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Vitamin D status and risk of anemia in patients with cardiovascular disease

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ZUSAMMENFASSUNG

Vitamin-D-Versorgung und Risiko einer Anämie bei Patienten mit kardiovaskulärer Erkrankung

Das Vorliegen eines Vitamin-D-Mangels sowie einer Anämie ist bei Patienten mit kardiovaskulärer Erkrankung (CVD) allgemein verbreitet. Beide Erkrankungen sind unabhängige Risikofaktoren für gesteigerte Morbidität und Mortalität. Epidemiologische Studien weisen auf eine inverse Assoziation zwischen der Vitamin-D-Versorgung und dem Risiko einer Anämie bei verschiedenen Personengruppen hin. Erste nicht-randomisierte Interventionsstudien zeigen einen Anstieg des Hämoglobin (Hb)-Spiegels durch die Gabe von Vitamin-D-Metaboliten. Jedoch werden noch immer adäquat designte, randomisierte kontrollierte Studien (RCT) mit Vitamin D benötigt, um zu klären, ob die Gabe von Vitamin D einen positiven Effekt auf das Vorliegen einer Anämie hat. Sollte sich dies bestätigen, könnte eine Vitamin-D-Supplementierung eine vielversprechende präventive oder therapeutische Option sein, um die Prävalenz eines Vitamin-D-Mangels sowie die Anämie-Prävalenz zu senken. Folglich war das Ziel der vorliegenden Arbeit 1) die Untersuchung der Assoziationen zwischen Vitamin-D-Metaboliten und Anämie bei Patienten mit CVD und zu ermitteln, ob 2) eine tägliche Vitamin-D-Supplementierung bei Patienten mit CVD die Hb-Werte verbessern und somit das Risiko einer Anämie verringern kann.

In zwei Querschnittsstudien (KAPITEL EINS und KAPITEL ZWEI) konnten signifikante, unabhängige, inverse Assoziationen zwischen den Vitamin-D-Metaboliten 25-Hydroxyvitamin D (25OHD) und 1,25-Dihydroxyvitamin D (1,25$[OH]_2$D) und dem Risiko einer Anämie bei herzchirurgischen Patienten sowie Patienten, die für eine Koronarangiographie vorgesehen waren, gezeigt werden. In beiden Studien konnte eine stärkere Assoziation zwischen dem Vorliegen einer Anämie und niedrigen 1,25$(OH)_2$D Spiegeln (<40 pmol/l) als defizitären 25OHD Werten (<30 nmol/l) nachgewiesen werden. In zwei Sekundäranalysen zweier RCTs wurden die Effekte eines täglichen Vitamin D_3 Supplements auf die Hb-Werte und das Risiko einer Anämie im Vergleich zu Placebo in Patienten mit Bluthochdruck für acht Wochen (2,800 IE täglich; KAPITEL DREI) und in herzinsuffizienten Patienten für 36 Monate (4,000 IE täglich; KAPITEL VIER) untersucht. Beide Studien zeigten, dass eine tägliche Vitamin-D-Supplementierung zu keiner Verbesserung der Hb-Werte bei Patienten mit CVD führt. Vergleichbare Ergebnisse wurden in Subgruppenanalysen bei Patienten mit initialen 25OHD Werten <30 nmol/l und bei Patienten mit initialer eGFR <60 mL/min/1.73 m^2 gefunden. Die Vitamin-D-Gabe konnte die Anämie-Prävalenz bei Patienten mit Bluthochdruck nicht signifikant verändern (7,5% Baseline vs. 7,5% Studienende), wohingegen die Anämie-Prävalenz bei herzinsuffizienten Patienten in der Vitamin-D-Gruppe um 12,9% anstieg.

Zusammenfassend spricht sich diese Arbeit, trotz der vielversprechenden Ergebnisse der Querschnittsstudien, gegen einen klinisch relevanten positiven Effekt von Vitamin-D-Supplementen auf den Hb-Spiegel bei Patienten mit CVD aus. Da niedrige 1,25$(OH)_2$D Spiegel stärker mit dem Risiko einer Anämie assoziiert sind als defizitäre 25OHD Spiegel und erste Interventionsstudien mit der Gabe von 1,25$(OH)_2$D vielversprechende Ergebnisse zeigten, sollten zukünftige RCTs die Gabe aktiver Vitamin-D-Metabolite bei anämischen und Vitamin-D-defizitären Patienten genauer untersuchen.

SUMMARY

Vitamin D status and risk of anemia in patients with cardiovascular disease

Vitamin D deficiency and anemia are prevalent in patients with cardiovascular disease (CVD), and both are independent risk factors for increased morbidity and mortality. Epidemiological studies suggest an inverse association between vitamin D status and anemia risk in several populations. Earlier non-randomized intervention studies reported an increase in hemoglobin (Hb) values by administration of vitamin D metabolites. However, adequately powered, randomized controlled trials (RCT) with vitamin D are still warranted to assess whether vitamin D has beneficial effects on anemia. If confirmed, vitamin D supplementation could be a promising preventive or therapeutic option to decrease the prevalence of vitamin D deficiency and anemia. Thus, the overall aim of this thesis was 1) to determine the associations between vitamin D metabolites and anemia in patients with CVD and 2) to determine if daily vitamin D supplementation can improve Hb levels and therefore anemia risk in CVD patients.

Two cross-sectional studies (CHAPTER ONE and CHAPTER TWO) showed significant independent inverse associations between the vitamin D metabolites 25-hydroxyvitamin D (25OHD) and 1,25-dihydroxyvitamin D (1,25[OH]$_2$D) and anemia risk in cardiac surgical patients and patients referred for coronary angiography. In both studies, the association of anemia was stronger with low circulating 1,25(OH)$_2$D levels (<40 pmol/l) than it was with deficient circulating 25OHD levels (<30 nmol/l). In two secondary analyses of RCTs the effects of a daily vitamin D$_3$ supplement on Hb levels and anemia risk versus placebo were examined in hypertensive patients for eight weeks (2,800 IU daily; CHAPTER THREE) and in heart failure patients for 36 months (4,000 IU daily; CHAPTER FOUR). Both investigations indicate that a daily vitamin D supplement does not improve Hb values in patients with CVD. Comparable results were shown in the subgroups of patients with initial 25OHD levels <30 nmol/l and in patients with initial eGFR values <60 mL/min/1.73 m^2. In hypertensive patients, vitamin D treatment did not influence anemic status significantly (7.5% baseline vs. 7.5% study termination), whereas in patients with heart failure anemia status significantly increased by 12.9% in the vitamin D group.

In conclusion, despite the promising results of the cross-sectional studies, this thesis argues against Hb-improving effects of vitamin D$_3$ supplements that are of clinical relevance in patients with CVD. However, since 1,25(OH)$_2$D concentrations show a stronger independent association with anemia risk than 25OHD levels and first interventional studies using 1,25(OH)$_2$D administration showed promising results, future studies should explore the administration of active vitamin D metabolites in anemic and vitamin D deficient patients in more detail.

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ABBREVIATIONS

$1,25(OH)_2D$	1,25-Dihydroxyvitamin D
25OHD	25-Hydroxyvitamin D
ACE	Angiotensin-converting Enzyme
AT	Angiotensin Receptor
BMI	Body Mass Index
CAD	Coronary Artery Disease
CALCITOP	Calcitriol and clinical outcome in cardiac surgical patients
CI	Confidence Interval
CKD	Chronic Kidney Disease
COPD	Chronic Obstructive Pulmonary Disease
CRP	C-reactive Protein
CVD	Cardiovascular Disease
DBP	Vitamin D-Binding Protein
eGFR	Estimated Glomerular Filtration Rate
EPO	Erythropoietin
ESA	Erythropoiesis-stimulating Agents
EVITA	Effect of Vitamin D on Mortality in Heart Failure
FGF-23	Fibroblast Growth Factor-23
Hb	Hemoglobin
HF	Heart Failure
Htc	Hematocrit
IOM	Institute of Medicine
IU	International Unit
LURIC	Ludwigshafen Risk and Cardiovascular Health Study
LVEF	Left Ventricular Ejection Fraction
MCH	Mean Corpuscular Hemoglobin
MCHC	Mean Corpuscular Hemoglobin Concentration
MCV	Mean Corpuscular Volume

MI	Myocardial Infarction
OR	Odds Ratio
PAOD	Peripheral Arterial Occlusive Disease
PTH	Parathyroid Hormone
RCT	Randomized Controlled Trial
RDW	Red Blood Cell Distribution Width
sTfR	Soluble Transferrin Receptor
UV	Ultraviolet
VDR	Vitamin D Receptor

GENERAL INTRODUCTION

Vitamin D is a fat soluble vitamin with two dominant forms: vitamin D_2 (ergocalciferol) and vitamin D_3 (cholecalciferol). For humans, there are three ways to achieve adequate levels of vitamin D supply [1-3]:

- through diet (vitamin D_2 and vitamin D_3)
- through supplements (only vitamin D_3 supplements are available in Germany)
- and through skin exposure to solar ultraviolet (UV) B radiation (wavelength 290-315 nm).

When the skin is exposed to UVB radiation, the liver-derived precursor 7-dehydrocholesterol is converted to pre-vitamin D, which in turn isomerizes to vitamin D [4]. The extent of this endogenous vitamin D synthesis is influenced by different factors like latitude and seasons. In Europe, the endogenous skin synthesis contributes up to 90% of the total human vitamin D status in June and July [5]. However, the endogenous synthesis may be completely missing in January. Therefore, dietary intake of vitamin D comprises 10% to 100% of circulating levels of vitamin D, depending on the season. Vitamin D_2 is naturally present in fungi, such as mushrooms and yeast. Vitamin D_3 is found in food of animal origin such as eggs and oily fish e.g. eel, herring, and salmon.

Once in the circulation, vitamin D is predominantly (~88%) transported by the vitamin D-binding protein (DBP) [6]. The remaining ~12% is loosely bound to albumin, and only ~0.03% of 25OHD is found in its free form [6,7]. The biologically inactive vitamin D is then converted by a hepatic hydroxylase (25-hydroxylase [CYP2R1-hydroxylase]) into 25-hydroxyvitamin D (25OHD). In a second hydroxylation, 25OHD is converted mainly in the kidney into its active hormonal form 1,25-dihydroxyvitamin D ($1,25[OH]_2D$) by a 1α-hydroxylase (CYP27B1-hxdroxylase). This endocrine production of $1,25(OH)_2D$ is tightly controlled by parameters of bone and mineral metabolism, e.g. parathyroid hormone (PTH) and the phosphaturic hormone fibroblast growth factor (FGF)-23 [1]. While PTH stimulates the 1α-hydroxylation, FGF-23 and $1,25(OH)_2D$ itself inhibit this enzymatic reaction. Major endocrine $1,25(OH)_2D$ actions include regulation of the bone and mineral metabolism by regulating calcium and phosphate homeostasis. The active vitamin D can also act in a paracrine and autocrine fashion because 1α-hydroxylase expression has been reported in several extrarenal cells, including cells of the cardiovascular system [8].

The unbound active vitamin D can enter target cells and interact with the ligand-binding domain of the cytosolic vitamin D receptor (VDR) [9,10]. The expression of the VDR has been identified in almost all human tissues. Directly or indirectly, 3% of the human genome is regulated by the vitamin D endocrine system and the number of genes regulated by $1,25(OH)_2D$ is still growing [11].

Degradation of 1,25(OH)$_2$D and other vitamin D metabolites is initiated by 24-hydroxylation (24-hydroxylase [CYP24A1-hydroxylase]). This enzymatic process, which can be induced by VDR activation itself, can thereby prevent vitamin D intoxication as part of an autoregulatory loop [8].

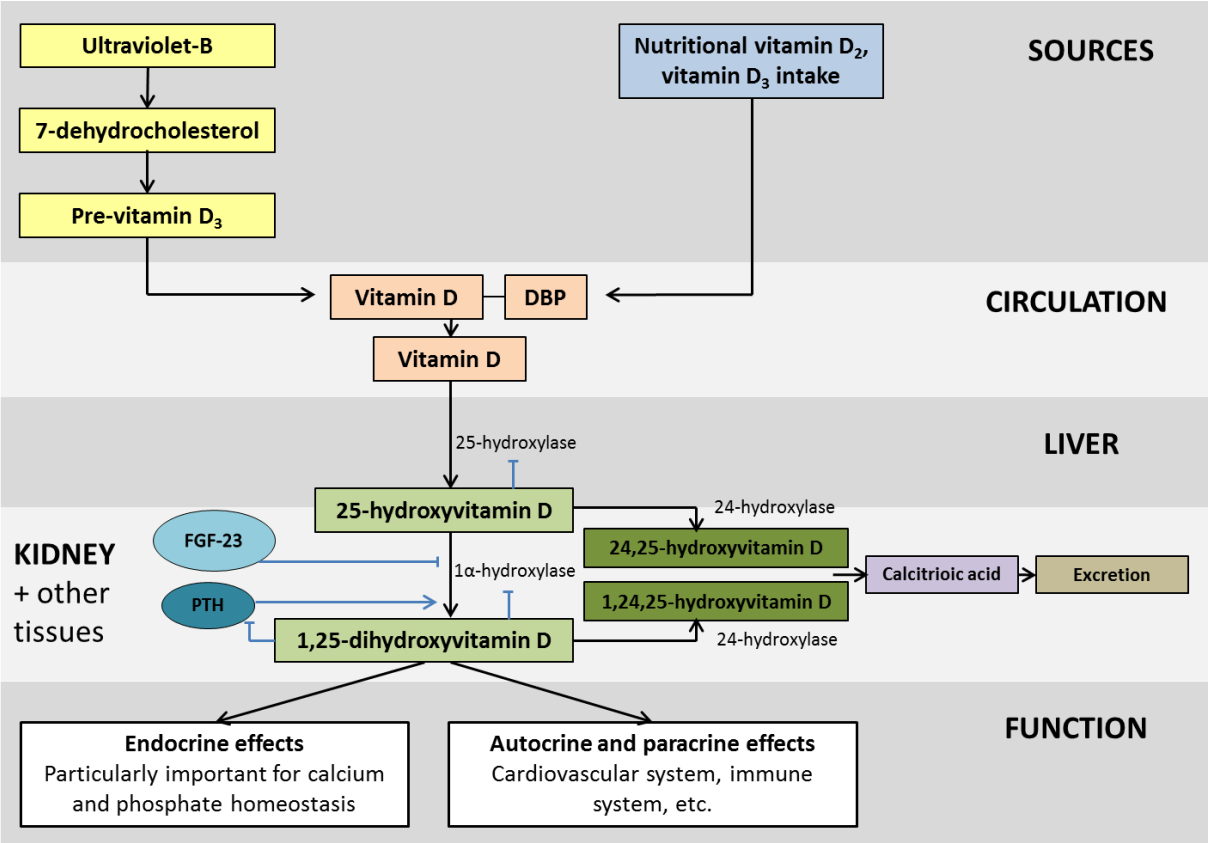

Figure 1: Human metabolism of Vitamin D (adapted from [12])

The best indicator for defining human vitamin D status is the circulating 25OHD level [13], because it is the major circulating vitamin D metabolite that best reflects vitamin D supply from all sources, e.g. skin synthesis, dietary and supplemental intake [14]. As noted before, circulating 25OHD levels are influenced by many different factors like geographic factors (latitude and altitude) and individual factors (skin pigmentation, outdoor activities, clothing, dietary habits, sunscreen use and body mass index) [15-19].

