-dose vitamin D_3 supplementation strategies. In patients with chronic kidney disease, we have found a dosing regimen of 50,000 IU D_3 once weekly for 12 weeks to be safe and effective in raising blood 25(OH)D concentrations.[224,225] In the CKD population, caution should be exercised in administering mega-doses of vitamin D due to reduced renal handling and consequent elevations in FGF-23 concentrations which are associated with adverse patient outcomes.[110,226] In clinical populations with fat malabsorption, such as cystic fibrosis and inflammatory bowel disease, or those in a catabolic state such as critical

illness, a higher dose may be tolerated and necessary. In a study of adults with cystic fibrosis, our group used a bolus dose of 250,000 IU D_3 to rapidly replete serum 25(OH)D concentrations.[227] Both vitamin D dosing regimens used in chapter 6 of this dissertation were effective in raising 25(OH)D concentrations to levels ≥ 30 ng/mL in critically ill adults.[185] However, there is currently no universal dosing regimen, and some recent studies have found high-dose vitamin D therapy to be association with increased risk of falls and elevated bone turnover markers.[40,228] Caution should be exercised in use of high-dose vitamin D and participants should be assessed regularly to ensure the safety of the dosing strategies.

In addition to the magnitude of the dose, consideration should be given to the frequency of administration. Vitamin D can be given daily, weekly, or monthly, with the dose titrated appropriately, but longer frequencies are unlikely to be effective in maintaining 25(OH)D concentrations in the blood, even if the original dose was very high.[39] For example, the healthy study population used in chapter 5 was given a one-time oral bolus dose of 250,000 IU vitamin D_3, and 25(OH)D concentration increased significantly after one week, but the levels were not sustained three months later.[169]

In addition to the frequency of administration, the timing of vitamin D dosing should be considered as well. Hepcidin concentrations vary diurnally, and are responsive to iron supplementation.[229] Therefore, the timing of the dose of vitamin D and the assessment of hepcidin should be consistent. Care should also be taken to exclude participants taking iron supplementation from the study as iron supplements affect hepcidin concentration and could therefore cloud the effect of vitamin D on hepcidin concentrations. Finally, the duration of a study aiming to more fully evaluate the effect of

vitamin D on iron metabolism should account for the red blood cell life cycle, and therefore last at least 120 days.[230]

Iron and vitamin D biomarkers

At minimum, studies aiming to evaluate the effect of vitamin D on iron metabolism and anemia must include 25(OH)D concentrations and hemoglobin measures. Additional biomarkers of iron metabolism which should also ideally be included are serum iron and total iron binding capacity (from which transferrin saturation may be calculated), serum ferritin, sTfR, and hepcidin. The ratio markers log(sTfR concentrations/ferritin) concentrations or sTfR/log(ferritin) have been suggested for use in distinguishing iron deficiency anemia from anemia of inflammation and may be used in the screening process to exclude those with only iron deficiency anemia from the study.[73,90]

An additional vitamin D biomarker that may be considered is free 25(OH)D, an assay for which is now commercially available. It has been suggested that extra-renal tissues which express CYP27B1, such as monocytes, may more readily utilize free 25(OH)D for conversion to the active form to carry out subsequent genomic functions.[20,21] Therefore, measurement of free vitamin D may provide a more accurate assessment of vitamin D bioactivity, though further research in this area is needed.

Other mechanisms of action of vitamin D

In addition to the hepcidin-lowering effects of vitamin D seen in this work and others,[95,96] other potential mechanisms of action of vitamin D in the regulation of iron

metabolism have been proposed and warrant further evaluation. To comprehensively characterize the role of vitamin D in iron homeostasis, studies should also evaluate the effect of vitamin D on other markers involved in the pathophysiology of anemia of inflammation.

Though not observed in chapter 5, other studies have indicated that vitamin D may reduce pro-inflammatory cytokines implicated in the pathophysiology of anemia of inflammation.[96,231,232] Elevations in IL-6 and IL-1β may stimulate hepcidin expression,[65,233] and TNF-α and IFN-γ have been shown to up-regulate DMT1 leading to increased uptake of iron by macrophages.[90,234] These cytokines may also promote iron retention by down-regulating the expression of ferroportin.[234] Furthermore, TNF-α and IFN-γ may impair erythropoiesis by further inhibiting the production of erythropoietin in the kidney and inhibiting the differentiation and proliferation of erythroid progenitor cells.[235,236] Therefore, vitamin D, by lowering inflammatory cytokines, often elevated in chronic diseases with a high burden of anemia of inflammation, may improve iron recycling and anemia, and should be considered for evaluation in future studies.

As described in chapter 2, there is also evidence that vitamin D may influence erythropoiesis by increasing erythroid progenitor proliferation and enhancing erythropoietin.[100,101,237] Further, as noted above, some studies have shown that vitamin D supplementation may reduce ESA resistance or ESA requirements in CKD patients.[105,119,120] Therefore, it would be of interest to measure erythropoietin in future studies to assess the effect of vitamin D treatment on this marker involved in the regulation of erythropoiesis when evaluating the larger role of vitamin D in iron metabolism.

Finally, another pathway by which vitamin D may influence iron metabolism in the context of anemia of inflammation is through effects on redox biology. Oxidative stress is associated with several chronic disease processes including those with high rates of anemia and vitamin D deficiency such as CKD and CVD.[238-240] Moreover, oxidative stress has been linked to anemia in CKD.[241,242] Increases in oxidative stress may lead to red blood cell damage and increased erythrophagocytosis.[243] Potentially compounding this, traditional treatments for anemia including iron and ESAs have been shown to increase oxidative stress in CKD patients.[244,245] Recently, it was shown that vitamin D may have a role in mediating oxidative stress through glutathione (GSH), the predominant intracellular antioxidant. Jain, et al,[246] showed that vitamin D can up-regulate glutathione (GSH) *in vitro* and Izquierdo, et al,[247] found in patients on hemodialysis, treatment with paricalcitol (a vitamin D receptor agonist) significantly increased GSH concentrations in the blood and decreased concentrations of inflammatory cytokines including IL-6. Data from our group has shown that serum 25(OH)D was positively associated with GSH and inversely associated with its redox potential (E_h GSSG) among generally healthy adults in the same cohort utilized in chapter 3.[248] Therefore, increasing GSH and reducing oxidative stress is another potential mechanism of action of vitamin D related to iron metabolism and anemia, which warrants further investigation.

In addition to considering the effect of vitamin D on other markers involved in the pathophysiology of anemia of inflammation described above, one may also apply discovery-based metabolomics methods to interventional studies of vitamin D to further

characterize the metabolic effects of vitamin D and possibly identify additional vitamin D-mediated pathways involved in iron metabolism.

Interplay of vitamin D with other regulators of iron metabolism

As discussed in chapter 2, other calciotropic hormones may be involved in the regulation of iron metabolism. PTH has been found to be inversely associated with hemoglobin, erythropoietin, erythroid progenitor formation and positively associated with fibrosis of the bone marrow and erythropoietin resistance.[111-113,249] FGF-23 has been shown to be a negative regulator of erythropoiesis in a mouse model,[107] and is inversely associated with hemoglobin concentrations in humans.[110] Classically, in response to low serum calcium concentrations, PTH stimulates the expression of 1α-hydroxylase to produce $1,25(OH)_2D$ and increase calcium absorption. FGF-23 responds to elevations in serum phosphate to down regulate the expression of 1α-hydroxylase and lower $1,25(OH)_2D$ concentrations.[24] However, as more is learned about the functions of the hormones, the understanding of the PTH-vitamin D-FGF-23 axis continues to evolve. The interplay between these hormones in the regulation of iron metabolism will be an interesting area of future research.

In addition to the interrelation of calciotropic hormones in the regulation of iron metabolism, the interplay between other hepcidin regulators should be considered in future studies of iron homeostasis. The results from this work and others suggest that vitamin D is a negative regulator of hepcidin.[95,96] Other regulators of hepcidin include BMP-6 and ERFE. Expression of BMP-6 is induced in response to increases in iron stores, and BMP-6 in turn stimulates hepcidin transcription.[250] ERFE is a hormone

produced in erythroblasts in response to erythropoietin, and acts on the liver to suppress hepcidin expression.[64] To comprehensively examine the role of vitamin D on iron metabolism, its interplay with these additional regulators of hepcidin should be considered in future trials of vitamin D therapy on hemoglobin concentrations and anemia risk.

Lifestyle factors and functional outcomes

In addition to evaluation of biochemical pathways involved in iron metabolism which may be influenced by vitamin D, lifestyle factors and functional outcomes should be considered as well. Such factors potentially relevant to the vitamin D and anemia association include assessment of social stress, physical activity, and physical function.

Social stress has been shown to impact inflammation status and chronic disease outcomes,[251,252] and racial differences in levels of perceived social stress have been described.[253] It may therefore be useful to assess social stress given the potential influence of this factor on characteristics of the vitamin D and anemia association including inflammation and racial differences.

Other factors that may be informative to assess in a trial testing the effect of vitamin D supplementation on hemoglobin concentrations are physical activity and physical capacity. Among the consequences of anemia are fatigue and decreased physical capacity. Therefore, if vitamin D can improve hemoglobin concentrations, it would be interesting to extend this finding to determine if improvements in hemoglobin lead to improvements physical function. There is evidence to suggest that 25(OH)D concentrations are positively associated with physical activity and physical capacity.[254,255]

Better vitamin D status has been shown to be associated with better performance on a 6 minute walk test, greater gait speed, better performance with timed chair stands, and greater handgrip strength in elderly adults.[256] A recent study in patients undergoing cardiovascular rehabilitation found that those who were vitamin D deficient at baseline had significantly worse performance on a 6 minute walk test at baseline and less improvement following rehab compared to those who were not vitamin D deficient.[257] However, whether the influence of vitamin D on physical performance is mediated by changes in hemoglobin has not been investigated.

Conclusions

This dissertation research provides preliminary evidence for a role for vitamin D in the regulation of iron metabolism. We found that 25(OH)D concentrations < 20 ng/mL were associated with lower hemoglobin concentrations and increased odds of anemia, particularly anemia of inflammation. The mechanism of action underlying this association appeared to involve down-regulatory effects of vitamin D on hepcidin. In both healthy and critically ill adults, treatment with high-dose vitamin D resulted in an acute reduction in circulating hepcidin concentrations, and in critically ill adults, high-dose vitamin D_3 therapy resulted in increases in hemoglobin concentrations over time.

These results should be confirmed in larger, longer-term randomized controlled trials testing the effectiveness of vitamin D in improving hemoglobin concentrations in racially diverse populations with a high prevalence of vitamin D deficiency and anemia of inflammation. To comprehensively evaluate this proposed role for vitamin D in the regulation of iron metabolism, future studies should consider additional mechanisms and

factors linking vitamin D to iron metabolism, as well as potential racial differences in the association between vitamin D status and anemia. The functional consequences of the effect of vitamin D on hemoglobin should also be explored to determine if improvements in hemoglobin may translate to additional benefits, such as improved physical capacity.

The potential efficacy of vitamin D in improving hemoglobin concentrations may have important clinical implications as an alternative or complementary therapy for anemia of inflammation. In addition, given the high prevalence of chronic disease and anemia in the general population, our findings that 25(OH)D concentrations $\geq$ 20 ng/mL were associated with higher hemoglobin concentrations and may be protective against anemia in two generally healthy adult populations, suggest that the results of this work have potential public health implications as well.

REFERENCES

1. Holick MF. Vitamin D deficiency. *N Engl J Med.* 2007;357(3):266-281.
2. Deluca HF. History of the discovery of vitamin D and its active metabolites. *Bonekey Rep.* 2014;3:479.
3. Hewison M. An update on vitamin D and human immunity. *Clin Endocrinol (Oxf).* 2012;76(3):315-325.
4. Monlezun DJ, Camargo CA, Jr., Mullen JT, Quraishi SA. Vitamin D status and the risk of anemia in community-dwelling adults: results from the National Health and Nutrition Examination Survey 2001-2006. *Medicine.* 2015;94(50):e1799.
5. Atkinson MA, Melamed ML, Kumar J, et al. Vitamin D, race, and risk for anemia in children. *J Pediatr.* 2014;164(1):153-158 e151.
6. Coutard A, Garlantezec R, Estivin S, Andro M, Gentric A. Association of vitamin D deficiency and anemia in a hospitalized geriatric population: denutrition as a confounding factor. *Ann Hematol.* 2013;92(5):615-619.
7. Hirani V, Cumming RG, Blyth F, et al. Cross-sectional and longitudinal associations between the active vitamin D metabolite (1,25 dihydroxyvitamin D) and haemoglobin levels in older Australian men: the Concord Health and Ageing in Men Project. *Age.* 2015;37(1):9749.
8. Patel NM, Gutierrez OM, Andress DL, Coyne DW, Levin A, Wolf M. Vitamin D deficiency and anemia in early chronic kidney disease. *Kidney Int.* 2010;77(8):715-720.

9. Perlstein TS, Pande R, Berliner N, Vanasse GJ. Prevalence of 25-hydroxyvitamin D deficiency in subgroups of elderly persons with anemia: association with anemia of inflammation. *Blood.* 2011;117(10):2800-2806.

10. Shin JY, Shim JY. Low vitamin D levels increase anemia risk in Korean women. *Clin Chim Acta.* 2013;421:177-180.

11. Sim JJ, Lac PT, Liu IL, et al. Vitamin D deficiency and anemia: a cross-sectional study. *Ann Hematol.* 2010;89(5):447-452.

12. Zittermann A, Jungvogel A, Prokop S, et al. Vitamin D deficiency is an independent predictor of anemia in end-stage heart failure. *Clin Res Cardiol.* 2011;100(9):781-788.

13. Zittermann A, Kuhn J, Dreier J, et al. Association of 25-hydroxyvitamin D with anemia risk in patients scheduled for cardiac surgery. *Int J Lab Hematol.* 2014;36(1):29-36.

14. Clinton SK. Vitamin D. In: Stipanuk MH and Caudill MA, eds. *Biochemical, Physiological, and Molecular Aspects of Human Nutrition*. 3rd ed. St. Louis, MO: Saunders, an imprint of Elsevier; 2013:703-717.

15. Holick MF. Photobiology of Vitamin D. In: Feldman D, ed. *Vitamin D.* Vol 1. 3rd ed. London, UK: Academic Press; 2011:13-22.

16. Christakos S, Dhawan P, Verstuyf A, Verlinden L, Carmeliet G. Vitamin D: metabolism, molecular mechanism of action, and pleiotropic effects. *Physiol Rev.* 2016;96(1):365-408.

17. Jones G. Pharmacokinetics of vitamin D toxicity. *Am J Clin Nutr.* 2008;88(2):582S-586S.

18. Wacker M, Holick MF. Vitamin D - effects on skeletal and extraskeletal health and the need for supplementation. *Nutrients.* 2013;5(1):111-148.

19. Chun RF, Lauridsen AL, Suon L, et al. Vitamin D-binding protein directs monocyte responses to 25-hydroxy- and 1,25-dihydroxyvitamin D. *J Clin Endocrinol Metab.* 2010;95(7):3368-3376.

20. Chun RF, Peercy BE, Orwoll ES, Nielson CM, Adams JS, Hewison M. Vitamin D and DBP: the free hormone hypothesis revisited. *J Steroid Biochem Mol Biol.* 2014;144 Pt A:132-137.

21. Shieh A, Chun RF, Ma C, et al. Effects of high-dose vitamin D2 versus vitamin D3 on total and free 25-hydroxyvitamin D and markers of calcium balance. *J Clin Endocrinol Metab.* 2016:jc20161871.

22. Hii CS, Ferrante A. The non-genomic actions of vitamin D. *Nutrients.* 2016;8(3):135.

23. Mizwicki MT, Norman AW. Vitamin D Sterol/VDR Conformational Dynamics and Nongenomic Actions. In: Feldman D, ed. *Vitamin D.* Vol 1. 3rd ed. London, UK: Academic Press; 2011:271-297.

24. Blau JE, Collins MT. The PTH-Vitamin D-FGF23 axis. *Rev Endocr Metab Disord.* 2015;16(2):165-174.

25. Liu PT, Stenger S, Li H, et al. Toll-like receptor triggering of a vitamin D-mediated human antimicrobial response. *Science.* 2006;311(5768):1770-1773.