To this day, the optimal 25OHD concentration for maximizing the health benefits of vitamin D is still being debated [7]. Various cut-offs regarding the optimal vitamin D ranges have been proposed in the scientific community. The Institute of Medicine (IOM) classifies values between 50-125 nmol/l as being adequate [20]. This recommendation is based on the fact that the IOM committee found 25OHD levels >50 nmol/l to be needed for good bone health for practically all individuals and that serum levels of 50 nmol/l are sufficient to meet the vitamin D

requirements in 97.5% of the general population. In detail, the IOM classifies vitamin D values <30 nmol/l as deficient, 30-49.9 nmol/l as insufficient, 50-125 nmol/l as adequate and >125 nmol/l as potentially harmful. However, since vitamin D deficiency has been associated with a large number of conditions additionally to its classical effects on bone health, recommendations regarding higher cut-offs have been proposed. An international expert panel has recommended a target 25OHD range of 75-100 nmol/l [21]. Of note, this recommendation concerns adult patients with, or individuals at risk for, classical applications of vitamin D such as osteoporosis, chronic kidney disease (CKD) and endocrinopathies. In addition, these recommendations apply to adult patients >18 years at risk for diseases in which the role of vitamin D is emerging, e.g. autoimmune diseases, cancer and cardiovascular disease (CVD) [21]. The Endocrine Society Practice Guidelines also define sufficient 25OHD levels as being above 75 nmol/l to maximize the effect of vitamin D on calcium, bone and muscle metabolism [22]. Again, the Task Force supports its argument with numerous epidemiological studies suggesting that 25OHD levels >75 nmol/l may have additional health benefits such as reducing the risk of common cancers, autoimmune and infectious diseases, type 2 diabetes mellitus and CVD [22]. However, according to the German Nutrition Society (DGE), the evidence regarding the well-known associations between vitamin D supply and falls and fractures is the only evidence to be regarded as ‘convincing‘. Additionally, the DGE sees ‘likely’ evidence regarding all-cause mortality among the elderly. For other diseases such as cancer, type 2 diabetes mellitus, hypertension and CVD, there is either ‘probable’ evidence that there is no association with the supply of vitamin D or the evidence regarding an inverse association or lack of an association with vitamin D supply was considered to be ‘possible’ or ‘insufficienct’ [23]. Based on these findings, the DGE considers 25OHD levels of ≥50 nmol/l an indicator of an optimal vitamin D status. In conclusion, there is broad agreement to prevent and treat 25OHD levels <50 nmol/l [24].

Vitamin D deficiency is claimed as a significant worldwide health problem [25]. Hagenau et al. [26] reported a global 25OHD level of 54 nmol/l, which is close to the insufficiency range. In a systematic review of 195 studies in 44 different countries, 37.3% of the studies reported mean values below 50 nmol/l [27]. In the European Union, 13% and 40.4% of individuals were found to have 25OHD concentrations <30 nmol/l and <50 nmol/l, respectively [28]. A national health survey in German adults showed that mean serum 25OHD level was 45.6 nmol/l [29] with 61.6% of the individuals having serum 25OHD levels <50 nmol/l. Compared to healthy controls, various groups of patients are at a higher risk of being vitamin D deficient. In Germany, approximately 25% of the all-cause patients even have 25OHD values <30 nmol/l [30]. In cardiac surgical patients, the prevalence of 25OHD values <30 nmol/l is between 15% and 38% [31-33]. Regarding heart failure patients, the prevalence varies between 56% and 87.8% for 25OHD values <50 nmol/l [34-36] and between 28% and 66.7% for 25OHD values even

below 25 nmol/l [37,38]. Short-term solar UVB exposure and/or use of oral supplementation are effective methods for improving vitamin D status. The German, Austrian and Swiss Societies for Nutrition (D_A_CH) recommend a daily intake of 20 µg (800 International Units [IU]) vitamin D for children, adolescents and adults when endogenous synthesis is missing. In contrast, the median vitamin D intake of adults living in Germany is 2-4 µg [23].

According to the World Health Organization (WHO), anemia is considered a condition in which the number of red blood cells or the oxygen-carrying hemoglobin (Hb) is insufficient to meet physiological needs and is defined as an Hb concentration less than 13 g/dL for men and less than 12 g/dL for women. With an estimated prevalence of 24.8%, it affects nearly one out of four of the global population [39]. The prevalence varies by several clinical characteristics: Older age, female gender, race and the occurrence of several diseases (e.g. heart failure, renal impairment, diabetes mellitus) have a profound influence on prevalence estimates [40-43]. In patients undergoing cardiac surgery the prevalence of anemia varies between 22.0% and 54.4% [44-46]. In heart failure patients, the prevalence rises up to 67.0% [47]. Of note, the prevalence correlates with the severity of the heart failure. In new-onset heart failure patients, the prevalence of anemia occurs only in 17.3% [48].

A growing body of evidence underscores the independent impact of anemia on a variety of clinical, functional and economic indicators [49]. In the elderly and various groups of patients with chronic diseases such as hemodialysis, heart failure, cardiac and non-cardiac surgery, anemia is an independent risk factor for increased morbidity and mortality. In several studies, anemia was associated with a 20% to 50% increased mortality risk in heart failure patients [50-52]. In cardiac surgical patients, preoperative anemia was significantly associated with infections [53,54], stroke [44,54], renal failure [44,54], prolonged ventilatory support [53,54], intensive care unit and in-hospital stay [45,55] as well as in-hospital and all-cause mortality [44,53,54]. Of note, in patients with heart failure, a 1-g/dL increase in Hb was associated with a 14.2% reduction in risk of hospitalization and a 15.8% reduction in mortality risk [56]. Therefore, treatment of anemia can improve patients outcome [49]. Current anemia treatment strategies include administration of erythropoietin (EPO) and other erythropoiesis-stimulating agents (ESAs), intravenous iron therapy, concurrent treatment with ESA and iron as well as blood transfusions. However, the use of blood transfusions is accompanied by several risks. In detail, blood transfusion is an independent risk factor for several clinical complications including prolonged ventilator support [45], increased length of hospital stay [57], infection [58] and mortality [59]. Therefore, other preventive or therapeutic options to decrease the prevalence of anemia are warranted.

There is evidence from several observational studies that indicate an inverse association between vitamin D status and anemia risk in several populations [60-68], including patients

with CVD, e.g. heart failure patients [34] and patients scheduled for cardiac surgery [69]. In heart failure patients, mean Hb values were significantly reduced in the lower tertiles of both vitamin D metabolites (25OHD [<18 nmol/l] and 1,25$(OH)_2$D [<40 pmol/l]). The odds ratios (OR) for anemia of the lowest tertiles of 25OHD and 1,25$(OH)_2$D were 2.69 (1.46-5.00) and 4.08 (2.18-7.62) compared with their respective highest tertiles (>32 nmol/l; >70 pmol/l) [34]. In cardiac surgical patients, the ORs for anemia in the groups with severe (<12.5 nmol/l) and moderate (12.5-29.99 nmol/l) vitamin D deficiency were 1.49 (1.04-2.12) and 1.24 (1.01-1.52), respectively, compared with patients with vitamin D levels of 50-100 nmol/l. Sensitivity analyses showed a stronger association when the reference category was restricted to patients with 25OHD levels of 75-100 nmol/l (1.70 [1.09-2.63] and 1.41 [1.02-1.96] for severe and moderate vitamin D deficiency, respectively) [69]. Interestingly enough, there is some evidence that circulating levels of 1,25$(OH)_2$D is a better predictor of anemia than circulating 25OHD [34]. However, sufficient data on the relationship between Hb levels and the active vitamin D metabolite 1,25$(OH)_2$D is lacking.

In line with the observational studies, first non-randomized interventional studies in patients with CKD demonstrated an increase in Hb concentration after 12 months of intravenous 1,25$(OH)_2$D administration and therefore improved control of anemia with reduced need for EPO [70,71]. In another study in hemodialysis patients, intravenous administration of 1,25$(OH)_2$D decreased the weekly needed EPO dose by 50% [72]. The supplementation of high-dose vitamin D_2 (50,000 IU monthly) was associated with ESA-dose reductions in hemodialysis patients. Yet, mean Hb levels remained unchanged [73,74]. However, these earlier studies have some limitations since they included only small numbers of patients and no control group.

Several experimental studies point to a relationship between low vitamin D levels and the development of anemia: (i) 1,25$(OH)_2$D may stimulate erythropoiesis in red blood precursor cells by increasing EPO sensitivity. In addition, 1,25$(OH)_2$D can upregulate proliferation of hematopoietic progenitor cells.[75,76] (ii) 1,25$(OH)_2$D is able to suppress the release of tumor necrosis factor-α [77,78] and interleukin-6 [77] and may therefore reduce the inflammatory milieu, leading to anemia of chronic disease.[62] (iii) Vitamin D may influence folate and iron absorption by increasing intestinal proton-coupled folate transporter.[79,80] The renal activation of 25OHD into 1,25$(OH)_2$D is catalyzed by a cytochrome P450-dependent ferrodoxin reductase.[81]

Therefore, studies investigating the relationship between anemia risk with both vitamin D metabolites, 25OHD and 1,25$(OH)_2$D, as well as adequately powered randomized controlled trials (RCT) with vitamin D supplementation are warranted to assess the association between vitamin D status and anemia risk and to proof if the association is causal.

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PURPOSE OF THE THESIS

The objective of this thesis was to answer the following questions:

1) Is there an independent association of the vitamin D metabolites 25OHD and $1,25(OH)_2D$ with Hb levels and anemia risk in different groups of patients with CVD?

2) Does a vitamin D supplementation improve Hb levels and therefore anemia control in different groups of patients with CVD?

To answer these questions, four clinical studies were performed:

Two cross-sectional studies (CHAPTER ONE and CHAPTER TWO) aimed to answer question 1) by investigating the associations between the vitamin D metabolites 25OHD and $1,25(OH)_2D$ and anemia risk in cardiac surgical patients and patients referred for coronary angiography.

In two secondary analyses of RCTs, question 2) was tried to be answered (CHAPTER THREE and CHAPTER FOUR). For that, the effect of a daily vitamin D supplementation versus placebo was investigated in hypertensive patients for eight weeks (2,800 IU/d) and in heart failure patients for 36 months (4,000 IU/d).

CHAPTER ONE

Independent association of circulating vitamin D metabolites with anemia risk in patients scheduled for cardiac surgery

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Abstract

Background: Preoperative anemia is considered an independent risk factor of poor clinical outcome in cardiac surgical patients. Low vitamin D status may increase anemia risk.

Methods: We investigated 3,615 consecutive patients scheduled for cardiac surgery to determine the association between preoperative anemia (hemoglobin [Hb] <12.5 g/dL) and circulating levels of the vitamin D metabolites 25-hydroxyvitamin D (25OHD) and 1,25-dihydroxyvitamin D ($1,25[OH]_2D$).

Results: Of the study cohort, 27.8% met the criteria for anemia. In patients with deficient 25OHD levels (<30 nmol/l) mean Hb concentrations were 0.5 g/dL lower than in patients with adequate 25OHD levels (50.0–125 nmol/l; P<0.001). Regarding $1,25(OH)_2D$, mean Hb concentrations were 1.2 g/dL lower in the lowest $1,25(OH)_2D$ category (<40 pmol/l) than in the highest $1,25(OH)_2D$ category (>70 pmol/l; P<0.001). In multivariable–adjusted logistic regression analyses, the odds ratios for anemia of the lowest categories of 25OHD and $1,25(OH)_2D$ were 1.48 (95%CI:1.19-1.83) and 2.35 (95%CI:1.86-2.97), compared with patients who had adequate 25OHD levels and $1,25(OH)_2D$ values in the highest category, respectively. Anemia risk was greatest in patients with dual deficiency of 25OHD and $1,25(OH)_2D$ (multivariable-adjusted OR = 3.60 (95%CI:2.40-5.40). Prevalence of deficient 25OHD levels was highest in anemia of nutrient deficiency, whereas low $1,25(OH)_2D$ levels were most frequent in anemia of chronic kidney disease.

Conclusion: This cross-sectional study demonstrates an independent inverse association between vitamin D status and anemia risk. If confirmed in clinical trials, preoperative administration of vitamin D or activated vitamin D (in case of chronic kidney disease) would be a promising strategy to prevent anemia in patients scheduled for cardiac surgery.

Introduction

Anemia is a worldwide public health problem. With an estimated prevalence of 24.8%, it affects nearly one out of four of the global population [1]. The prevalence of anemia in patients undergoing cardiac surgery varies between 22.0% and 54.4% [2-5].

There is evidence from several observational studies that preoperative anemia is an independent predictor of poor clinical outcome in cardiac [2-4, 6-8] and non-cardiac [9] surgical patients. Different peri- and postoperative complications such as infections [3, 6, 7], stroke [2, 7] and renal failure [2, 7, 10], as well as prolonged ventilatory support [3, 7] and intensive care unit and in-hospital stay [3, 8, 9], were significantly associated with preoperative anemia. In addition, preoperative anemia is an independent risk factor of in-hospital and all-cause mortality [2, 6, 7, 9].

It has recently been shown that 25-hydroxyvitamin D (25OHD) deficiency is independently associated with lower hemoglobin (Hb) levels and higher anemia risk in patients scheduled for cardiac surgery [5]. The association between vitamin D deficiency and anemia is supported by various observational studies in different groups of patients [11-14]. Data in end-stage heart failure patients also indicate that circulating levels of the active vitamin D hormone, 1,25-dihydroxyvitamin D (1,25$[OH]_2$D), is a better predictor of anemia than circulating 25OHD [14]. In line with these findings, first interventional studies in hemodialysis patients demonstrated an increase in Hb levels after intravenous administration of 1,25$(OH)_2D_3$, resulting in improved control of anemia [15, 16]. Experimental studies have provided evidence for potential mechanisms regarding how vitamin D may contribute to anemia prevention [17, 18]. Briefly, 1,25$(OH)_2$D may influence bone marrow by stimulating erythropoiesis in red cell precursor cells via increased erythropoietin (EPO) sensitivity. Moreover, 1,25$(OH)_2$D has the capacity to up-regulate proliferation of progenitor cells, independent of the presence of EPO.