26. Zasloff M. Fighting infections with vitamin D. *Nat Med.* 2006;12(4):388-390.

27. Rolf L, Muris AH, Hupperts R, Damoiseaux J. Illuminating vitamin D effects on B cells--the multiple sclerosis perspective. *Immunology.* 2016;147(3):275-284.

28. Yin K, Agrawal DK. Vitamin D and inflammatory diseases. *J Inflamm Res.* 2014;7:69-87.

29. Zhang Y, Leung DY, Richers BN, et al. Vitamin D inhibits monocyte/macrophage proinflammatory cytokine production by targeting MAPK phosphatase-1. *J Immunol.* 2012;188(5):2127-2135.

30. Dickie LJ, Church LD, Coulthard LR, Mathews RJ, Emery P, McDermott MF. Vitamin D3 down-regulates intracellular Toll-like receptor 9 expression and Toll-like receptor 9-induced IL-6 production in human monocytes. *Rheumatology (Oxford).* 2010;49(8):1466-1471.

31. Calton EK, Keane KN, Newsholme P, Soares MJ. The impact of vitamin D levels on inflammatory status: a systematic review of immune cell studies. *PloS one.* 2015;10(11):e0141770.

32. IOM (Institute of Medicine). *Dietary Reference Intakes for Calcium and Vitamin D.* Washington (DC): The National Academies Press. 2011.

33. Holick MF, Binkley NC, Bischoff-Ferrari HA, et al. Evaluation, treatment, and prevention of vitamin D deficiency: an Endocrine Society Clinical Practice Guideline. *J Clin Endocrinol Metab.* 2011;96(7):1911-1930.

34. Schleicher RL, Sternberg MR, Lacher DA, et al. The vitamin D status of the US population from 1988 to 2010 using standardized serum concentrations of 25-hydroxyvitamin D shows recent modest increases. *Am J Clin Nutr.* 2016;104(2):454-461.

35. Wacker M and Holick MF. Sunlight and vitamin D: a global perspective for health. *Dermatoendrocrinol*. 2013;5(1):51-108.

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

37. Vaes AM, Brouwer-Brolsma EM, van der Zwaluw NL, et al. Food sources of vitamin D and their association with 25-hydroxyvitamin D status in Dutch older adults. *J Steroid Biochem Mol Biol.* 2016. [Epub ahead of print]. doi: 10.1016/j.jsbmb.2016.10.004

38. Bikle DD. Vitamin D insufficiency/deficiency in gastrointestinal disorders. *J Bone Miner Res*. 2007;22 Suppl 2:V50-54.

39. Ish-Shalom S, Segal E, Salganik T, Raz B, Bromberg IL, Vieth R. Comparison of daily, weekly, and monthly vitamin D3 in ethanol dosing protocols for two months in elderly hip fracture patients. *J Clin Endocrinol Metab.* 2008;93(9):3430-3435.

40. Kearns MD, Alvarez JA, Tangpricha V. Large, single-dose, oral vitamin d supplementation in adult populations: a systematic review. *Endocr Pract.* 2014;20(4):341-351.

41. Pepper KJ, Judd SE, Nanes MS, Tangpricha V. Evaluation of vitamin D repletion regimens to correct vitamin D status in adults. *Endocr Pract.* 2009;15(2):95-103.

42. Vande Griend JP, McQueen RB, Linnebur SA, Vondracek SF. Prescription ergocalciferol dosing for vitamin D repletion: a retrospective evaluation. Pharmacotherapy. 2012;32(2):135-141.

43. Holick MF, Binkley NC, Bischoff-Ferrari HA, et al. Evaluation, treatment, and prevention of vitamin D deficiency: an Endocrine Society clinical practice guideline. *J Clin Endocrinol Metab*. 2011;96(7):1911-1930.

44. Tripkovic L, Lambert H, Hart K, et al. Comparison of vitamin D2 and vitamin D3 supplementation in raising serum 25-hydroxyvitamin D status: a systematic review and meta-analysis. *Am J Clin Nutr.* 2012;95(6):1357-1364.

45. von Drygalski A, Adamson JW. Iron metabolism in man. *JPEN.* 2013;37(5):599-606.

46. Camaschella C. Iron-Deficiency Anemia. *N Engl J Med.* 2015;372(19):1832-1843.

47. McLean E, Cogswell M, Egli I, Wojdyla D, de Benoist B. Worldwide prevalence of anaemia, WHO Vitamin and Mineral Nutrition Information System, 1993-2005. *Public Health Nutr.* 2009;12(4):444-454.

48. Beck KL, Conlon CA, Kruger R, Coad J. Dietary determinants of and possible solutions to iron deficiency for young women living in industrialized countries: a review. *Nutrients.* 2014;6(9):3747-3776.

49. Hallberg L. Perspectives on nutritional iron deficiency. *Annu Rev Nutr.* 2001;21:1-21.

50. Anderson GJ, Frazer DM, McLaren GD. Iron absorption and metabolism. *Curr Opin Gastroenterol.* 2009;25(2):129-135.

51. Ganz T. Systemic iron homeostasis. *Physiol Rev.* 2013;93(4):1721-1741.

52. Hallberg L, Hulten L, Gramatkovski E. Iron absorption from the whole diet in men: how effective is the regulation of iron absorption? *Am J Clin Nutr.* 1997;66(2):347-356.

53. Cook JD. Adaptation in iron metabolism. *Am J Clin Nutr.* 1990;51(2):301-308.

54. Hentze MW, Muckenthaler MU, Andrews NC. Balancing acts: molecular control of mammalian iron metabolism. *Cell.* 2004;117(3):285-297.

55. Munoz M, Garcia-Erce JA, Remacha AF. Disorders of iron metabolism. Part 1: molecular basis of iron homoeostasis. *J Clin Pathol.* 2011;64(4):281-286.

56. Han O. Molecular mechanism of intestinal iron absorption. *Metallomics.* 2011;3(2):103-109.

57. Krause A, Neitz S, Magert HJ, et al. LEAP-1, a novel highly disulfide-bonded human peptide, exhibits antimicrobial activity. *FEBS Lett.* 2000;480(2-3):147-150.

58. Park CH, Valore EV, Waring AJ, Ganz T. Hepcidin, a urinary antimicrobial peptide synthesized in the liver. *J Biol Chem.* 2001;276(11):7806-7810.

59. Pigeon C, Ilyin G, Courselaud B, et al. A new mouse liver-specific gene, encoding a protein homologous to human antimicrobial peptide hepcidin, is overexpressed during iron overload. *J Biol Chem.* 2001;276(11):7811-7819.

60. Ruchala P, Nemeth E. The pathophysiology and pharmacology of hepcidin. *Trends Pharmacol Sci.* 2014;35(3):155-161.

61. Nemeth E, Tuttle MS, Powelson J, et al. Hepcidin regulates cellular iron efflux by binding to ferroportin and inducing its internalization. *Science.* 2004;306(5704):2090-2093.

62. Nemeth E, Ganz T. Regulation of iron metabolism by hepcidin. *Annu Rev Nutr.* 2006;26:323-342.

63. Ganz T, Nemeth E. Hepcidin and iron homeostasis. *Biochim Biophys Acta.* 2012;1823(9):1434-1443.

64. Kautz L, Jung G, Valore EV, Rivella S, Nemeth E, Ganz T. Identification of erythroferrone as an erythroid regulator of iron metabolism. *Nat Genet.* 2014;46(7):678-684.

65. Nemeth E, Rivera S, Gabayan V, et al. IL-6 mediates hypoferremia of inflammation by inducing the synthesis of the iron regulatory hormone hepcidin. *J Clin Invest.* 2004;113(9):1271-1276.

66. Nemeth E, Ganz T. Anemia of inflammation. *Hematol Oncol Clin North Am.* 2014;28(4):671-681, vi.

67. Crichton RR. Iron. In: Stipanuk MH and Caudill MA, eds. *Biochemical, Physiological, and Molecular Aspects of Human Nutrition*. 3rd ed. St. Louis, MO: Saunders, an imprint of Elsevier; 2013:801-827.

68. Hallberg L, Hulthen L. Perspectives on iron absorption. *Blood Cells Mol Dis.* 2002;29(3):562-573.

69. IOM (Institute of Medicine). *Dietary Reference Intakes for Vitamin A, Vitamin K, Arsenic, Boron, Chromium, Copper, Iodine, Iron, Manganese, Molybdenum, Nickel, Silicon, Vanadium, and Zinc*. Washington (DC): National Academies Press (US). 2001.

70. World Health Organization. Dept. of Nutrition for Health and Development. Iron Deficiency Anaemia: Assessment, Prevention, and Control. A Guide for Programme Managers. Geneva: World Health Organization. 2001.

71. Looker AC, Dallman PR, Carroll MD, Gunter EW, Johnson CL. Prevalence of iron deficiency in the United States. *JAMA.* 1997;277(12):973-976.

72. Iron. In: Gibson RS, ed. *Principles of Nutrition Assessment*. 2nd ed. New York: Oxford University Press; 2005.

73. Skikne BS, Punnonen K, Caldron PH, et al. Improved differential diagnosis of anemia of chronic disease and iron deficiency anemia: a prospective multicenter evaluation of soluble transferrin receptor and the sTfR/log ferritin index. *Am J Hematol.* 2011;86(11):923-927.

74. Smith DJ, Anderson GJ, Lamont IL, Masel P, Bell SC, Reid DW. Accurate assessment of systemic iron status in cystic fibrosis will avoid the hazards of inappropriate iron supplementation. *J Cyst Fibros.* 2013;12(3):303-304.

75. Kiss JE. Laboratory and genetic assessment of iron deficiency in blood donors. *Clin Lab Med.* 2015;35(1):73-91.

76. Balarajan Y, Ramakrishnan U, Ozaltin E, Shankar AH, Subramanian SV. Anaemia in low-income and middle-income countries. *Lancet.* 2011;378(9809):2123-2135.

77. Stoltzfus RJ, Mullany L, Black RE. Iron Deficiency Anaemia. In: Ezzati M, Lopez AD, Rodgers A, Murray CJL, ed. *Comparative Quantification of Health Risks: Global and Regional Burden of Disease Attributable to Selected Major Risk Factors.* Vol 1. Geneva: WHO; 2004.

78. Munoz M, Garcia-Erce JA, Remacha AF. Disorders of iron metabolism. Part II: iron deficiency and iron overload. *J Clin Pathol.* 2011;64(4):287-296.

79. Salgia RJ, Brown K. Diagnosis and management of hereditary hemochromatosis. *Clin Liver Dis.* 2015;19(1):187-198.

80. De Benoist B ME, Egli I, Cogswell M. *Worldwide Prevalence of Anaemia 1993-2005: WHO Global Database on Anemia.* Geneva: WHO; 2008.

81. Kassebaum NJ, Jasrasaria R, Naghavi M, et al. A systematic analysis of global anemia burden from 1990 to 2010. *Blood.* 2014;123(5):615-624.

82. Dong X, Mendes de Leon C, Artz A, Tang Y, Shah R, Evans D. A population-based study of hemoglobin, race, and mortality in elderly persons. *J Gerontol A Biol Sci Med Sci.* 2008;63(8):873-878.

83. Patel KV, Longo DL, Ershler WB, et al. Haemoglobin concentration and the risk of death in older adults: differences by race/ethnicity in the NHANES III follow-up. *Br J Haematol.* 2009;145(4):514-523.

84. Zakai NA, McClure LA, Prineas R, et al. Correlates of anemia in American blacks and whites: the REGARDS Renal Ancillary Study. *Am J Epidemiol.* 2009;169(3):355-364.

85. Robinson BE. Epidemiology of chronic kidney disease and anemia. *J Am Med Dir Assoc.* 2006;7(9 Suppl):S3-6; quiz S17-21.

86. Hayden SJ, Albert TJ, Watkins TR, Swenson ER. Anemia in critical illness: insights into etiology, consequences, and management. *Am J Resp Crit Care Med.* 2012;185(10):1049-1057.

87. Sarnak MJ, Tighiouart H, Manjunath G, et al. Anemia as a risk factor for cardiovascular disease in The Atherosclerosis Risk in Communities (ARIC) study. *J Am Coll Cardiol.* 2002;40(1):27-33.

88. Gifford AH, Miller SD, Jackson BP, et al. Iron and CF-related anemia: expanding clinical and biochemical relationships. *Pediatr Pulmonol.* 2011;46(2):160-165.

89. Goodhand JR, Kamperidis N, Rao A, et al. Prevalence and management of anemia in children, adolescents, and adults with inflammatory bowel disease. *Inflamm Bowel Dis.* 2012;18(3):513-519.

90. Weiss G, Goodnough LT. Anemia of chronic disease. *N Engl J Med.* 2005;352(10):1011-1023.

91. Retter A, Wyncoll D, Pearse R, et al. Guidelines on the management of anaemia and red cell transfusion in adult critically ill patients. *Br J Haematol.* 2013;160(4):445-464.

92. National Kidney Foundation. KDOQI Clinical Practice Guidelines and Clinical Practice Recommendations for Anemia in Chronic Kidney Disease. *Am J Kidney Dis.* 2006;47(5):S11–S145.

93. Jin HJ, Lee JH, Kim MK. The prevalence of vitamin D deficiency in iron-deficient and normal children under the age of 24 months. *Blood Res.* 2013;48(1):40-45.

94. Bacchetta J, Chun RF, Gales B, et al. Antibacterial responses by peritoneal macrophages are enhanced following vitamin D supplementation. *PloS one.* 2014;9(12):e116530.

95. Bacchetta J, Zaritsky JJ, Sea JL, et al. Suppression of iron-regulatory hepcidin by vitamin D. *J Am Soc Nephrol.* 2014;25(3):564-572.

96. Zughaier SM, Alvarez JA, Sloan JH, Konrad RJ, Tangpricha V. The role of vitamin D in regulating the iron-hepcidin-ferroportin axis in monocytes. *J Clin Transl Endocrinol.* 2014;1(1):19-25.

97. Silva B, Faustino P. An overview of molecular basis of iron metabolism regulation and the associated pathologies. *Biochim Biophys Acta.* 2015;1852(7):1347-1359.

98. Nairz M, Haschka D, Demetz E, Weiss G. Iron at the interface of immunity and infection. *Front Pharmacol.* 2014;5:152.

99. Poggiali E, Migone De Amicis M, Motta I. Anemia of chronic disease: a unique defect of iron recycling for many different chronic diseases. *Eur J Intern Med.* 2014;25(1):12-17.

100. Alon DB, Chaimovitz C, Dvilansky A, et al. Novel role of 1,25(OH)(2)D(3) in induction of erythroid progenitor cell proliferation. *Exp Hematol.* 2002;30(5):403-409.

101. Aucella F, Scalzulli RP, Gatta G, Vigilante M, Carella AM, Stallone C. Calcitriol increases burst-forming unit-erythroid proliferation in chronic renal failure. A synergistic effect with r-HuEpo. *Nephron Clin Pract.* 2003;95(4):c121-127.

102. Refaat B, Ashour TH, El-Shemi AG. Ribavirin induced anaemia: the effect of vitamin D supplementation on erythropoietin and erythrocyte indices in normal Wistar rat. *Int J Clin Exp Med.* 2014;7(9):2667-2676.

103. Kiss Z, Ambrus C, Almasi C, et al. Serum 25(OH)-cholecalciferol concentration is associated with hemoglobin level and erythropoietin resistance in patients on maintenance hemodialysis. *Nephron Clin Pract.* 2011;117(4):c373-378.

104. Kumar VA, Kujubu DA, Sim JJ, Rasgon SA, Yang PS. Vitamin D supplementation and recombinant human erythropoietin utilization in vitamin D-deficient hemodialysis patients. *J Nephrol.* 2011;24(1):98-105.

105. Rianthavorn P, Boonyapapong P. Ergocalciferol decreases erythropoietin resistance in children with chronic kidney disease stage 5. *Pediatr Nephrol.* 2013;28(8):1261-1266.

106. Afsar B, Agca E, Turk S. Comparison of erythropoietin resistance in hemodialysis patients using calcitriol, cinacalcet, or paricalcitol. *J Clin Pharmacol.* 2015;55(11):1280-1285.

107. Coe LM, Madathil SV, Casu C, Lanske B, Rivella S, Sitara D. FGF-23 is a negative regulator of prenatal and postnatal erythropoiesis. *J Biol Chem.* 2014;289(14):9795-9810.