The present study therefore aimed to investigate the association of preoperative 25OHD and 1,25$(OH)_2$D levels with the risk of anemia in patients scheduled for cardiac surgery.

Subjects and Methods

Patients

For this cross-sectional study, we considered 3,852 adult patients who were scheduled for cardiac surgery at our institution between February 2012 and December 2013 (geographic latitude 52˚N). We excluded 237 patients with incomplete data sets, e.g. missing 25OHD levels, $1,25(OH)_2D$ levels and/or Hb levels, leaving a total of 3,615 patients for data analysis. The vast majority of patients were Caucasians. This investigation is a secondary analysis of the CALCITOP-study which was approved by the Ethics Committee Ruhr University Bochum at Bad Oeynhausen, was registered at clinicaltrials.gov as NCT02192528, and was recently published [19]. Participants provided their written confirmed consent. All patient information was anonymized and de-identified prior to analysis.

Study Design

For analysis, we used routinely collected and filed demographic and clinical patient data (THGQIMS, Münster, Germany) and biochemical patient data (Lauris, SWISSLAB, Berlin, Germany). Blood samples were collected and analyzed on the last day before cardiac surgery. We assessed levels of 25OHD, $1,25(OH)_2D$ and Hb, as well as other biochemical parameters (creatinine, C-reactive protein [CRP], leukocytes, erythrocytes, hematocrit [Htc], mean corpuscular volume [MCV], mean corpuscular hemoglobin [MCH], thrombocytes, red blood cell distribution width). In addition, we assessed patient characteristics like age, gender, season of blood sampling, body mass index, smoking, left ventricular ejection fraction [LVEF], concomitant diseases (myocardial infarction [MI], chronic obstructive pulmonary disease [COPD], stroke, hemofiltration, peripheral arterial occlusive disease [PAOD], hypertension, diabetes, hyperlipidemia) and medication use (diuretics, angiotensin-converting enzyme-inhibitors, angiotensin receptor-blockers, aspirin, clopidogrel, glucocorticoids).

Biochemical Analysis

Circulating $1,25(OH)_2D_3$ levels were measured by a liquid chromatography/tandem mass spectrometry method provided by Immundiagnostik (Bensheim, Germany). Since the detection limit of the method is 14 pmol/l, we considered values below this limit as 13.0 pmol/l. Circulating 25OHD levels (sum of $25[OH]D_2$ and $25[OH]D_3$) were analyzed by the autoanalyzer Liaison (DiaSorin, Stillwater, MN, USA). The measuring range was between 10 and 375 nmol/l. Values below the detection limit of 10.0 nmol/l were considered 9.9 nmol/l. CRP and creatinine levels were measured using the Architect Autoanalyzer (Abbott, Wiesbaden, Germany). Estimated glomerular filtration rate (eGFR) was calculated using the Modification of Diet in Renal Disease [20]. Blood Hb, Htc, erythrocytes, leukocytes, thrombocytes, MCV and MCH were measured

by automated procedures using the Abbott CellDyn 3500 hematology analyzer (Abbott, Wiesbaden, Germany). According to earlier classifications [2, 5], Hb concentrations <12.5 g/dL were considered as anemic, which is the average threshold value of the World Health Organization's gender-based definition (<13 g/dL in men and <12.0 g/dL in women). The following reference values were used for other hematological parameters: erythrocytes: males: 4.5-6.9 10^{12}/l, females: 4.0-5.2 10^{12}/l; Htc: males: 41-53%, females: 36-46%; MCH: 26-34 pg Hb/erythrocytes; MCV: 80-94 μm^3. Together with low Hb levels, low MCV values can be an indicator of iron deficiency, whereas high MCV values can be an indicator of folate and/or vitamin B_{12} deficiency [21]. Therefore, we used earlier approaches to classify anemia hierarchically according to subtypes [5, 22]: anemia because of nutritional deficiency (iron deficiency: MCV <80 μm^3; folate or vitamin B12 deficiency: MCV: >94 μm^3), anemia of chronic kidney disease (CKD; eGFR: <60 ml/min per 1.73 m^2), anemia of inflammation (CRP >10 mg/l) and unexplained anemia if none of these subtypes were present.

Statistics

Categorical variables are reported as a percentage of observations. Continuous variables are presented as a mean and standard deviation. Clinical and demographic parameters of anemic and non-anemic patients were compared using Student´s *t* test, Chi-squared test and ANOVA. All continuous variables were normally distributed (Kolmogorov-Smirnov test). According to the Institute of Medicine [23], the following 25OHD cutoff values were used for classifying vitamin D status: deficiency (<30 nmol/l), inadequacy (30-49.9 nmol/l), adequacy (50-125 nmol/l) and potentially harmful (>125 nmol/l). Based on earlier classifications (14, 24), $1,25(OH)_2D$ values were divided into three categories (<40 pmol/l; 40-70 pmol/l; >70 pmol/l).

To assess the independent association of 25OHD and $1,25(OH)_2D$ categories with anemia, we performed logistic regression analysis (unadjusted and multivariable-adjusted). Results are expressed as odds ratios (ORs) with a 95% confidence interval. We considered the groups with adequate 25OHD levels (50-125 nmol/l) and $1,25(OH)_2D$ levels >70 pmol/l as a reference group. The following covariates were included in multivariable models: model 1: unadjusted data; model 2: adjusted for age, gender and season of blood sampling (winter: January through March; spring: April through June; summer: July through September; fall: October through December); model 3: model 2 plus adjustments for body mass index, smoking, LVEF, concomitant diagnoses (MI, COPD, stroke, hemofiltration, PAOD, hypertension, diabetes and hyperlipidemia) and medications (diuretics, angiotensin-converting enzyme-inhibitors, angiotensin receptor-blockers, aspirin, clopidogrel, glucocorticoids); model 4: model 3 plus adjustments for kidney function (eGFR in ml/min per 1.73 m^2) and inflammatory processes (CRP in mg/dL). In sensitivity analysis, we also used the following 25OHD classification: <50 nmol/l, 50-125 nmol/l and >125 nmol/l. We considered the P-values <0.05 as statistically

significant. P-values are two-sided. Analyses were performed using SPSS version 21.0 (SPSS, Inc., Chicago, IL, USA).

Results

Of the study cohort, 26.1% were 25OHD deficient (<30.0 nmol/l), 35.4% had inadequate 25OHD values (30-49.9 nmol/l), 36.7% had adequate 25OHD values (50-125 nmol/l) and 1.7% had values >125 nmol/l. Regarding $1,25(OH)_2D$, 29.4% had values below 40 pmol/l, 40.6% values between 40 and 70 pmol/l and 30.0% had values >70 pmol/l. Baseline characteristics of the study cohort are listed in Table 1, broken down by anemia status. Of the study cohort, 27.8% met the criteria for anemia. Compared with non-anemic patients, circulating 25OHD and $1,25(OH)_2D$ levels were significantly lower in anemic patients. Anemic patients were significantly older, more frequently female but less often smokers than non-anemic patients. In addition, anemic patients had significantly lower LVEF values and suffered more often from COPD, hemofiltration, PAOD, diabetes and hyperlipidemia than non-anemic patients. In addition, anemia was associated with reduced Hb, Htc, MCV, MCH and erythrocyte values, but MCV, MCH and erythrocyte values were still within the respective reference range. CRP was significantly elevated in the anemic group.

Tables 2 and 3 illustrate the hematological parameters broken down by 25OHD status and $1,25(OH)_2D$ categories, respectively. Anemia risk was greatest in the groups with the lowest 25OHD and $1,25(OH)_2D$ concentrations. In detail, mean Hb concentrations were 0.5 g/dL lower in patients with deficient levels of 25OHD levels (<30 nmol/l) than in patients with adequate 25OHD levels (50.0–125 nmol/l; P<0.001). Regarding $1,25(OH)_2D$, mean Hb concentrations were 1.2 g/dL lower in patients in the lowest $1,25(OH)_2D$ category (<40 pmol/l) than in the highest $1,25(OH)_2D$ category (>70 pmol/l; P<0.001). Hematological parameters, such as erythrocytes, Htc, MCH and MCV, were all lowest in patients with low $1,25(OH)_2D$ levels. MCV and MCH were lowest in patients with deficient 25OHD levels, whereas erythrocytes and Htc were lowest in patients with 25OHD levels >125 nmol/l. MCV differed significantly between 25OHD categories, but not between $1,25(OH)_2D$ categories.

In Tables 4 and 5, the ORs for anemia are given by categories of 25OHD and $1,25(OH)_2D$. In the unadjusted model, the ORs for patients in the lowest categories of 25OHD and $1,25(OH)_2D$ were significantly higher = 1.79 (95% CI: 1.49-2.15) and 3.73 (95% CI: 3.05-4.56), compared with the respective reference group. In the fully adjusted models, the ORs for anemia were attenuated, but remained significant. Compared with the respective reference group, the OR for the category of 25OHD deficient patients was = 1.48 (95% CI: 1.19-1.83) and was = 2.35 (95% CI: 1.86-2.97) for the lowest $1,25(OH)_2D$ category.

In patients with dual deficiency of 25OHD and $1,25(OH)_2D$ (n=336), the fully adjusted OR for anemia was compared with patients who had adequate 25OHD levels and $1,25(OH)_2D$ levels in the highest category (n=438) = 3.60 (95% CI: 2.40-5.40; P<0.001). In sensitivity analysis,

the fully adjusted OR of patients with 25OHD values <50 nmol/l was = 1.21 (95% CI: 1.01-1.45), compared with the reference group of 50 – 125 nmol/l.

The prevalence of deficient 25OHD levels was highest in anemia of nutrient deficiency, whereas low 1,25$(OH)_2$D levels were most frequent in anemia of CKD (Figures 1 and 2).

Table 1: **Characteristics of the study cohort by anemia status**

	Anemic (<12.5 g/dl) n = 1,006	Non-anemic (≥12.5 g/dl) n = 2,609	*P* value
Age (years)	72±10.7	67±11.1	<0.001
Gender (% males)	47.5	74.7	<0.001
BMI (kg/m^2)	27.5±5.3	27.8±4.3	0.114
Smokers (%)	21.2	31.8	<0.001
LVEF (%)	56.3±12.5	57.8±12.1	0.001
Concomitant diseases			
MI (%)	19.2	16.8	0.096
COPD (%)	11.2	7.8	0.001
Stroke (%)	6.3	4.6	0.051
Hemofiltration (%)	5.3	0.4	<0.001
PAOD (%)	9.7	6.6	0.002
Hypertension (%)	80.9	78.8	0.183
Diabetes (%)	34.6	22.9	<0.001
Hyperlipidemia (%)	62.6	66.4	0.035
Medications			
Diuretics (%)	57.8	36.7	<0.001
ACE-Inhibitors (%)	47.6	45.2	0.192
AT-blockers (%)	12.9	11.4	0.206
Aspirin (%)	50.7	44.9	0.002
Clopidogrel (%)	9.2	6.7	0.013
Glucocorticoids(%)	4.0	2.9	0.114
Biochemical parameters			
eGFR (ml/min/1.73 m^2)	64.1±26.2	78.6±20.6	<0.001
CRP (mg/dl)	2.3±4.7	0.7±1.6	<0.001
25OHD (nmol/l)	46.9±30.4	49.3±26.0	0.026
1,25(OH)$_2$D (pmol/l)	46.7±26.8	60.6±27.5	<0.001
Hemoglobin (g/dl)	11.1±1.1	14.3±1.1	<0.001
Leukocytes (10^9/l)	8.2±3.8	7.8±3.1	0.003
Erythrocytes (10^{12}/l)	3.8±0.5	4.6±0.4	<0.001
Hematocrit (%)	33.5±3.2	41.9±3.3	<0.001
MCV (µm^3)	89.3±6.3	90.4±4.5	<0.001
MCH (pg Hb/red blood cell)	29.6±2.6	30.8±1.8	<0.001
Thrombocytes (10^9/l)	249±100.8	231±72.7	<0.001

RDW (%)	14.0±2.2	12.4±1.2	<0.001

ACE = Angiotensin-converting-enzyme; AT = Angiotensin receptor; BMI = Body Mass Index; COPD = chronic obstructive pulmonary disease; CRP = C-reactive protein; eGFR = estimated globular filtration rate; Hb = Hemoglobin; LVEF = Left ventricular ejection fraction; MCH = mean corpuscular/cellular hemoglobin; MCV = mean corpuscular/cell volume; MI = myocardial infarction; PAOD = peripheral artery occlusive disease; RDW = Red Blood Cell Distribution Width ([S_v x 100]/MCV)

Table 2: **Hematological parameters by cutoffs of 25-Hydroxyvitamin D**

25OHD	<30 nmol/l n = 945	30-49.9 nmol/l n = 1281	50-125 nmol/l n = 1328	>125 nmol/l n = 61	*P* value for trend
Hemoglobin (g/dl)	13.0±2.0	13.5±1.8	13.5±1.7	12.8±1.8	<0.001
Erythrocytes (10^{12}/l)	4.3±0.6	4.4±0.6	4.4±0.6	4.2±0.5	<0.001
Hematocrit (%)	38.7±5.4	39.8±5.0	39.9±4.6	38.2±4.8	<0.001
MCV (μm^3)	89.7±5.9	90.2±4.9	90.3±4.5	90.8±5.9	0.034
MCH (pg Hb/Erythrocytes)	30.2±2.5	30.6±2.0	30.6±1.8	30.4±2.5	<0.001

Hb = Hemoglobin; MCH = mean corpuscular/cellular hemoglobin; MCV = mean corpuscular/cell volume

Table 3: **Hematological parameters by cutoffs of 1,25-Dihydroxyvitamin D**

$1,25(OH)_2D$	<40 pmol/l n = 1064	40-70 pmol/l n = 1468	>70 pmol/l n = 1083	*P* value for trend
Hemoglobin (g/dl)	12.7±1.9	13.5±1.7	13.9±1.6	<0.001
Erythrocytes (10^{12}/l)	4.2±0.6	4.4±0.6	4.6±0.5	<0.001
Hematocrit (%)	37.6±5.3	39.9±4.8	40.8±4.3	<0.001
MCV (μm^3)	89.9±5.6	90.3±4.9	90.0±4.8	0.101
MCH (pg Hb/Erythrocytes)	30.2±2.3	30.6±2.0	30.6±2.0	<0.001

Hb = Hemoglobin; MCH = mean corpuscular/cellular hemoglobin; MCV = mean corpuscular/cell volume

Table 4: **Unadjusted and adjusted odds ratio (OR) for anemia by cutoffs of 25-Hydroxyvitamin D**