108. Vadakke Madathil S, Coe LM, Casu C, Sitara D. Klotho deficiency disrupts hematopoietic stem cell development and erythropoiesis. *Am J Pathol.* 2014;184(3):827-841.

109. Scialla JJ, Xie H, Rahman M, et al. Fibroblast growth factor-23 and cardiovascular events in CKD. *J Am Soc Nephrol.* 2014;25(2):349-360.

110. Scialla JJ, Wolf M. Roles of phosphate and fibroblast growth factor 23 in cardiovascular disease. *Nat Rev Nephrol.* 2014;10(5):268-278.

111. Icardi A, Paoletti E, De Nicola L, Mazzaferro S, Russo R, Cozzolino M. Renal anaemia and EPO hyporesponsiveness associated with vitamin D deficiency: the potential role of inflammation. *Nephrol Dial Transplant.* 2013;28(7):1672-1679.

112. Russo D, Morrone L, Di Iorio B, et al. Parathyroid hormone may be an early predictor of low serum hemoglobin concentration in patients with not advanced stages of chronic kidney disease. *J Nephrol.* 2014.

113. Gillespie IA, Macdougall IC, Richards S, et al. Factors precipitating erythropoiesis-stimulating agent responsiveness in a European haemodialysis cohort: case-crossover study. *Pharmacoepidemiol Drug Saf.* 2015;24(4):414-426.

114. Ernst JB, Becker T, Kuhn J, Gummert JF, Zittermann A. Independent association of circulating vitamin D metabolites with anemia risk in patients scheduled for cardiac surgery. *PloS one.* 2015;10(4):e0124751.

115. Lee JA, Hwang JS, Hwang IT, Kim DH, Seo JH, Lim JS. Low vitamin D levels are associated with both iron deficiency and anemia in children and adolescents. *Pediatr Hematol Oncol.* 2015;32(2):99-108.

116. Smith EM, Alvarez JA, Martin GS, Zughaier SM, Ziegler TR, Tangpricha V. Vitamin D deficiency is associated with anaemia among African Americans in a US cohort. *Br J Nutr.* 2015;113(11):1732-1740.

117. Ganji V, Zhang X, Tangpricha V. Serum 25-hydroxyvitamin D concentrations and prevalence estimates of hypovitaminosis D in the U.S. population based on assay-adjusted data. *J Nutr*. 2012;142(3):498-507.

118. Albitar S, Genin R, Fen-Chong M, Serveaux MO, Schohn D, Chuet C. High-dose alfacalcidol improves anaemia in patients on haemodialysis. *Nephrol Dial Transplant.* 1997;12(3):514-518.

119. Goicoechea M, Vazquez MI, Ruiz MA, Gomez-Campdera F, Perez-Garcia R, Valderrabano F. Intravenous calcitriol improves anaemia and reduces the need for erythropoietin in haemodialysis patients. *Nephron.* 1998;78(1):23-27.

120. Lin CL, Hung CC, Yang CT, Huang CC. Improved anemia and reduced erythropoietin need by medical or surgical intervention of secondary hyperparathyroidism in hemodialysis patients. *Ren Fail.* 2004;26(3):289-295.

121. Riccio E, Sabbatini M, Bruzzese D, et al. Effect of paricalcitol vs calcitriol on hemoglobin levels in chronic kidney disease patients: a randomized trial. *PloS one.* 2015;10(3):e0118174.

122. Sooragonda B, Bhadada SK, Shah VN, Malhotra P, Ahluwalia J, Sachdeva N. Effect of vitamin D replacement on hemoglobin concentration in subjects with concurrent iron-deficiency anemia and vitamin D deficiency: a randomized, single-blinded, placebo-controlled trial. *Acta Haematol.* 2015;133(1):31-35.

123. Atkinson MA, Kim JY, Roy CN, Warady BA, White CT, Furth SL. Hepcidin and risk of anemia in CKD: a cross-sectional and longitudinal analysis in the CKiD cohort. *Pediatr Nephrol.* 2015;30(4):635-643.

124. Pasricha SR, Atkinson SH, Armitage AE, et al. Expression of the iron hormone hepcidin distinguishes different types of anemia in African children. *Sci Transl Med.* 2014;6(235):235re233.

125. Kendrick J, Targher G, Smits G, Chonchol M. 25-Hydroxyvitamin D deficiency is independently associated with cardiovascular disease in the Third National Health and Nutrition Examination Survey. *Atherosclerosis.* 2009;205(1):255-260.

126. Lappe JM, Travers-Gustafson D, Davies KM, Recker RR, Heaney RP. Vitamin D and calcium supplementation reduces cancer risk: results of a randomized trial. *Am J Clin Nutr.* 2007;85(6):1586-1591.

127. Yamshchikov AV, Desai NS, Blumberg HM, Ziegler TR, Tangpricha V. Vitamin D for treatment and prevention of infectious diseases: a systematic review of randomized controlled trials. *Endocr Pract.* 2009;15(5):438-449.

128. Roy CN, Andrews NC. Anemia of inflammation: the hepcidin link. *Curr Opin Hematol.* Mar 2005;12(2):107-111.

129. Brigham KL. Predictive health: the imminent revolution in health care. *J Am Geriatr Soc.* 2010;58 Suppl 2:S298-302.

130. Whitt MC, Levin S, Ainsworth BE, Dubose KD. Evaluation of a two-part survey item to assess moderate physical activity: the Cross-Cultural Activity Participation Study. *J Women's Health.* 2003;12(3):203-212.

131. Levey AS, Bosch JP, Lewis JB, Greene T, Rogers N, Roth D. A more accurate method to estimate glomerular filtration rate from serum creatinine: a new prediction equation. Modification of Diet in Renal Disease Study Group. *Ann of Intern Med.* 1999;130(6):461-470.

132. Pearson TA, Mensah GA, Alexander RW, et al. Markers of inflammation and cardiovascular disease: application to clinical and public health practice: A statement for healthcare professionals from the Centers for Disease Control and Prevention and the American Heart Association. *Circulation.* 2003;107(3):499-511.

133. Ross AC, Manson JE, Abrams SA, et al. The 2011 report on dietary reference intakes for calcium and vitamin D from the Institute of Medicine: what clinicians need to know. *J Clini Endocrinol Metab.* 2011;96(1):53-58.

134. Hollowell JG, van Assendelft OW, Gunter EW, et al. Hematological and iron-related analytes--reference data for persons aged 1 year and over: United States, 1988-94. *Vital Health Stat 11.* 2005(247):1-156.

135. Mellibovsky L, Diez A, Perez-Vila E, et al. Vitamin D treatment in myelodysplastic syndromes. *Br J Haematol.* 1998;100(3):516-520.

136. Feairheller DL, Park JY, Sturgeon KM, et al. Racial differences in oxidative stress and inflammation: in vitro and in vivo. *Clin Transl Sci.* 2011;4(1):32-37.

137. Nazmi A, Victora CG. Socioeconomic and racial/ethnic differentials of C-reactive protein levels: a systematic review of population-based studies. *BMC Public Health.* 2007;7:212.

138. WHO. The global prevalence of anaemia in 2011. Geneva: World Health Organization; 2015.

139. Haider BA, Olofin I, Wang M, et al. Anaemia, prenatal iron use, and risk of adverse pregnancy outcomes: systematic review and meta-analysis. *BMJ.* 2013;346:f3443.

140. Stoltzfus RJ. Iron-deficiency anemia: reexamining the nature and magnitude of the public health problem. Summary: implications for research and programs. *J Nutr.* 2001;131(2S-2):697S-700S; discussion 700S-701S.

141. Dreyfuss ML, Stoltzfus RJ, Shrestha JB, et al. Hookworms, malaria and vitamin A deficiency contribute to anemia and iron deficiency among pregnant women in the plains of Nepal. *J Nutr.* 2000;130(10):2527-2536.

142. Siridamrongvattana S, Van Hoa N, Sanchaisuriya K, et al. Burden of anemia in relation to thalassemia and iron deficiency among Vietnamese pregnant women. *Acta Haematol.* 2013;130(4):281-287.

143. Nguyen PH, Gonzalez-Casanova I, Nguyen H, et al. Multicausal etiology of anemia among women of reproductive age in Vietnam. *Eur J Clin Nutr.* 2015;69(1):107-113.

144. Bener A, Al-Hamaq AO, Saleh NM. Association between vitamin D insufficiency and adverse pregnancy outcome: global comparisons. *Int J Women's Health.* 2013;5:523-531.

145. Smith EM, Tangpricha V. Vitamin D and anemia: insights into an emerging association. *Curr Opin Endocrinol, Diabetes Obes.* 2015;22(6):432-438.

146. Laillou A, Wieringa F, Tran TN, et al. Hypovitaminosis D and mild hypocalcaemia are highly prevalent among young Vietnamese children and women and related to low dietary intake. *PloS one.* 2013;8(5):e63979.

147. Khan NC, Hoan PV. Vietnam recommended dietary allowances 2007. *Asia Pac J Clin Nutr* 2008;409-15.

148. Nguyen PH, Lowe AE, Martorell R, et al. Rationale, design, methodology and sample characteristics for the Vietnam pre-conceptual micronutrient supplementation trial (PRECONCEPT): a randomized controlled study. *BMC Public Health.* 2012;12:898.

149. Tran DT. FFQ for adult in rural Vietnam. In: *An examination of the relationship between low body mass index and micronutrient malnutrition and the risk of*

morbidity in adults aged 18 to 60 in rural Vietnam [PhD Thesis]. Australia, The University of Newcastle; 2009.

150. NIN MOH. *Vietnamese Food Composition Table.* Hanoi: Medical Publishing House; 2007.

151. Yen H. *555 Vietnamese Dishes - Cooking Technique and Nutrient Contents.* Hanoi: Tu dien Bach khoa Publishing House; 2009.

152. Erhardt JG, Estes JE, Pfeiffer CM, Biesalski HK, Craft NE. Combined measurement of ferritin, soluble transferrin receptor, retinol binding protein, and C-reactive protein by an inexpensive, sensitive, and simple sandwich enzyme-linked immunosorbent assay technique. *J Nutr.* 2004;134(11):3127-3132.

153. WHO. Basic laboratory methods in medical parasitology. Geneva: World Health Organization; 1991.

154. Cogill B. Anthropometric Indicators Measurement Guide. Washington, DC: Food and Nutrition Technical Assistance (FANTA) Project, FHI 360; 2003.

155. Gwatkin DR Rustein S, Johnsin K, Pande RP, Wagstaff A. *Socio-economic differences in health, nutrition, and population in Bangladesh.* Washington, D.C.: The World Bank's Health and Population Advisory Service; 2000.

156. Coates J, Swindale A, Bilinsky P. *Household Food Insecurity Access Scale (HFIAS) for Measurement of Household Food Access: Indicator Guide (v. 3).* Washington, D.C.: Food and Nutrition Technical Assistance Project, Academy for Educational Development; 2007.

157. Thurnham DI, McCabe LD, Haldar S, Wieringa FT, Northrop-Clewes CA, McCabe GP. Adjusting plasma ferritin concentrations to remove the effects of

subclinical inflammation in the assessment of iron deficiency: a meta-analysis. *Am J Clin Nutr*. 2010;92(3):546-555.

158. Dien le N, Thang NM, Bentley ME. Food consumption patterns in the economic transition in Vietnam. *Asia Pac J Clin Nutr.* 2004;13(1):40-47.

159. Thang NM, Popkin BM. Patterns of food consumption in Vietnam: effects on socioeconomic groups during an era of economic growth. *Eur J Clin Nutr.* 2004;58(1):145-153.

160. IOM (Institute of Medicine). *Dietary Reference Intakes for Energy, Carbohydrate, Fiber, Fat, Fatty Acids, Cholesterol, Protein, and Amino Acids (Macronutrients).* Washington, DC: The National Academies Press; 2005.

161. Nguyen PH, Strizich G, Lowe A, et al. Food consumption patterns and associated factors among Vietnamese women of reproductive age. *Nutr J.* 2013;12:126.

162. Nguyen PH, Nguyen H, Gonzalez-Casanova I, et al. Micronutrient intakes among women of reproductive age in Vietnam. *PloS one.* 2014;9(2):e89504.

163. Hanieh S, Ha TT, Simpson JA, et al. Maternal vitamin D status and infant outcomes in rural Vietnam: a prospective cohort study. *PloS one.* 2014;9(6):e99005.

164. Nguyen HT, von Schoultz B, Nguyen TV, et al. Vitamin D deficiency in northern Vietnam: prevalence, risk factors and associations with bone mineral density. *Bone.* 2012;51(6):1029-1034.

165. Hsu CY, McCulloch CE, Curhan GC. Epidemiology of anemia associated with chronic renal insufficiency among adults in the United States: results from the

Third National Health and Nutrition Examination Survey. *J Am Soc Nephrol.* 2002;13(2):504-510.

166. LaClair RE, Hellman RN, Karp SL, et al. Prevalence of calcidiol deficiency in CKD: a cross-sectional study across latitudes in the United States. *Am J Kidney Dis.* 2005;45(6):1026-1033.

167. Groenveld HF, Januzzi JL, Damman K, et al. Anemia and mortality in heart failure patients a systematic review and meta-analysis. *J Am Coll Cardiol.* 2008;52(10):818-827.

168. Kim DH, Sabour S, Sagar UN, Adams S, Whellan DJ. Prevalence of hypovitaminosis D in cardiovascular diseases (from the National Health and Nutrition Examination Survey 2001 to 2004). *Am J Cardiol.* 2008;102(11):1540-1544.

169. Kearns MD, Binongo JN, Watson D, et al. The effect of a single, large bolus of vitamin D in healthy adults over the winter and following year: a randomized, double-blind, placebo-controlled trial. *Eur J Clin Nutr.* 2015;69(2):193-197.

170. Butterfield AM, Luan P, Witcher DR, et al. A dual-monoclonal sandwich ELISA specific for hepcidin-25. *Clin Chem.* 2010;56(11):1725-1732.

171. Troutt JS, Rudling M, Persson L, et al. Circulating human hepcidin-25 concentrations display a diurnal rhythm, increase with prolonged fasting, and are reduced by growth hormone administration. *Clin Chem.* 2012;58(8):1225-1232.

172. Thomas CE, Guillet R, Queenan RA, et al. Vitamin D status is inversely associated with anemia and serum erythropoietin during pregnancy. *Am J Clin Nutr.* 2015;102(5):1088-1095.

173. Carvalho C, Isakova T, Collerone G, et al. Hepcidin and disordered mineral metabolism in chronic kidney disease. *Clin Nephrol.* 2011;76(2):90-98.

174. Ernst JB, Tomaschitz A, Grubler MR, et al. Vitamin D Supplementation and Hemoglobin Levels in Hypertensive Patients: A Randomized Controlled Trial. *Int J Endocrinol.* 2016;2016:6836402.

175. Corwin HL, Gettinger A, Pearl RG, et al. The CRIT Study: Anemia and blood transfusion in the critically ill--current clinical practice in the United States. *Crit Care Med.* 2004;32(1):39-52.

176. Vincent JL, Baron JF, Reinhart K, et al. Anemia and blood transfusion in critically ill patients. *JAMA.* 2002;288(12):1499-1507.

177. Dejam A, Malley BE, Feng M, et al. The effect of age and clinical circumstances on the outcome of red blood cell transfusion in critically ill patients. *Crit Care.* 2014;18(4):487.

178. Pieracci FM, Stovall RT, Jaouen B, et al. A multicenter, randomized clinical trial of IV iron supplementation for anemia of traumatic critical illness*. *Crit Care Med.* 2014;42(9):2048-2057.

179. Zarychanski R, Turgeon AF, McIntyre L, Fergusson DA. Erythropoietin-receptor agonists in critically ill patients: a meta-analysis of randomized controlled trials. *CMAJ.* 2007;177(7):725-734.

180. Sihler KC, Napolitano LM. Anemia of inflammation in critically ill patients. *J Intensive Care Med.* 2008;23(5):295-302.

181. Smith EM, Alvarez JA, Kearns MD, et al. High-dose vitamin D3 reduces circulating hepcidin concentrations: A pilot, randomized, double-blind, placebo-controlled trial in healthy adults. *Clin Nutr.* Jun 27 2016 [Epub ahead of print].

182. Jeng L, Yamshchikov AV, Judd SE, et al. Alterations in vitamin D status and anti-microbial peptide levels in patients in the intensive care unit with sepsis. *J Transl Med.* 2009;7:28.