25OHD nmol/l	N	Anemia (%)	Model 1 OR (95% CI)	Model 2 OR (95% CI)	Model 3 OR (95% CI)	Model 4 OR (95% CI)
<30	945	337 (35.7)	1.79 (1.49-2.15)	1.63 (1.34-1.98)	1.50 (1.22-1.84)	1.48 (1.19-1.83)
30-49.9	1281	331 (25.8)	1.13 (0.94-1.34)	1.06 (0.88-1.28)	1.06 (0.87-1.28)	1.05 (0.85-1.28)
50-125	1328	314 (23.6)	1.0 (reference)	1.0 (reference)	1.0 (reference)	1.0 (reference)
>125	61	24 (39.3)	2.10 (1.23-3.56)	2.27 (1.30-3.96)	1.69 (0.92-3.10)	1.34 (0.71-2.52)

Model 1: unadjusted
Model 2: adjusted for age, gender and season of blood drawing
Model 3: adjusted as in model 2 and for concomitant diseases, medications, smoking, body mass index and left ventricular ejection fraction
Model 4: adjusted as in model 3 and for kidney function (eGFR) and inflammatory process (CRP)

Table 5: **Unadjusted and adjusted odds ratio (OR) for anemia by cutoffs of 1,25-Dihydroxyvitamin D**

$1,25(OH)_2D$ pmol/l	N	Anemia (%)	Model 1 OR (95% CI)	Model 2 OR (95% CI)	Model 3 OR (95% CI)	Model 4 OR (95% CI)
<40	1064	452 (42.5)	3.73 (3.05-4.56)	3.50 (2.83-4.33)	2.97 (2.38-3.71)	2.35 (1.86-2.97)
40-70	1468	375 (25.5)	1.73 (1.42-2.11)	1.69 (1.37-2.08)	1.60 (1.29-1.98)	1.52 (1.22-1.89)
>70	1083	179 (16.5)	1.0 (reference)	1.0 (reference)	1.0 (reference)	1.0 (reference)

Model 1: unadjusted
Model 2: adjusted for age, gender and season of blood drawing
Model 3: adjusted as in model 2 and for concomitant diseases, medications, smoking, body mass index and left ventricular ejection fraction
Model 4: adjusted as in model 3 and for kidney function (eGFR) and inflammation (CRP)

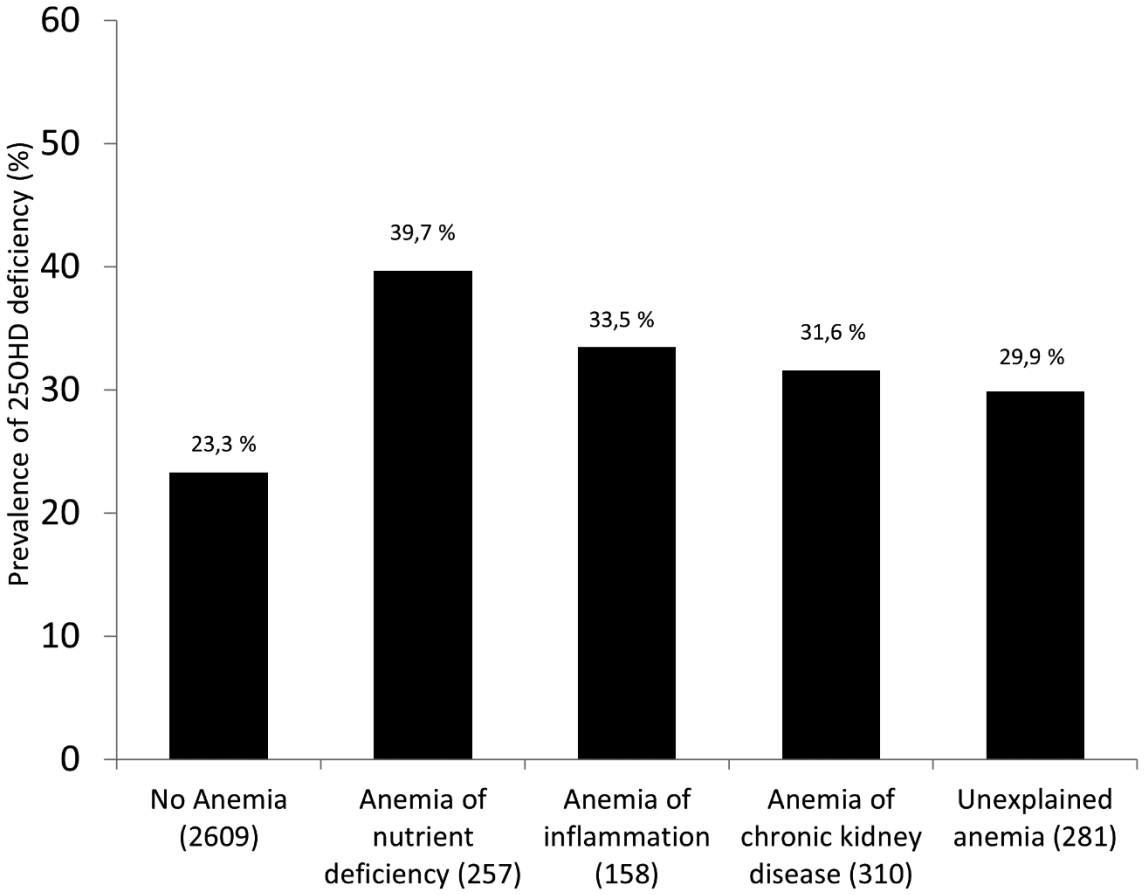

Figure 1: Prevalence of deficient 25OHD levels according to subtypes of anemia.

Figure legend: There were significant differences between subgroups (*P*<0.001). Number of patients in brackets.

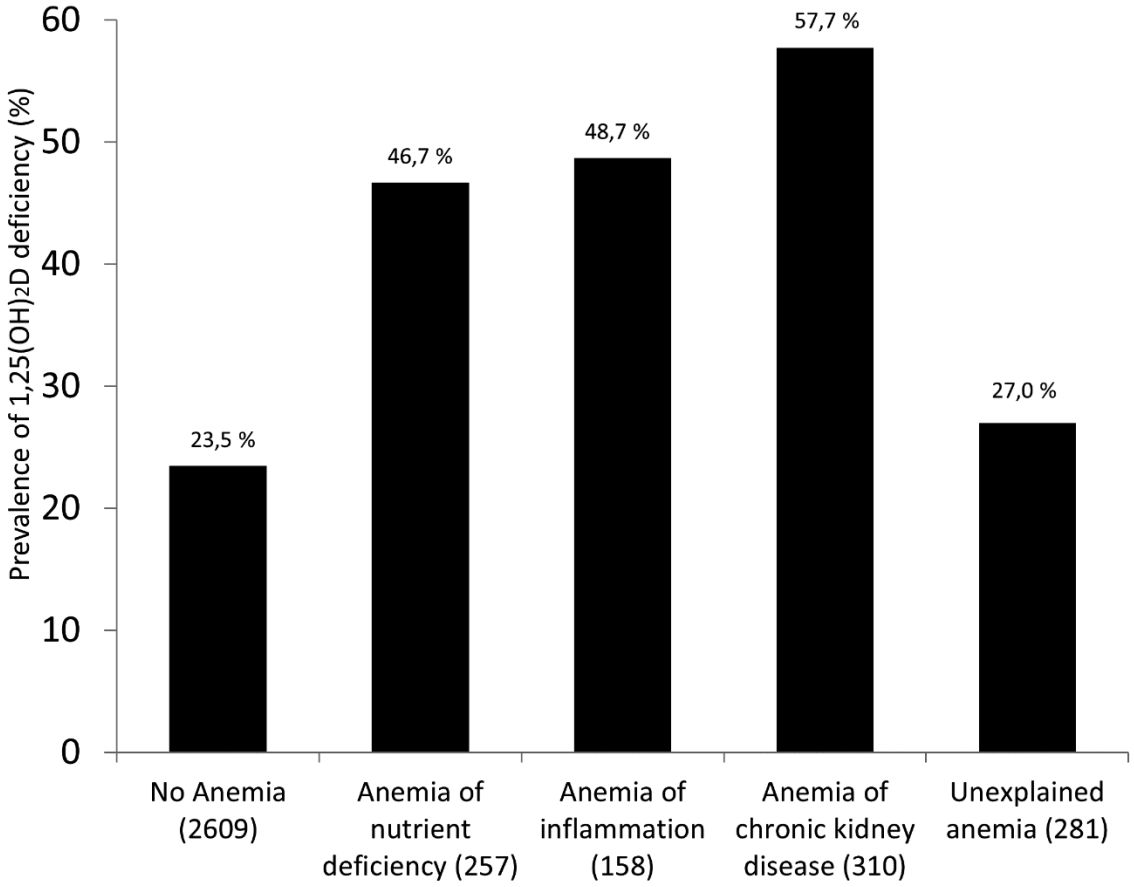

Figure 2: Prevalence of $1,25(OH)_2D$ levels <40 pmol/l according to subtypes of anemia.

Figure legend: There were significant differences between subgroups (*P*<0.001). Number of patients in brackets.

Discussion

This cross-sectional study demonstrates that low 25OHD and 1,25$(OH)_2$D levels are independently associated with low Hb levels and anemia risk in patients scheduled for cardiac surgery. Patients with 25OHD values <30 nmol/l and 1,25$(OH)_2$D values <40 pmol/l had the highest risk for anemia, whereas the risk was lowest in patients with adequate 25OHD levels (50-125 nmol/l) and 1,25$(OH)_2$D levels above 70 pmol/l. Circulating 1,25$(OH)_2$D was a better predictor of anemia than circulating 25OHD.

Our data confirm earlier studies of an independent inverse association between circulating 25OHD and anemia risk [5, 11, 12, 22, 25-30]. However, the association of 1,25$(OH)_2$D with anemia risk has rarely been studied, and the few available studies on this topic have primarily been performed in patients with CKD and end-stage heart failure [13] [14]. In end-stage heart failure [14], Hb concentrations were 1.6 g/dL higher in patients with values >70 pmol/l compared to patients with values <40 pmol/l. These earlier findings are in general agreement with the data of the present study.

There are several suggested causes why low 1,25$(OH)_2$D may play a role in the development of anemia. Vitamin D modulates the level of systemic cytokine production. Therefore, vitamin D may reduce the inflammatory milieu that leads to anemia of chronic disease [11]. In addition, vitamin D may influence folate and iron absorption by increasing intestinal proton-coupled folate transporter [31] [32]. Iron participates in the renal activation of 25OHD into 1,25$(OH)_2$D [33]; the enzymatic conversion is catalyzed by a cytochrome P450-dependent ferrodoxin reductase [34]. 1,25$(OH)_2$D can also stimulate erythropoiesis in red blood cell precursor cells by increasing EPO sensitivity and can up-regulate proliferation of progenitor cells [17, 18]. First interventional studies support the hypothesis that 1,25$(OH)_2$D administration and probably also high-dose bolus application of vitamin D is effective in enhancing Hb levels. Briefly, two studies in patients with CKD demonstrated an increase in mean Hb concentration after 12 months of intravenous 1,25$(OH)_2$D administration by 1.0 mg/dL [15] and 1.2 mg/dL [16], and therefore improved control of anemia with reduced need for EPO. However, results must be considered preliminary since no control group was included in the two studies. In another study in hemodialysis patients, intravenous administration of 1,25$(OH)_2$D decreased the weekly EPO dose requirement by 50% [35]. Similarly, high-dose monthly oral vitamin D_2 administration (50,000 IU) was associated with non-significant [36] and significant [37] reductions in doses of erythropoiesis-stimulating agents (ESA), while mean Hb levels remained unchanged. Interestingly enough, it has been demonstrated that high-dose oral bolus administration of vitamin D is even able to increase circulating 1,25$(OH)_2$D levels in CKD patients [38].

Data obtained in the United States demonstrate that blood transfusion is the most common procedure performed during hospitalization (11.0% of hospital stays with a procedure) [39]. With a range of 35.8% to 60.0%, the transfusion rate in patients undergoing a cardiovascular procedure is very high [40-46]. However, the use of blood transfusion involves several risks. Blood transfusion is an independent risk factor for clinical complications like prolonged ventilatory support [3, 7], increased length of hospital stay [45, 46], infection [43-45, 47] and mortality [40-43, 45]. The increased risk of blood transfusion has been attributed to the presence of leucocytes in allogeneic blood transfusion thereby causing immunomodulatory events [48]. In addition, duration of blood storage influences clinical outcome with higher incidence of prolonged mechanical ventilation, renal failure, sepsis and in-hospital mortality in patients receiving older blood products (>14 days) [49]. Besides the use of leukocyte-depleted red blood cells and short storage time, reduction or even avoidance of the use of blood transfusion might be another interesting approach. Anemia is the most important risk factor for transfusion [50]. If confirmed in randomized controlled trials, administration of vitamin D or its active form $1,25(OH)_2D$ could be a promising preventive or therapeutic option to decrease the prevalence of anemia in elective cardiac surgical patients. Of note, CKD patients (stages IV and V) should preferably be treated with activated vitamin D.

In our study, prevalence of deficient 25OHD levels was highest in anemia of nutrient deficiency, whereas low $1,25(OH)_2D$ levels were most frequent in anemia of CKD. With regard to CKD, this may be due to low GFR, resulting in reduced $1,25(OH)_2D$ synthesis. However, it may also be that CKD-induced increased 24-hydroxylase activity [51] leads to enhanced conversion of 25OHD and $1,25(OH)_2D$ into their 24-hydroxylated inactive forms. It is well known that $1,25(OH)_2D$ levels are suppressed in CKD. However, low $1,25(OH)_2D$ levels were also present in anemia of inflammation and anemia of nutrient deficiency. It has recently been shown that low $1,25(OH)_2D$ levels are independently associated not only with poor kidney function but also with low 25OHD levels, high CRP levels, diabetes mellitus and high values of the cardio-surgical risk marker EuroSCORE [19]. It may therefore well be that the anemia subtype ‘nutrient deficiency’ may only be a surrogate for poor health status and that circulating $1,25(OH)_2D$ levels are also suppressed by other, currently unknown factors.

Our study has some strengths but also some limitations. Its strengths are measurement of the active vitamin D hormone, $1,25(OH)_2D$, in a large cohort of patients and adjustments for several demographical and clinical variables. One inherent limitation is the observational study design, which prevents us from concluding that the association between 25OHD and $1,25(OH)_2D$ deficiency and anemia is causal. It is another limitation that we were unable to assess the use of ESA or to measure EPO concentrations. However, since the association of low $1,25(OH)_2D$ levels with anemia was highest in anemia of CKD, it is rather unlikely that ESA were frequently

used in our study cohort. Finally, no data on parathyroid hormone, magnesium or fibroblast growth factor 23 levels were available, parameters that are involved in $1,25(OH)_2D$ regulation and therefore play a central role in vitamin D metabolism.