183. Kempker JA, West KG, Kempker RR, et al. Vitamin D status and the risk for hospital-acquired infections in critically ill adults: a prospective cohort study. *PloS one.* 2015;10(4):e0122136.

184. Amrein K, Schnedl C, Holl A, et al. Effect of high-dose vitamin D3 on hospital length of stay in critically ill patients with vitamin D deficiency: the VITdAL-ICU randomized clinical trial. *JAMA.* 2014;312(15):1520-1530.

185. Han JE, Jones JL, Tangpricha V, et al. High dose vitamin D administration in ventilated intensive care unit patients: a pilot double blind randomized placebo controlled trial. *J Clin Transl Endocrinol.* 2016;4:59-65.

186. Madar AA, Stene LC, Meyer HE, Brekke M, Lagerlov P, Knutsen KV. Effect of vitamin D3 supplementation on iron status: a randomized, double-blind, placebo-controlled trial among ethnic minorities living in Norway. *Nutr J.* 2016;15(1):74.

187. Ernst JB, Zittermann A, Pilz S, et al. Independent associations of vitamin D metabolites with anemia in patients referred to coronary angiography: the LURIC study. *Eur J Nutr.* Jan 8 2016.

188. Walsh TS, Boyd JA, Watson D, et al. Restrictive versus liberal transfusion strategies for older mechanically ventilated critically ill patients: a randomized pilot trial. *Crit Care Med.* 2013;41(10):2354-2363.

189. Zilberberg MD, Stern LS, Wiederkehr DP, Doyle JJ, Shorr AF. Anemia, transfusions and hospital outcomes among critically ill patients on prolonged acute mechanical ventilation: a retrospective cohort study. *Crit Care.* 2008;12(2):R60.

190. Hebert PC, Wells G, Blajchman MA, et al. A multicenter, randomized, controlled clinical trial of transfusion requirements in critical care. Transfusion Requirements in Critical Care Investigators, Canadian Critical Care Trials Group. *New Engl J Med.* 1999;340(6):409-417.

191. Thomas J, Jensen L, Nahirniak S, Gibney RT. Anemia and blood transfusion practices in the critically ill: a prospective cohort review. *Heart Lung.* 2010;39(3):217-225.

192. Flegal KM, Kruszon-Moran D, Carroll MD, Fryar CD, Ogden CL. Trends in Obesity Among Adults in the United States, 2005 to 2014. *JAMA.* 2016;315(21):2284-2291.

193. U.S. Department of Health and Human Services and U.S. Department of Agriculture. *2015-2020 Dietary Guidelines for Americans*. 8th ed. Dec 2015. Available at http://health.gov/dietaryguidelines/2015/guidelines/.

194. von Drygalski A, Biller J. Anemia in cystic fibrosis: incidence, mechanisms, and association with pulmonary function and vitamin deficiency. *Nutr Clin Pract.* 2008;23(5):557-563.

195. Thomas R, Kanso A, Sedor JR. Chronic kidney disease and its complications. *Prim Care.* 2008;35(2):329-344, vii.

196. Charytan DM, Fishbane S, Malyszko J, McCullough PA, Goldsmith D. Cardiorenal Syndrome and the Role of the Bone-Mineral Axis and Anemia. *Am J Kidney Dis.* 2015;66(2):196-205.

197. Guagnozzi D, Lucendo AJ. Anemia in inflammatory bowel disease: a neglected issue with relevant effects. *World J Gastroenterol.* 2014;20(13):3542-3551.

198. Del Pinto R, Pietropaoli D, Chandar AK, Ferri C, Cominelli F. Association between inflammatory bowel disease and vitamin D deficiency: a systematic review and meta-analysis. *Inflamm Bowel Dis.* 2015;21(11):2708-2717.

199. Yetley EA. Assessing the vitamin D status of the US population. *Am J Clin Nutr*. 2008;88(2):558S-564S.

200. Fulgoni VL 3rd, Keast DR, Bailey RL, Dwyer J. Food, fortificatnts, and supplements: Where do Americans get their nutrients? *J Nutr*. 2011;141(10):1847-1854.

201. Black LJ, Seamans KM, Cashman KD, Kiely M. An updated systematic review and meta-analysis of the efficacy of vitamin D food fortification. *J Nutr*. 2012;142(6):1102-1108.

202. O'Neil CE, Keast DR, Fulgoni VL, Nicklas TA. Food sources of energy and nutrients among adults in the US: NHANES 2003-2006. *Nutrients.* 2012;4(12):2097-2120.

203. Newmark HL, Heaney RP, Lachance PA. Should calcium and vitamin D be added to the current enrichment program for cereal-grain products. *Am J Clin Nutr*. 2004;80(2):264-270.

204. Hayes A and Cashman KD. Food-based solutions for vitamin D deficiency: putting policy into practice and the key role for research. *Proc Nutr Soc*. 2016. [Epub ahead of print]. DOI:10.1017/S0029665116000756

205. Nissenson AR, Goodnough LT, Dubois RW. Anemia: not just an innocent bystander? *Arch Intern Med.* 2003;163(12):1400-1404.

206. Kovesdy CP, Trivedi BK, Kalantar-Zadeh K, Anderson JE. Association of anemia with outcomes in men with moderate and severe chronic kidney disease. *Kidney Int.* 2006;69(3):560-564.

207. Odden MC, Whooley MA, Shlipak MG. Association of chronic kidney disease and anemia with physical capacity: the heart and soul study. *J Am Soc Nephrol.* 2004;15(11):2908-2915.

208. Gifford AH. Hemoglobin </= 12.9 g/dl predicts risk of antibiotic treatment in cystic fibrosis. *J Cyst Fibros.* 2014;13(1):114-115.

209. Reid DW, Withers NJ, Francis L, Wilson JW, Kotsimbos TC. Iron deficiency in cystic fibrosis: relationship to lung disease severity and chronic Pseudomonas aeruginosa infection. *Chest.* 2002;121(1):48-54.

210. Fishbane S, Nissenson AR. Anemia management in chronic kidney disease. *Kidney Int.* 2010(117):S3-9.

211. Lawler EV, Gagnon DR, Fink J, et al. Initiation of anaemia management in patients with chronic kidney disease not on dialysis in the Veterans Health Administration. *Nephrol Dial Transplant.* 2010;25(7):2237-2244.

212. Pfeffer MA, Burdmann EA, Chen C-Y, et al. A Trial of Darbepoetin Alfa in Type 2 Diabetes and Chronic Kidney Disease. *N Engl J Med.* 2009;361(21):2019-2032.

213. Regidor DL, Kopple JD, Kovesdy CP, et al. Associations between changes in hemoglobin and administered erythropoiesis-stimulating agent and survival in hemodialysis patients. *J Am Soc Nephrol.* 2006;17(4):1181-1191.

214. Thomas A, Peterson LE. Reduction of costs for anemia-management drugs associated with the use of ferric citrate. *Int J Nephrol Renovasc Dis.* 2014;7:191-201.

215. Miskulin DC, Majchrzak K, Tighiouart H, et al. Ergocalciferol Supplementation in Hemodialysis Patients With Vitamin D Deficiency: A Randomized Clinical Trial. *J Am Soc Nephrol.* 2015.

216. Lea JP, Norris K, Agodoa L. The role of anemia management in improving outcomes for African-Americans with chronic kidney disease. *Am J Nephrol.* 2008;28(5):732-743.

217. Gutierrez OM, Isakova T, Andress DL, Levin A, Wolf M. Prevalence and severity of disordered mineral metabolism in Blacks with chronic kidney disease. *Kidney Int.* 2008;73(8):956-962.

218. Gifford AH, Moulton LA, Dorman DB, et al. Iron homeostasis during cystic fibrosis pulmonary exacerbation. *Clin Transl Sci.* 2012;5(4):368-373.

219. O'Connor T M, McGrath DS, Short C, O'Donnell M J, Sheehy M, Bredin CP. Subclinical anaemia of chronic disease in adult patients with cystic fibrosis. *J Cyst Fibros.* 2002;1(1):31-34.

220. Pond MN, Morton AM, Conway SP. Functional iron deficiency in adults with cystic fibrosis. *Respir Med.* 1996;90(7):409-413.

221. Fischer R, Simmerlein R, Huber RM, Schiffl H, Lang SM. Lung disease severity, chronic inflammation, iron deficiency, and erythropoietin response in adults with cystic fibrosis. *Pediatr Pulmonol.* 2007;42(12):1193-1197.

222. Kendrick J, Targher G, Smits G, Chonchol M. 25-Hydroxyvitamin D deficiency and inflammation and their association with hemoglobin levels in chronic kidney disease. *Am J Nephrol.* 2009;30(1):64-72.

223. Jones KS, Assar S, Harnpanich D, et al. $25(OH)D_2$ half-life is shorter than $25(OH)D_3$ half-life and is influenced by DBP concentration and genotype. *J Clin Endocrinol Metab.* 2014;99(9):3373-3381.

224. Alvarez JA, Law J, Coakley KE, et al. High-dose cholecalciferol reduces parathyroid hormone in patients with early chronic kidney disease: a pilot, randomized, double-blind, placebo-controlled trial. *Am J Clin Nutr.* 2012;96(3):672-679.

225. Chandra P, Binongo JN, Ziegler TR, et al. Cholecalciferol (vitamin D3) therapy and vitamin D insufficiency in patients with chronic kidney disease: a randomized controlled pilot study. *Endocr Pract.* 2008;14(1):10-17.

226. Kidney Disease: Improving Global Outcomes (KDIGO) CKD Work Group. KDIGO 2012 Clinical Practice Guideline for the Evaluation and Management of Chronic Kidney Disease. Kidney Int Suppl. 2013;3(1):1-150.

227. Grossmann RE, Zughaier SM, Kumari M, et al. Pilot study of vitamin D supplementation in adults with cystic fibrosis pulmonary exacerbation: a randomized, controlled trial. *Dermato-endocrinol.* 2012;4(2):191-197.

228. Bischoff-Ferrari HA, Dawson-Hughes B, Orav EJ, et al. Monthly high-dose vitamin D treatment for the prevention of functional decline: a randomized clinical trial. *JAMA Intern Med.* 2016;176(2):175-183

229. Moretti D, Goede JS, Zeder C, et al. Oral iron supplements increase hepcidin and decrease iron absorption from daily or twice-daily doses in iron-depleted young women. *Blood.* 2015;126(17):1981-1989.

230. Korolnek T, Hamza I. Macrophages and iron trafficking at the birth and death of red cells. *Blood.* 2015;125(19):2893-2897.

231. Alvarez JA, Zughaier SM, Law J, et al. Effects of high-dose cholecalciferol on serum markers of inflammation and immunity in patients with early chronic kidney disease. *Eur J Clin Nutr.* 2013;67(3):264-269.

232. Grossmann RE, Zughaier SM, Liu S, Lyles RH, Tangpricha V. Impact of vitamin D supplementation on markers of inflammation in adults with cystic fibrosis hospitalized for a pulmonary exacerbation. *Eur J Clin Nutr.* 2012;66(9):1072-1074.

233. Lee P, Peng H, Gelbart T, Wang L, Beutler E. Regulation of hepcidin transcription by interleukin-1 and interleukin-6. *Proc Natl Acad Sci USA.* 2005;102(6):1906-1910.

234. Ludwiczek S, Aigner E, Theurl I, Weiss G. Cytokine-mediated regulation of iron transport in human monocytic cells. *Blood.* 2003;101(10):4148-4154.

235. Taniguchi S, Dai C-H, Price JO, Krantz SB. Interferon γ downregulates stem cell factor and erythropoietin receptors but not insulin-like growth factor-I receptors in human erythroid colony-forming cells. *Blood*. 1997;90(6):2244-52.

236. Wang CQ, Udupa KB, Lipschitz DA. Interferon-γ exerts its negative regulatory effect primarily on the earliest stages of murine erythroid progenitor cell development. *J Cell Physiol.* 1995;162(1):134-138.

237. Sun CC, Vaja V, Babitt JL, Lin HY. Targeting the hepcidin-ferroportin axis to develop new treatment strategies for anemia of chronic disease and anemia of inflammation. *Am J Hematol.* 2012;87(4):392-400.

238. Annuk M, Zilmer M, Lind L, Linde T, Fellström B. Oxidative stress and endothelial function in chronic renal failure. *J Am Soc Nephrol.* 2001;12(12):2747-2752.

239. Cachofeiro V, Goicochea M, de Vinuesa SG, Oubina P, Lahera V, Luno J. Oxidative stress and inflammation, a link between chronic kidney disease and cardiovascular disease. *Kidney Int.* 2008;74(S111):S4-S9.

240. Terawaki H, Yoshimura K, Hasegawa T, et al. Oxidative stress is enhanced in correlation with renal dysfunction: Examination with the redox state of albumin. *Kidney Int.* 2004;66(5):1988-1993.

241. Celik G, Yontem M, Bilge M, Cilo M, Unaldi M. The relationship between the antioxidant system and anaemia in haemodialysis patients. *J Int Med Res.* 2011;39(5):1954-1960.

242. Kato A, Odamaki M, Hishida A. Blood 8-hydroxy-2'-deoxyguanosine is associated with erythropoietin resistance in haemodialysis patients. *Nephrol Dial Transplant.* 2003;18(5):931-936.

243. Rybka J, Kedziora-Kornatowska K, Banas-Lezanska P, et al. Interplay between the pro-oxidant and antioxidant systems and proinflammatory cytokine levels, in relation to iron metabolism and the erythron in depression. *Free Radic Biol Med.* 2013;63:187-194.

244. Ganguli A, Kohli HS, Khullar M, Lal Gupta K, Jha V, Sakhuja V. Lipid peroxidation products formation with various intravenous iron preparations in chronic kidney disease. *Ren Fail.* 2009;31(2):106-110.

245. Vaziri ND, Zhou XJ. Potential mechanisms of adverse outcomes in trials of anemia correction with erythropoietin in chronic kidney disease. *Nephrol Dial Transplant.* 2009;24(4):1082-1088.

246. Jain SK, Micinski D. Vitamin D upregulates glutamate cysteine ligase and glutathione reductase, and GSH formation, and decreases ROS and MCP-1 and IL-8 secretion in high-glucose exposed U937 monocytes. *Biochem Biophys Res Commun.* 2013;437(1):7-11.

247. Izquierdo MJ, Cavia M, Muniz P, et al. Paricalcitol reduces oxidative stress and inflammation in hemodialysis patients. *BMC Nephrol.* 2012;13:159.

248. Alvarez JA, Chowdhury R, Jones DP, et al. Vitamin D status is independently associated with plasma glutathione and cysteine thiol/disulphide redox status in adults. *Clin Endocrinol (Oxf).* 2014;81(3):458-66.

249. Romero JR, Youte R, Brown EM, et al. Parathyroid hormone ablation alters erythrocyte parameters that are rescued by calcium-sensing receptor gene deletion. *Eur J Haematol.* 2013;91(1):37-45.

250. Frazer DM, Anderson GJ. The regulation of iron transport. *BioFactors.* 2014;40(2):206-214.

251. McDade TW, Hawkley LC, Cacioppo JT. Psychosocial and behavioral predictors of inflammation in middle-aged and older adults: the Chicago health, aging, and social relations study. *Psychosom Med.* 2006;68(3):376-381.

252. Krieger N, Sidney S. Racial discrimination and blood pressure: the CARDIA Study of young black and white adults. *Am J Public Health.* 1996;86(10):1370-1378.

253. Das A. How does race get "under the skin"?: inflammation, weathering, and metabolic problems in late life. *Soc Sci Med.* 2013;77:75-83.

254. Al-Eisa ES, Alghadir AH, Gabr SA. Correlation between vitamin D levels and muscle fatigue risk factors based on physical activity in healthy older adults. *Clin Interv Aging.* 2016;11:513-522.

255. Houston DK, Cesari M, Ferrucci L, et al. Association between vitamin D status and physical performance: the InCHIANTI study. *J Gerontol A Biol Sci Med Sci.* 2007;62(4):440-446.

256. Toffanello ED, Perissinotto E, Sergi G, et al. Vitamin D and physical performance in elderly subjects: the Pro.V.A study. *PLoS One.* 2012;7(4):e34950.

257. Ucay O, Pouche M, Guiraud T, Besnier F, Pathak A, Labrunee M. Vitamin D deficiency related to physical capacity during cardiac rehabilitation. *Ann Phys Rehabil Med.* 2016. [Epub ahead of print].