In conclusion, our study demonstrates that 25OHD and $1,25(OH)_2D$ levels are independently associated with low Hb concentrations and high anemia risk in cardiac surgical patients. Circulating $1,25(OH)_2D$ was a better predictor of anemia than circulating 25OHD. Future studies have to clarify whether the association is causal and whether preoperative administration of vitamin D or $1,25(OH)_2D$ is able to successfully prevent or treat anemia in patients scheduled for cardiac surgery.

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CHAPTER TWO

Independent associations of vitamin D metabolites with anemia in patients referred to coronary angiography: The LURIC Study

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Abstract

Purpose: Anemia and vitamin D deficiency are both frequent in adult patients. Whether low vitamin D metabolite levels are an independent risk factor for different subtypes of anemia remains to be studied in detail.

Methods: In 3,299 patients referred for coronary angiography, we investigated the association of 25-hydroxyvitamin D (25OHD) and 1,25-dihydroxyvitamin D ($1,25[OH]_2D$) with anemia (hemoglobin [Hb] <12.5 g/dL) of specific subtypes.

Results: Compared with patients with 25OHD levels in the adequate range (50-125 nmol/l), patients with deficient 25OHD concentrations (<30 nmol/l; 33.6% of patients) had 0.6 g/dL lower Hb levels. Hb values were 1.3 g/dL lower in patients with $1,25(OH)_2D$ levels <40 pmol/l (5.4% of patients), compared with patients in the highest $1,25(OH)_2D$ category (>70 pmol/l). Of the participants, 16.7% met the criteria for anemia. In multivariate-adjusted regression analyses, the odds ratios for anemia in the lowest 25OHD and $1,25(OH)_2D$ categories were 1.52 (95%CI:1.15-2.02) and 3.59 (95%CI:2.33-5.52), compared with patients with 25OHD levels in the adequate range and patients with $1,25(OH)_2D$ levels >70 pmol/l. The probability of anemia was highest in patients with combined 25OHD and $1,25(OH)_2D$ deficiency (multivariable-adjusted odds ratio 5.11 [95%CI:2.66-9.81]). Patients with anemia of chronic kidney disease had the highest prevalence of 25OHD deficiency and $1,25(OH)_2D$ concentrations of <40 pmol/l.

Conclusions: Low 25OHD and $1,25(OH)_2D$ concentrations are independently associated with anemia. Patients with poor kidney function are most affected. Interventional trials with are warranted to prove whether administration of plain or activated vitamin D can prevent anemia.

Introduction

With an estimated global prevalence rate of approximately 25% anemia is a common health problem [1]. In various medical conditions such as hemodialysis, heart failure, cardiac and non-cardiac surgery, anemia is an independent risk factor for increased morbidity and mortality [2].

Low vitamin D status, e.g. 25-hydroxyvitamin D (25OHD) levels <50 nmol/l, is also frequently seen in elderly patients [3]. Recent observational studies have indicated an association between vitamin D status and anemia risk in community-dwelling older people [4,5], Asian children and adolescents [6] and different groups of patients [7-11]. There is however some evidence that the active vitamin D hormone 1,25-dihydroxyvitamin D ($1,25[OH]_2D$) is a better predictor of anemia than circulating 25OHD [4,7,10]. In line with these data, first interventional studies in hemodialysis patients testing intravenous $1,25(OH)_2D$ administration showed an increase in Hb levels, leading to improved control of anemia and reduced need for erythropoietin (EPO) [12,13].

According to experimental studies, vitamin D (i) can stimulate erythropoiesis in red cell precursor cells via increased EPO sensitivity [14], (ii) reduces pro-inflammatory cytokine production [11], (iii) enhances cellular folate uptake [15], and (iv) may increase intestinal iron absorption [16], whereas the enzymatic activation of 25OHD into $1,25(OH)_2D$ may be suppressed by low iron availability [17].

Since large studies on the association between vitamin D metabolites and specific subtypes of anemia are almost completely lacking, we aimed to examine in patients referred to coronary angiography the associations between the vitamin D metabolites 25OHD and $1,25(OH)_2D$ with anemia (Hb <12.5 g/dL) of specific subtypes.

Methods

Study Population

The Ludwigshafen Risk and Cardiovascular Health Study (LURIC) is a prospective cohort study of 3,316 Caucasian patients referred to coronary angiography [18]. Patients were recruited between July 1997 and January 2000 at a single tertiary care center in southwestern Germany (Herzzentrum Ludwigshafen). Inclusion criteria were as follows: Caucasian origin, availability of a coronary angiogramm and clinical stability with the exception of acute coronary syndromes. Exclusion criteria were: any acute illness other than acute coronary syndromes, any chronic disease where non-cardiac disease predominated and a history of malignancy within the past five years. In 3,299 study participants, serum concentrations of 25OHD and $1,25(OH)_2D$ as well as hematological parameters were available. These patients were included in our analysis. The study was approved by the ethics committee at the 'Ärztekammer Rheinland-Pfalz' (Mainz, Germany). All participants gave their informed written consent.

Data Assessment

For data analysis, 25OHD, $1,25(OH)_2D$, parathyroid hormone (PTH), fibroblast growth factor 23 (FGF-23), Hb, hematocrit, erythrocytes, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), iron, ferritin, transferrin, soluble transferrin receptor, folic acid, vitamin B12, C-reactive protein (CRP), hepcidin, creatinine and cystatin C were included. In addition, patient characteristics like age, sex, body mass index (BMI), smoking, concomitant diseases and medication use were considered.

Biochemical Analysis

Fasting venous blood samples were drawn in the morning, before coronary angiography was performed. Basic biochemical parameters were immediately measured as described [18]. One milliliter aliquots of serum were shock frozen in liquid nitrogen and stored at -80°C for later use. Serum concentrations of 25OHD and $1,25(OH)_2D$ were determined using radioimmunoassays (DiaSorin Antony, France; Stillwater, MN, USA and Nichols Institute Diagnostika GmbH, Bad Nauheim, Germany, respectively). FGF-23 and hepcidin were measured by enzyme-linked immunosorbent assays (Immundiagnostik AG, Bensheim, Germany and DRG Instruments GmbH, Marburg, Germany, respectively). Hb was measured by cyanmethemoglobin method (Technicon H1, Technicon GmbH, Bad Vilbel, Germany; Advia 120 Bayer Diagnostics, Tarrytown, USA). Transferrin was determined by immunoturbidimetry, serum iron was determined by a colorimetric ferrozine assay on a Hitachi 717 analyzer, ferritin and PTH were analyzed by electrochemiluminescence immunoassay on an Elecsys 2010 Roche and

creatinine was determined by the method of Jaffé at 37°C with a Hitachi 717 autoanalyzer and commercial kits (Roche, Mannheim, Germany). CRP, cystatin C and soluble transferrin receptor were measured by immunonephelometry (N Latex CRP mono, Dade Behring, Marburg, Germany). Folic acid and vitamin B12 concentrations were analyzed using ion capture immunoassay and microparticle enzyme immunoassay, respectively (Abbott AXYM autosampler, Abbott Diagnostics, Wiesbaden, Germany).

According to earlier classifications [8,10], Hb concentrations <12.5 g/dL were considered as anemic. This is the average threshold value of the World Health Organization´s gender based definition (<13 g/dL in men; <12 g/dL in women). We used earlier approaches to classify anemia hierarchically according to subtypes [5,8,10]: anemia of nutrient deficiency was present when subjects met one of these criteria: iron deficiency (≥ 2 of the following: serum ferritin <12 ng/mL, transferrin saturation <15 % and MCV <80 μm^3); folate deficiency (serum folate <2.6 µg/L) or vitamin B12 deficiency (serum levels <200 ng/L); anemia of chronic kidney disease (CKD; eGFR <60 ml/min per 1.73 m^2), anemia of inflammation (CRP >1 mg/dL or serum iron <60 µg/dL) and unexplained anemia if none of these subtypes were present.

Estimated glomerular filtration rate (eGFR) was calculated using the following equation: GFR (mL/min/1.73 m^2) = 177.6*[creatinine (mg/dL]$^{-0.65}$*[cystatin C (mg/L)]$^{-0.57}$*age$^{-0.20}$*0.82 (if female) [19]. Transferrin saturation (%) was calculated using the following equation: (serum iron/5.5)/(transferrin/100)*3.98.

Statistics

Categorical variables are reported as percentage of observations and continuous variables as mean and standard deviation. We tested normal distribution of the data using the Kolmogorov-Smirnov test. Normal distribution was considered if probability values were greater than 0.05. Clinical parameters were compared using Chi-squared test. Since several demographic and biochemical parameters were non-normally distributed, the non-parametric Mann-Whitney-U-test and Kruskal-Wallis-test were used, when appropriate. According to the Institute of Medicine [20], 25OHD status was classified as follows: deficiency (<30 nmol/l), inadequacy (30-49.9 nmol/l), adequacy (50-125 nmol/l) and potentially harmful (>125 nmol/l). Based on earlier classifications [7,10], $1,25(OH)_2D$ values were divided into three categories (<40 pmol/l; 40-70 pmol/l; >70 pmol/l).

To assess the independent association of 25OHD and $1,25(OH)_2D$ categories with anemia, we performed logistic regression analyses (unadjusted and multivariable-adjusted). Results are expressed as odds ratios (ORs) with 95% confidence intervals (CI). We considered the groups with adequate 25OHD levels (50-125 nmol/l) and $1,25(OH)_2D$ levels >70 pmol/l as a reference group. The following covariates were included in multivariable models: model 1: unadjusted

data; model 2: adjusted for age, gender and season of blood sampling (winter: January-March; spring: April-June; summer: July-September; fall: October-December); model 3: model 2 plus adjustments for BMI, smoking, concomitant diagnoses (coronary artery disease [CAD], history of stroke, diabetes mellitus, myocardial infarction [MI]) and medications (antibiotics, ACE-inhibitors/AT-blockers, calcium-antagonists, glucocorticoids, statins, digitalis, diuretics); model 4: model 3 plus adjustments for kidney function (eGFR in ml/min per 1.73 m^2), inflammatory processes (CRP, hepcidin), iron status (ferritin, transferrin saturation) and nutritional status (folic acid, vitamin B12). Considering that there exists no universally accepted classification for $1,25(OH)_2D$, we performed sensitivity analysis by using tertiles of $1,25(OH)_2D$ (<70.5 pmol/l; 70.5-97.75 pmol/l; >97.75 pmol/l) and considered the highest tertile as reference group. Interrelationships between variables were assed using Spearman's rank correlation coefficient (r_s). We considered P-values <0.05 as statistically significant. P-values are two-sided. Analyses were performed using SPSS version 21.0 (SPSS, Inc., Chicago, IL, USA).

Results

Of the study cohort, 33.6% had deficient 25OHD levels (<30.0 nmol/l), 32.3% had inadequate 25OHD levels (30.0-49.9 nmol/l), 33.7% had 25OHD levels in the adequate range (50-125 nmol/l) and 0.4% had 25OHD levels above 125 nmol/l; 5.4% had $1,25(OH)_2D$ <40 pmol/l, 27.4% had $1,25(OH)_2D$ between 40 and 70 pmol/l and 67.2% had concentrations above 70 pmol/l. 25OHD and $1,25(OH)_2D$ were inversely correlated with PTH levels (25OHD: r_s= -0.25;P<0.001; $1,25(OH)_2D$: r_s= -0.05;P=0.008).

16.7% of participants met the criteria for anemia. In detail, 3.4% had anemia of nutritional deficiency (1.0% iron deficiency; 2.4% folate or vitamin B12 deficiency), 3.4% had anemia of CKD; 5.7% had anemia of inflammation, and 4.2% had unexplained anemia. In Table 1, baseline characteristics of the participants are presented, according to anemia status. Anemic participants were older, had a lower mean BMI, were more frequent women but less often smokers than non-anemic patients. While prevalence rates of arterial hypertension, peripheral vascular disease and history of venous thrombosis/pulmonary embolism did not differ between the two groups, anemic patients suffered significantly more often from all other concomitant diseases (CAD, history of stroke, diabetes mellitus, MI, cancer and acute infectious diseases). The intake of antibiotics, ACE-inhibitors/AT-blockers, calcium-antagonists, statins, digitalis and diuretics differed between anemic and non-anemic patients. Compared with non-anemic patients, circulating 25OHD and $1,25(OH)_2D$ levels were significantly lower and PTH and FGF-23 levels significantly higher in anemic patients. In addition, anemia was associated with reduced MCV, MCH and MCHC.

Hb values were lowest in the groups with the lowest 25OHD and $1,25(OH)_2D$ values. Compared with patients with adequate 25OHD (50-125 nmol/l) and $1,25(OH)_2D$ levels >70 pmol/l, Hb concentrations were 0.6 g/dL and 1.3 g/dL lower in patients with deficient 25OHD and $1,25(OH)_2D$ <40 pmol/l, respectively (both P-values <0.001). Iron, ferritin, transferrin, transferrin saturation, folic acid, vitamin B12 and eGFR were significantly lower and CRP and cystatin C significantly higher in patients with deficient 25OHD and $1,25(OH)_2D$ <40 pmol/l, compared with patients with adequate 25OHD and $1,25(OH)_2D$ >70 pmol/l (Supplemental Tables 1 and 2).

Table 2 presents the ORs for anemia across the categories of circulating 25OHD and $1,25(OH)_2D$. Compared with the respective reference groups, in those patients who had lowest 25OHD and $1,25(OH)_2D$, the ORs were 2.50 (95%CI:2.0-3.14) and 5.59 (95%CI:3.82-7.68) in the unadjusted models, respectively. In the fully adjusted models, the ORs for anemia in the lowest 25OHD and $1,25(OH)_2D$ categories were 1.52 (95%CI:1.15-2.02) and 3.59 (95%CI:2.33-5.52), respectively. In participants with combined 25OHD and $1,25(OH)_2D$

deficiency (n=115) the unadjusted and fully adjusted ORs for anemia were 8.13 (95%CI:5.30-12.47) and 5.11 (95%CI:2.66-9.81), compared with the adequate 25OHD and highest $1,25(OH)_2D$ category, respectively. Patients with anemia of CKD had the highest prevalence of 25OHD deficiency and $1,25(OH)_2D$ levels <40 pmol/l (Figures 1 and 2). Results did not change substantially if patients with an eGFR <60 ml/min per 1.73 m^2 were excluded (data not shown). The ORs for anemia subtypes were for 25OHD (reference: non-anemic patients) as follows: anemia of nutrient deficiency: 2.99 (95%CI:2.04-4.38); anemia of CKD: 3.27 (95%CI:2.23-4.79); anemia of inflammation: 2.16 (95%CI:1.61-2.91); unexplained anemia: 1.84 (95%CI:1.30-2.60). The corresponding ORs were for $1,25(OH)_2D$ (reference: non-anemic patients) as follows: anemia of nutrient deficiency: 5.35 (95%CI:3.14-9.11); anemia of CKD: 13.27 (95%CI:8.57-20.57); anemia of inflammation: 2.26 (95%CI:129-3.97); unexplained anemia: 0.99 (95%CI:0.40-2.46).

In sensitivity analysis, the fully adjusted OR for anemia in participants in the lowest $1,25(OH)_2D$ tertile (<70.5 pmol/l) was 1.82 (95%CI:1.37-2.42), compared to those in the highest tertile (>97.75 pmol/l). The association of 25OHD with anemia did not change substantially, when 25OHD levels were classified into tertiles (data not shown).

Table 1: Baseline Characteristics of the study cohort by anemia status

	Anemic (<12.5 g/dl) n = 552	Non-anemic (≥12.5 g/dl) n = 2747	*P* value
n (%)	16.7	83.3	
Age (years)	67±9.9	62±10.5	<0.001
Gender (% males)	42.0	75.2	<0.001
BMI (kg/m^2)	26.9±4.4	27.6±4.0	<0.001
Smokers (%)	57.4	65.3	<0.001
Concomitant diseases (%)			
CAD	84.2	76.6	<0.001
Stroke	15.6	7.8	<0.001
Arterial hypertension	62.0	57.8	0.069
Diabetes	24.3	16.1	<0.001
Myocardial Infarction	48.6	39.8	<0.001
Peripheral vascular disease	11.1	9.2	0.170
Cancer	12.2	6.3	<0.001
Venous thrombosis/pulmonary embolism	7.3	5.8	0.174
Acute infectious disease	13.1	8.9	0.002
Medication (%)			
Antibiotics	3.6	1.3	<0.001
ACE-Inhibitors/AT-Blockers	65.4	55.2	<0.001
Aspirin/other Antiplatelets	73.2	71.1	0.329
Beta-Blockers	65.2	63.1	0.343
Calcium-Antagonists	19.2	15.0	0.013
Glucocorticoids	2.7	2.0	0.260
Vitamin K-Antagonists	7.1	6.6	0.706
Statins	52.5	45.9	0.004
Digitalis	22.1	14.1	<0.001
Diuretics	38.9	26.3	<0.001
Vitamin Supplementations	2.9	2.4	0.461
25OHD (nmol/l)	35.9±22.3	44.9±24.2	<0.001
1,25(OH)$_2$D (pmol/l)	73.7±34.3	90.5±34.4	<0.001
PTH (ng/L)	39.0±43.0	33.0±24.3	<0.001
FGF-23 (RU/mL)	312.0±2273.9	86.1±316.1	<0.001
Hb (g/dL)	11.5±0.8	14.3±1.1	<0.001
Hct (%)	34.4±2.6	41.8±3.2	<0.001
Leukocytes (10^9/L)	7.0±2.4	7.1±2.1	0.027

Erythrocytes (10^{12}/L)	3.9±0.4	4.7±0.4	<0.001
MCV (μm^3)	88.2±5.8	89.4±4.5	<0.001
MCH (pg Hb/red blood cell)	29.6±2.3	30.6±1.7	<0.001
MCHC (g/L)	33.6±1.3	34.2±1.1	<0.001
Thrombocytes (10^9/L)	251.0±80.3	228.8±65.2	<0.001
Iron (µg/dL)	67.1±32.0	97.9±38.3	<0.001
Ferritin (ng/mL)	188.5±234.9	220.7±200.5	<0.001
Transferrin (mg/dL)	243.0±48.0	253.3±28.3	<0.001
Transferrin saturation (%)	20.6±10.9	28.5±11.9	<0.001
sTfR (mg/L)	1.4±0.7	1.3±0.5	0.279
Folic acid (µg/L)	8.0±3.0	8.2±2.8	0.071
Vitamin B12 (ng/L)	417.3±274.5	391.0±212.0	0.911
CRP (mg/dL)	2.0±3.2	0.8±1.5	<0.001
Hepcidin (ng/mL)	9.0±10.9	8.1±6.5	0.584
TNF-α (ng/L)	11.1±7.3	9.7±10.5	<0.001
eGFR (mL/min/1.73 m^2)	71.6±21.9	83.2±18.7	<0.001
Cystatin C (mg/L)	1.2±0.7	1.0±0.3	<0.001
Homoarginin (µmol/L)	2.0±0.9	2.7±1.1	<0.001

ACE = angiotensin-converting-enzyme; AT = angiotensin receptor; BMI = body mass index; CRP = C-reactive protein; CAD = coronary artery disease; eGFR = estimated globular filtration rate; FGF-23 = fibroblast growth factor 23; Hb = hemoglobin; MCH = mean corpuscular hemoglobin; MCHC = mean corpuscular hemoglobin concentration; MCV = mean corpuscular volume; PTH = parathyroid hormone; sTfR = soluble transferrin receptor; TNF-α = tumor necrosis factor-α

Table 2: **Unadjusted and adjusted odds ratio (OR) for anemia by cutoffs of 25-Hydroxyvitamin D and 1,25-Dihydroxyvitamin D**

Vitamin D	N	Anemia N (%)	Model 1 OR (95% CI)	Model 2 OR (95% CI)	Model 3 OR (95% CI)	Model 4 OR (95% CI)
25OHD						
<30 nmol/l	1107	281 (25.4)	2.50 (2.0-3.14)	1.81 (1.42-2.32)	1.56 (1.21-2.02)	1.52 (1.15-2.02)
30-49.9 nmol/l	1067	135 (12.7)	1.07 (0.83-1.38)	1.0 (0.76-1.29)	0.96 (0.73-1.26)	1.02 (0.76-1.36)
50-125 nmol/l	1111	133 (12.0)	1.0 (reference)	1.0 (reference)	1.0 (reference)	1.0 (reference)
>125 nmol/l	14	3 (21.4)	2.0 (0.6-7.28)	1.84 (0.48-7.0)	2.1 (0.55-7.95)	1.93 (0.46-8.17)
$1,25(OH)_2D$						
<40 pmol/l	178	77 (43.3)	5.59 (4.05-7.72)	5.41 (3.82-7.68)	4.45 (3.09-6.40)	3.59 (2.33-5.52)
40-70 pmol/l	905	209 (23.1)	2.20 (1.80-2.69)	1.88 (1.52-2.32)	1.69 (1.35-2.10)	1.68 (1.32-2.15)
>70 pmol/l	2216	266 (12.0)	1.0 (reference)	1.0 (reference)	1.0 (reference)	1.0 (reference)

Model 1: unadjusted
Model 2: adjusted for age, gender and season of blood drawing
Model 3: adjusted as in model 2 and for concomitant diseases, medications, smoking and BMI
Model 4: adjusted as in model 3 and for kidney function (eGFR), inflammatory process (CRP, hepcidin), iron status (ferritin, transferrin saturation) and nutritional status (folic acid, vitamin B12)

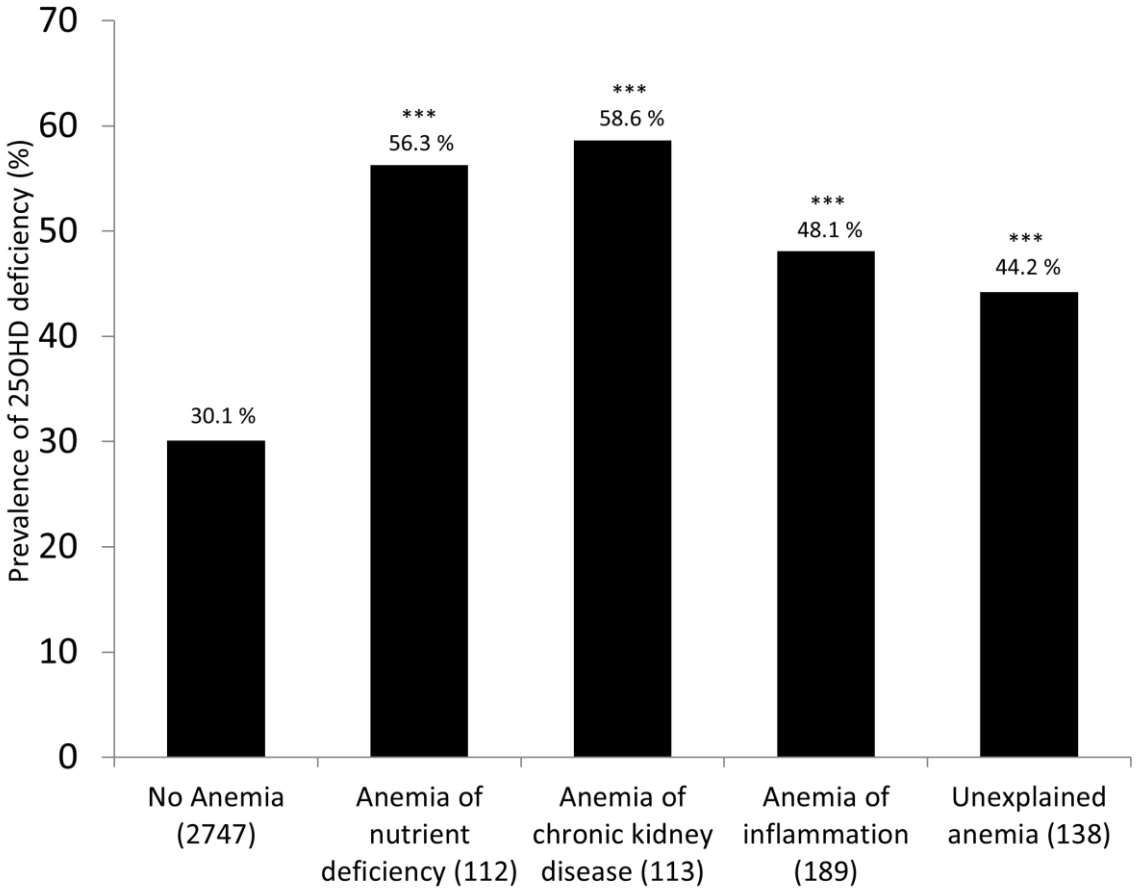

Figure 1: **Prevalence of deficient 25OHD levels according to subtypes of anemia**

Differences between subgroups were statistically significant (***P*<0.01; ****P*<0.001 vs. non-anemic participants).

Number of participants in brackets.

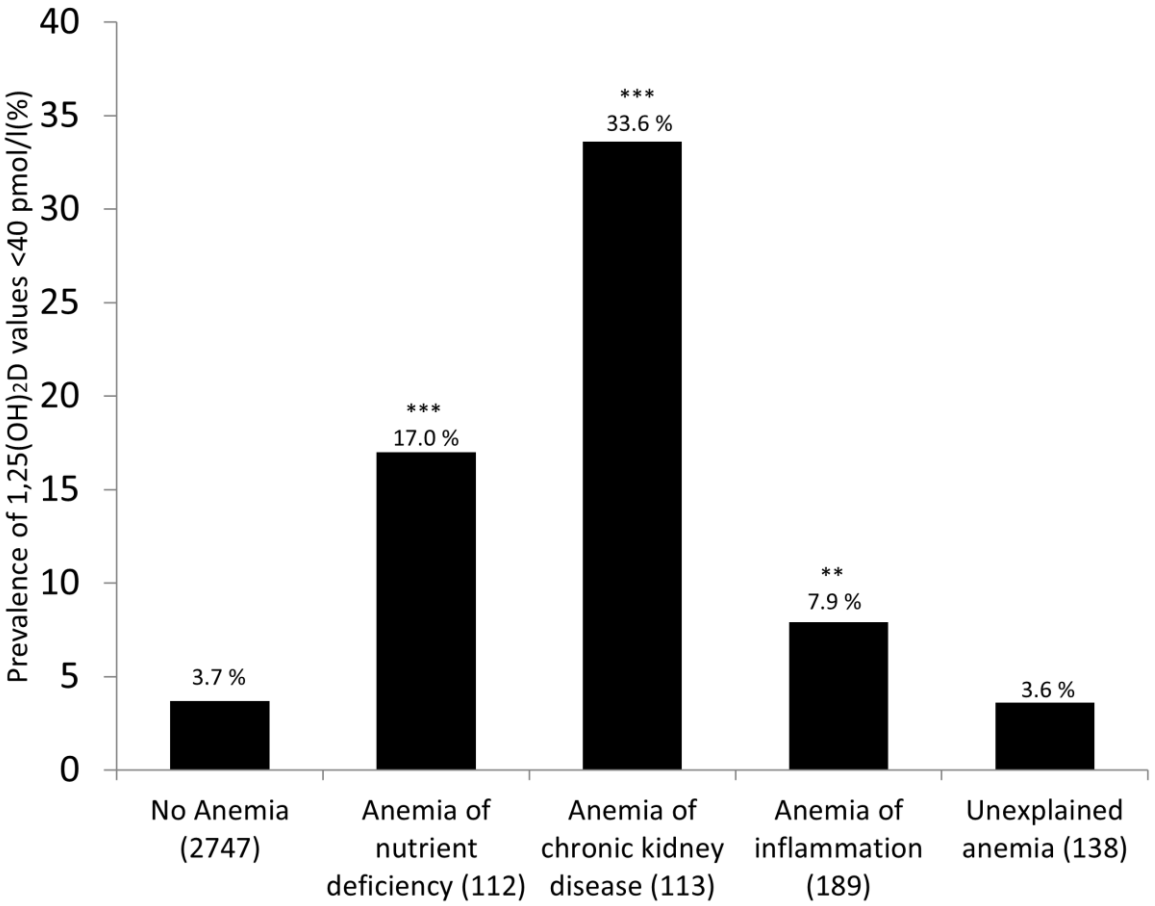

Figure 2: **Prevalence of $1,25(OH)_2D$ levels <40 pmol/l according to subtypes of anemia**

Differences between subgroups were statistically significant (***P*<0.01; ****P*<0.001 vs. non-anemic participants).

Number of participants in brackets.

Discussion

Our data demonstrate that in patients referred to coronary angiography, both, low 25OHD and $1,25(OH)_2D$ levels are independently associated with anemia. Of note, the association of anemia was stronger with low circulating $1,25(OH)_2D$ levels than with deficient circulating 25OHD levels, even if in sensitivity analysis 70.5 pmol/l instead of 40 pmol/l was used as cutoff for inadequate $1,25(OH)_2D$ levels.

Our findings are in line with recent studies demonstrating an independent association of anemia risk with low 25OHD and/or $1,25(OH)_2D$ concentrations in community-dwelling older people [4,5], Asian children and adolescents [6], heart failure patients [7], cardiac surgical patients [8,10] and CKD patients [9,11]. However, in a recent cross-sectional study examining 1,666 older men [4], only serum $1,25(OH)_2D$ levels, but not 25OHD levels were significantly associated with Hb levels. With respect to 25OHD, these data are not in line with our results. The differences may to some extent be due to the smaller number of individuals studied by Hirani et al.[4], leading to reduced statistical power to obtain significant results. Nevertheless, both, our study and the study by Hirani et al.[4] support the assumption that $1,25(OH)_2D$ is a more important predictor of anemia risk than 25OHD [7,10]. In line with this, first interventional studies in CKD patients demonstrated an increase in Hb concentrations during 12 months of intravenous $1,25(OH)_2D$ administration that was paralleled by reduced EPO need [12]. However, these earlier studies included only small numbers of patients and no control group.

In our study, patients with anemia of CKD had the highest prevalence of 25OHD deficiency and $1,25(OH)_2D$ levels <40 pmol/l. It is well known that CKD is associated with low $1,25(OH)_2D$ levels [21]. Moreover, EPO synthesis is suppressed and anemia is prevalent in CKD patients [22,23]. Whether $1,25(OH)_2D$ directly affects renal EPO synthesis is unknown. However, there is evidence that in CKD patients $1,25(OH)_2D$ can stimulate erythroid precursors proliferation [14], suggesting effects of $1,25(OH)_2D$ on the bone marrow. Similar to the high prevalence of low $1,25(OH)_2D$ levels, the prevalence of low 25OHD levels is higher in CKD patients compared with the general population [24]. It has been speculated that in CKD patients low 25OHD and low $1,25(OH)_2D$ levels result from increased 24-hydroxylase activity [25], thereby increasing 25OHD and $1,25(OH)_2D$ catabolism. In the bone marrow, levels of $1,25(OH)_2D$ are several 100-fold higher compared with plasma [26]. Normalizing tissue 25OHD may be necessary for providing adequate amounts of substrate for local tissue production of $1,25(OH)_2D$ [11]. Therefore, low 25OHD concentrations in CKD may lead to insufficient substrate availability for the extrarenal 1α-hydroxylase-induced synthesis of $1,25(OH)_2D$ in hematopoietic tissues. It is also noteworthy that in CKD patients $1,25(OH)_2D$ administration can increase 25OHD uptake of peripheral cells [27] suggesting that adequate circulating

$1,25(OH)_2D$ levels are necessary for sufficient local 25OHD availability. Consequently, both vitamin D metabolites may synergistically improve erythropoiesis.

Our data demonstrate that low $1,25(OH)_2D$ levels are primarily but not exclusively associated with poor kidney function. Low $1,25(OH)_2D$ were paralleled by elevated PTH and FGF-23 levels, the latter parameter being well-known for its suppressive effect on $1,25(OH)_2D$ synthesis [28]. Recent studies in cardiac surgical patients [29] indicate that circulating $1,25(OH)_2D$ levels are probably less homeostatically regulated than hitherto assumed. In that study, low $1,25(OH)_2D$ levels were associated not only with poor kidney function but also with low 25OHD levels, diabetes mellitus as well as high CRP levels and high values of the cardio-surgical risk marker EuroSCORE. In our study cohort, the prevalence of $1,25(OH)_2D$ levels <40 pmol/l (5.4%) was much lower than the prevalence of deficient 25OHD levels (33.6%). In patients with CKD stages IV and V and in some other groups of patients such as end-stage heart failure patients, the prevalence of $1,25(OH)_2D$ levels <40 pmol/l is much higher than in the present study [7,30], and this may at least in part also explain differences in the prevalence of anemia between these groups of patients. Altogether, results indicate that in clinical practice low levels of 25OHD and $1,25(OH)_2D$ may often both contribute to anemia.

Our study has some strengths and also some limitations. Strengths are (i) the large number of included patients (ii), the availability of data on 25OHD, $1,25(OH)_2D$ levels and specific anemia subtypes, and (iii) the opportunity of performing multivariable-adjusted analyses including important confounders (age, gender, season of blood drawing, BMI, smoking, concomitant diseases, medications). One limitation is the cross-sectional study design, which precludes us from concluding that there is a causal relationship between vitamin D metabolites and anemia. Despite careful adjustments of our analyses, we can therefore not exclude that low 25OHD and $1,25(OH)_2D$ are just simply a consequence of diseases or pathologic conditions that contribute to anemia. The study was restricted to Caucasians at intermediate to high cardiovascular risk and may thus not be generalizable for the overall population. In addition, we have no data on reticulocytes, EPO concentrations or use of erythropoiesis-stimulating agents. Moreover, the number of patients with 25OHD levels >125 nmol/l was rather small. Therefore, we cannot definitively rule out a U-shaped or inverse J-shaped association of circulating 25OHD levels with anemia. Consequently, high dose daily vitamin D supplements or bolus administration of vitamin D should be performed with caution and/or accompanied by 25OHD measurements. Finally, no generally accepted cutoffs for classifying circulating $1,25(OH)_2D$ values do exist. Hence, the use of different cut-offs influences the prevalence of low/inadequate circulating $1,25(OH)_2D$ levels substantially.

In conclusion, our data of a large cohort of patients referred to coronary angiography indicate an independent association of low circulating 25OHD and $1,25(OH)_2D$ concentrations with low

Hb levels and anemia. Patients with poor kidney function have the highest prevalence of low circulating 25OHD and $1,25(OH)_2D$ concentrations. Randomized controlled trials in patients with deficient 25OHD levels and/or low $1,25(OH)_2D$ levels using plain and/or activated vitamin D are warranted to clarify whether this association is causal.

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CHAPTER THREE

Vitamin D supplementation and hemoglobin levels in hypertensive patients: A randomized controlled trial

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Abstract

Purpose: Epidemiological evidence suggests that circulating 25-hydroxyvitamin D (25OHD) levels are inversely associated with hemoglobin (Hb) levels and anemia risk. We evaluated whether vitamin D supplementation improves Hb levels and reduces anemia risk in hypertensive patients.

Methods: Two hundred patients with 25OHD levels <75 nmol/l who attended the Styrian Vitamin D Hypertension Trial were included, of whom 188 completed the trial. Patients randomly received 2800 IU vitamin D3 daily or a matching placebo for eight weeks.

Results: Initially, the prevalence of anemic status (Hb levels <12.5 g/dl) and deficient 25OHD levels (<30 nmol/l) was 6.5% and 7.5%, respectively. All anemic patients had 25OHD levels >50 nmol/l. The mean (95% confidence interval) vitamin D effect on Hb levels was 0.04 (-0.14 to 0.22) g/dl (P=0.661). Moreover, vitamin D treatment did not influence anemic status significantly (P>0.999). Likewise, vitamin D had no significant effect on Hb levels in the subgroups of anemic patients or in patients with initial 25OHD levels <30 nmol/l.

Conclusion: In conclusion, a daily vitamin D supplement of 2800 IU for eight weeks did not improve Hb levels or anemic status in hypertensive patients. Future trials should focus on anemic patients with deficient 25OHD levels (e.g. <30 nmol/l) (clinicaltrials.gov [NCT02136771]).

Introduction

Anemia is a global health problem [1] and an independent risk factor for increased morbidity and mortality in various groups of patients, especially in patients with chronic diseases and in the elderly [2]. Low levels of 25-hydroxyvitamin D (25OHD) are also highly prevalent among these patients [3].

Recent epidemiological evidence suggests that circulating 25OHD is inversely associated with hemoglobin (Hb) levels [4-15]. The risk of anemia is highest at deficient 25OHD levels (i.e. <30 nmol/l) and lowest at 25OHD levels of 50 to 125 nmol/l [4,6,15].

In chronic kidney disease (CKD) patients, some non-randomized intervention studies could already show that intravenous administration of activated vitamin D (1,25-dihydroxyvitamin D3 = $1,25(OH)_2D_3$) is associated with an increase in Hb levels within 12 months of treatment and a reduced need for erythropoietin (EPO) [16,17]. Moreover, intravenous $1,25(OH)_2D_3$ administration was associated with a decreased weekly EPO dose of 50% [18]. Regarding Hb levels, similar results have been obtained in hemodialysis patients with oral alfacalcidol (1α-vitamin D_3) after 8 weeks [19] and also after 12 and 18 months of treatment [20]. In hemodialysis patients, high-dose oral vitamin D_2 (50,000 IU monthly) was associated with dose-reductions in erythropoiesis-stimulating agents (ESA), while Hb concentrations remained unchanged [21,22]. In children with CKD stage 5 and 25OHD levels <75 nmol/l, 12 weeks of vitamin D_2 treatment in conjunction with $1,25(OH)_2D_3$ was associated with a significantly reduced dose of ESA required to treat the children [23]. In anemic patients with preserved kidney function, however, one single intramuscular bolus of 600,000 IU vitamin D_3 did not influence Hb levels [24]. Nevertheless, it is noteworthy that in general populations the effect of high-dose bolus administration of vitamin D on clinical outcomes has been questioned [25].

The purpose of the present study was to determine the effect of a daily vitamin D_3 supplement on Hb levels in a group of hypertensive patients with preserved kidney function and inadequate 25OHD levels.

Methods

Study Design

This is a secondary analysis of the Styrian Vitamin D Hypertension Trial of a post-specified endpoint. The study was a randomized, double-blind, placebo-controlled, single-center trial which took place at the outpatient clinic at the Division of Endocrinology and Metabolism, Medical University of Graz, Austria. Major study results have already been published elsewhere [26]. The study was registered at EudraCT (No. 2009-018125-70) and clinicaltrials.gov (NCT02136771). All study participants gave written informed consent. The study was approved by the Ethics Committee of the Medical University of Graz, Austria.

Participants

Two hundred study participants (106 men and 94 women) were recruited from the clinics of the Department of Cardiology and the Department of Internal Medicine, Division of Endocrinology and Metabolism, Medical University of Graz, Austria from June 2011 to August 2014. Eligible study participants were adults aged 18 years or older with a serum 25OHD concentration below 75 nmol/l and arterial hypertension. Pregnant or lactating women were excluded and also patients with hypercalcemia (serum calcium >2.65 mmol/l), regular vitamin D intake >880 IU per day during the last four weeks before the study, estimated glomerular filtration rate (eGFR) <15 mL/min per 1.73 m^2, drug intake as part of another clinical study, acute coronary syndrome or cerebrovascular event in the previous two weeks, 24-hour systolic BP >160 mm Hg or <120 mm Hg, 24-hour diastolic BP >100 mm Hg, change in hypertensive treatment (drugs or lifestyle) in the previous four weeks or planned changes in antihypertensive treatment during the study, any disease with an estimated life expectancy of <1 year, any clinically significant acute disease requiring drug treatment, and chemotherapy or radiation therapy.

Intervention

Eligible study participants were randomly allocated to receive 2800 IU (70 µg) cholecalciferol as seven oily drops per day (Oleovit D3, Fresenius Kabi Austria, Graz, Austria) or a matching placebo for eight weeks. The dose of 2,800 IU vitamin D per day was chosen because a rule of thumb suggests that vitamin D supplementation of 1,000 IU increases 25OHD levels by approximately 25 nmol/l [27]. Given that a commonly used normal range of 25OHD is 75 to 150 nmol/l [28] we conclude that a supplementation of 2,800 IU daily may be sufficient to increase the 25OHD level of most study participants to target ranges without causing supra-physiological 25OHD levels. One hundred patients were assigned to the intervention group and 100 patients to the control group. Randomization was performed by web-based software

(www.randomizer.at) with a permuted block randomization (block size of 10 and stratification according to sex).

Endpoints

The primary outcome measures were Hb levels and anemia. In accordance with earlier classifications [4,6,29], Hb concentrations <12.5 g/dL were considered as anemic, which corresponded to the average threshold value of the World Health Organization gender-based definition (<13.0 g/dL in men and <12.0 g/dL in women).

Biochemical Measurements

Blood sampling was performed in the morning between 7 and 11 am after an overnight fast. All blood samples were either measured at least four hours after blood collection or immediately stored at -20°C until analysis. Measurement of 25OHD was performed by chemiluminescence assay (IDS-iSYS 25-hydroxyvitamin D assay; Immunodiagnostic Systems Ltd., Boldon, UK) on an IDS-iSYS multidiscipline automated analyzer. Lower and upper quantification limits were 17.5 nmol/l and 312.5 nmol/l, respectively. All hematological parameters were measured on a Sysmex® XE-5000 automated hematology analyzer (Sysmex America, Inc., Mundelein, IL, USA). eGFR was calculated using the Modification of Diet in Renal Disease Formula [30]. The measurements of other biochemical parameters have been described elsewhere [26]. In accordance with published data [31,32] we categorized 25OHD levels < 30 nmol/l as deficient, 30-49.9 nmol/l as insufficient and 50 to 74.9 nmol/l as borderline.

Statistics

Categorical variables are reported as a percentage of observations. Normally distributed continuous data are shown as means with standard deviation. We used the Kolmogorov-Smirnov test to check normal data distribution. Normal distribution was a consideration when probability values were >0.05. Variables with a skewed distribution are shown as medians with interquartile range. Change from baseline data are shown as means and 95% confidence interval (CI). We used the McNemar test and Fisher's exact test, respectively, to assess differences in anemic status within and between groups. The unpaired t test or chi-squared test was used for group comparisons at baseline. Skewed variables were normalized by logarithmic transformation before use in parametric statistical analysis. ANCOVA with adjustments for baseline values was used to test for differences in the outcome variables between the vitamin D and the placebo group at the follow-up visit. We considered P-values <0.05 (two-sided) as statistically significant. Analyses were performed using SPSS version 21.0 (SPSS, Inc., Chicago, IL, USA).

Results

Baseline characteristics of the study participants are shown in Table 1. In both groups, mean Hb values were clearly above the anemia threshold of 12.5 g/dL. At baseline, the proportion of anemia in the vitamin D and placebo groups was 9.0% and 4.0%, respectively (P=0.152). Regarding 25OHD levels, 6.0% of the vitamin D group and 9.0% of the placebo group had deficient levels. The proportion of insufficient 25OHD levels was 27.0% and 36.0%, respectively. In the remaining 67.0% and 55.0%, the vitamin D status was classified as borderline.

Of the 200 participants, 188 terminated the study as planned. Table 2 shows the treatment results on biochemical parameters. Circulating 25OHD increased on average by 35.4 nmo/l (95% CI: 31.2 to 39.6 nmol/l) in the treatment group and 8.1 nmol/l (95% CI: 4.6 to 11.7 nmol/l) in the placebo group (P<0.001). There was no significant vitamin D effect on Hb levels (Table 2). In both study groups, Hb levels remained almost constant. Moreover, vitamin D treatment did not influence anemic status significantly (P>0.999). In detail, the percentage of anemic subjects remained constant in the vitamin D group (P>0.999) and increased only slightly in the placebo group (P>0.999) (Figure 1). Vitamin D treatment had no effect on other hematological parameters (Table 2).

All anemic patients had initial 25OHD levels > 50 nmol/l. Vitamin D treatment had no effect on Hb levels and other hematological parameters in anemic patients (Table S1). Moreover, there was no vitamin D effect on Hb levels and other hematological parameters in the group of subjects with initial 25OHD levels <30 nmol/l (Table S2).

There was, however, a significant vitamin D effect on PTH levels, with suppressed PTH levels in the vitamin D group (Table 2). Vitamin D treatment did not influence serum calcium and phosphate levels.

Table 1: Baseline characteristics of the study groups

Characteristics	Vitamin D Group (n = 100)	Placebo Group (n = 100)	*P*-Value
Females (%)	46	48	0.777
Age (years)	60.5±10.9	59.7±11.4	0.607
Anemic subjects[1] (%)	9	4	0.152
BMI (kg/m^2)	30.4±4.4	30.4±6.2	0.967
Active smoker (%)	19	14	0.341
Concomitant diseases (%)			
Previous MI	8	5	0.390
Previous Stroke	9	7	0.602
Diabetes Mellitus	32	41	0.186
Biochemical parameters			
25OHD (nmol/l)	54.5±13.6	51.0±14.2	0.073
PTH (pmol/l)	5.2 (4.2-6.5)	5.5 (4.2-7.0)	0.931
Phosphate (mg/dL)	2.9±0.5	3.0±0.5	0.085
Calcium (mmol/L)	2.37±0.10	2.37±0.11	0.989
Hemoglobin (g/dL)	14.4±1.3	14.4±1.4	0.665
Hematocrit (%)	41.1±3.3	41.5±3.4	0.329
Leukocyte Counts (10^9/L)	5.9 (5.1-7.0)	6.0 (5.0-7.5)	0.817
Erythrocyte Counts (10^{12}/L)	4.8±0.4	4.9±0.4	0.237
MCV (µm^3)	86.1±4.4	85.7±4.0	0.457
MCH (pg Hb/red blood cell)	30.1±1.9	29.8±1.6	0.234
MCHC (g/L)	34.9±1.0	34.7±1.1	0.207
Platelets (10^9/L)	239±64	232±50	0.442
MPV (fl)	10.7±1.0	10.7±0.9	0.572
CRP (mg/L)	2.3 (1.13-3.8)	1.4 (0.9-3.4)	0.044
eGFR (ml/min/1.73 m^2)	80.0±17.9	77.2±17.9	0.272
Medications (%)			
Vitamin D supplement	5	9	0.268
ACE-inhibitor	25	38	0.048
AT II blocker	33	31	0.762
Diuretic	42	48	0.394
Beta-blocker	44	49	0.478
Statin	26	32	0.350
Calcium channel blocker	27	25	0.747

[1]Hemoglobin <12.5 g/dL

25OHD: 25-hydroxyvitamin D; ACE: angiotensin converting enzyme; AT: angiotensin; BMI: body mass index; CAD: coronary artery disease; CRP: C-reactive protein; eGFR: estimated globular filtration rate; MCV: mean corpuscular volume; MCH: mean corpuscular hemoglobin; MCHC: mean corpuscular hemoglobin concentration; MI: myocardial infarction; MPV: mean platelet volume; PTH: parathyroid hormone

Table 2: **Results of vitamin D treatment on hematological parameters, calcium and phosphate metabolism, and additional parameters in hypertensive subjects**

Characteristics	Vitamin D group (n=93)			Placebo group (n=95)			Treatment Effect	*P*-value[2]
	Baseline	Follow-Up (8 weeks)	Mean Change from Baseline[1]	Baseline	Follow-Up (8 weeks)	Mean Change from Baseline[1]		
Hematological Parameters								
Hemoglobin (g/dL)	14.4±1.3	14.3±1.3	-0.1 (-0.18 to 0.05)	14.4±1.4	14.3±1.3	-0.1 (-0.25 to 0.04)	0.04 (-0.14 to 0.22)	0.661
Hematokrit (%)	41.1±3.2	41.1±3.3	0.02 (-0.31 to 0.36)	41.5±3.5	41.3±3.2	-0.2 (-0.59 to 0.19)	0.16 (-0.33 to 0.65)	0.514
Erythrocytes (10^{12}/L)	4.8±0.4	4.8±0.4	-0.02 (-0.05 to 0.02)	4.9±0.4	4.8±0.4	-0.04 (-0.09 to 0.00)	0.01 (-0.05 to 0.07)	0.691
MCV (μm^3)	86.4±4.3	86.7±4.6	0.3 (-0.14 to 0.72)	85.6±4.0	86.0±4.0	0.3 (-0.09 to 0.77)	0.01 (-0.59 to 0.61)	0.971
MCH (pg Hb/RBC)	30.2±1.8	29.9±1.7	-0.04 (-0.16 to 0.08)	29.7±1.6	29.7±1.6	0.05 (-0.07 to 0.18)	-0.08 (-0.25 to 0.09)	0.890
MCHC (g/L)	35.0±1.0	34.8±1.1	-0.2 (-0.3 to 0.00)	34.7±1.1	34.7±1.1	-0.09 (-0.3 to 0.09)	-0.01 (-0.24 to 0.22)	0.938
Leucocytes (10^9/L)	5.9 (5.1-7.0)	6.0 (5.0-7.2)	0.04 (-0.18 to 0.26)	6.0 (5.0-7.5)	5.8 (5.0-6.9)	-0.1 (-0.31 to 0.12)	-0.13 (-0.17 to 0.42)	0.291
Platelets (10^9/L)	237±62	240±64	2.9 (-1.6 to 7.4)	231±50	232±53	1.7 (-3.8 to 7.1)	1.6 (-5.4 to 8.6)	0.651
Mean Platelet Volume (fl)	10.7±1.0	10.7±0.9	0.01 (-0.08 to 0.09)	10.8±0.9	10.8±0.9	-0.02 (-0.1 to 0.08)	0.01 (-0.12 to 0.13)	0.887
Calcium and Phosphate Metabolism								
25-Hydroxyvitamin D (nmol/l)	54.9±13.6	90.3±18.3	35.4 (31.2 to 39.6)	50.8±14.2	59.0±22.1	8.1 (4.6 to 11.7)	28.7 (23.5 to 34.2)	<0.001
Parathyroid Hormone (pmol/l)	5.2 (4.3-6.5)	4.8 (4.0-5.8)	-0.4 (-0.69 to -0.17)	5.4 (4.1-6.8)	5.3 (4.1-7.0)	0.18 (-0.13 to 0.49)	-0.60 (-0.99 to -0.22)	0.003
Phosphate (mg/mL)	2.9±0.5	3.0±0.5	0.1 (0.02 to 0.22)	3.0±0.5	3.1±0.5	0.09 (-0.02 to 0.19)	-0.01 (-0.14 to 0.11)	0.823
Calcium (mmol/l)	2.37±0.1	2.37±0.1	0.00 (-0.02 to 0.02)	2.37±0.1	2.35±0.1	-0.01 (-0.03 to 0.01)	-0.01 (-0.03 to 0.01)	0.259
Additional Parameters								
C-Reactive Protein (mg/L)	2.1 (0.9-3.8)	2.2 (1.0-4.2)	0.3 (-0.6 to 1.2)	1.4 (0.8-3.0)	1.4 (0.6-4.1)	0.2 (-0.2 to 0.6)	0.15 (-0.69 to 1.2)	0.598
eGFR (mL/min/1.73^2)	80.5±18.1	77.9±18.8	-2.7 (-5.0 to -0.4)	77.5±17.6	76.7±16.9	-0.9 (-3.0 to 1.3)	-1.3 (-4.3 to 1.7)	0.398

[1]Change from Baseline data are shown as means and 95% confidence interval

[2]ANCOVA with adjustments for baseline values was used to test for differences in the outcome variables between the vitamin D and the placebo group Abbreviations: MCV: mean corpuscular volume; MCH: mean corpuscular hemoglobin; Hb: hemoglobin; RBC: red blood cell; MCHC: mean corpuscular hemoglobin concentration; eGFR: estimated glomerular filtration rate

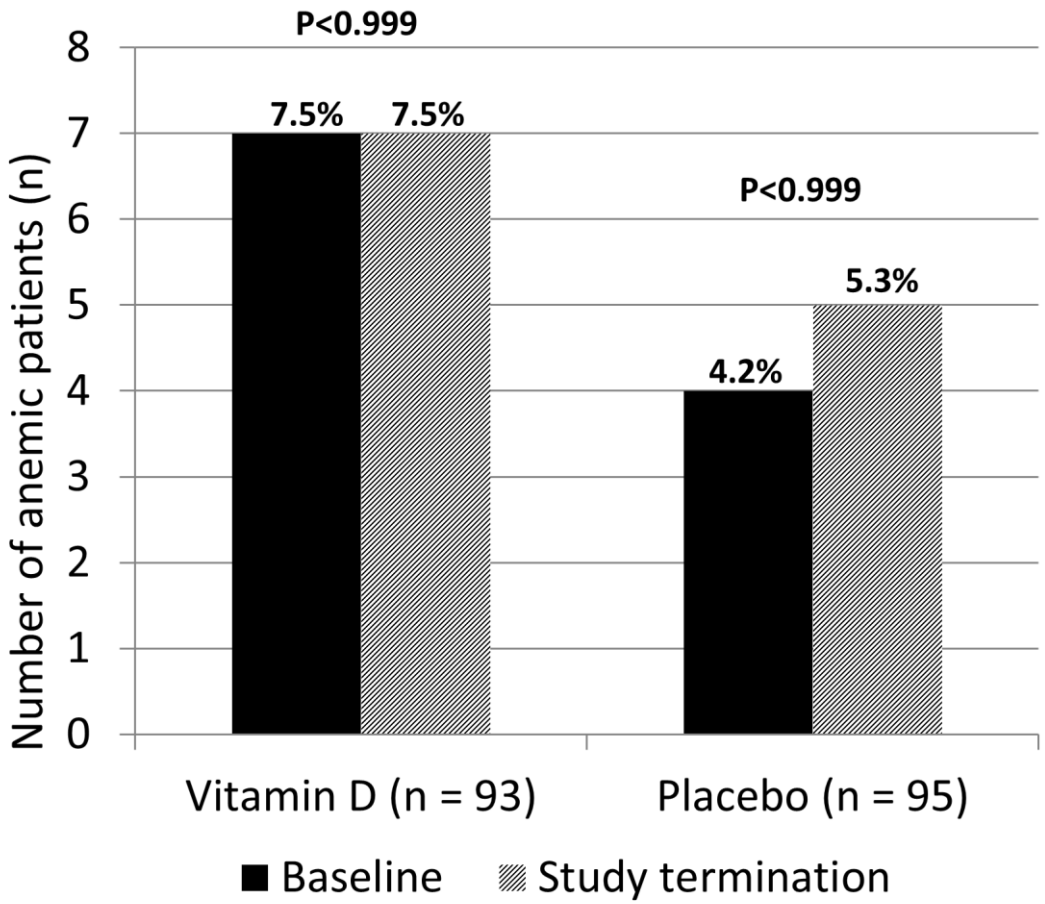

Figure 1: **Anemia proportion in participants of the Styrian Vitamin D Hypertension Trial at baseline and study termination (by study group)**. P-values indicate within study group results.

Table S1: **Effect of vitamin D treatment on hematological parameters in anemic hypertensive subjects**

Characteristics	Vitamin D group (n=7)			Placebo group (n=4)			Treatment Effect	P-value
	Baseline	Follow-Up (8 weeks)	Mean change from Baseline	Baseline	Follow-Up (8 weeks)	Mean change from Baseline		
Hematological Parameters								
Hemoglobin (g/dL)	11.8±0.7	11.8±0.9	-0.01 (-0.64 to 0.61)	10.8±1.3	10.9±1.0	0.15 (-1.74 to 2.04)	0.29 (-1.10 to 1.67)	0.643
Hematokrit (%)	34.5±3.0	34.3±3.3	-0.10 (-1.59 to 1.34)	31.6±3.2	32.4±2.0	0.83 (-3.46 to 5.11)	-0.15 (-3.35 to 3.06)	0.919
Erythrocytes (10^{12}/L)	3.89±0.6	3.91±0.7	0.01 (-0.19 to 0.21)	4.02±0.3	4.04±0.3	0.23 (-0.46 to 0.51)	0.00 (-0.38 to 0.38)	0.985
MCV (μm^3)	89.6±9.0	89.7±11.9	0.13 (-2.85 to 3.10)	78.5±5.0	80.3±3.9	1.75 (-1.75 to 5.20)	-4.14 (-8.50 to 0.23)	0.060
MCH (pg Hb/RBC)	28.8±1.2	28.5±1.3	-0.30 (-0.77 to 0.17)	26.8±2.4	27.0±2.1	0.28 (-1.35 to 1.90)	-0.28 (-1.93 to 1.37)	0.677
MCHC (g/L)	34.3±1.5	34.4±1.2	0.06 (-0.54 to 0.65)	34.0±1.5	33.6±1.3	-0.42 (-3.33 to 2.50)	0.62 (-0.81 to 2.06)	0.345

Table S2: **Effect of vitamin D treatment on hematological parameters in hypertensive subjects with initial 25OHD levels <30 nmol/l**

Characteristics	Vitamin D group (n=6)			Placebo group (n=8)			Treatment Effect	P-value
	Baseline	Follow-Up (8 weeks)	Mean change from Baseline	Baseline	Follow-Up (8 weeks)	Mean change from Baseline		
Hematological Parameters								
Hemoglobin (g/dL)	14.7±1.3	14.4±1.1	-0.28 (-0.82 to 0.26)	15.0±1.2	14.5±1.3	-0.46 (-0.80 to 0.11)	0.16 (-0.39 to 0.71)	0.543
Hematokrit (%)	41.7±3.4	40.6±2.7	-1.15 (-2.55 to 0.25)	43.3±2.9	41.7±3.1	-1.52 (-2.67 to 0.37)	0.15 (-1.49 to 1.80)	0.841
Erythrocytes (10^{12}/L)	4.76±0.3	4.63±0.2	-0.12 (-0.23 to 0.02)	5.04±0.5	4.84±0.4	-0.19 (-0.30 to 0.07)	-0.02 (-012 to 0.16)	0.750
MCV (μm^3)	87.6±4.4	87.4±4.2	-0.11 (-1.42 to 1.19)	86.0±4.8	86.1±4.8	0.11 (-2.02 to 2.25)	-0.05 (-2.62 to 2.53)	0.969
MCH (pg Hb/RBC)	30.9±2.2	31.0±2.2	0.18 (-0.56 to 0.92)	29.2±1.7	29.2±1.9	0.08 (-0.26 to 0.42)	0.06 (-0.70 to 0.81)	0.869
MCHC (g/L)	35.1±0.9	35.4±0.6	0.28 (-0.31 to 0.88)	34.4±1.2	34.66±1.1	0.14 (-0.77 to 1.04)	0.48 (-0.49 to 1.46)	0.299

Discussion

This study showed that a daily vitamin D supplement of 2800 IU for 8 weeks had no effect on Hb levels or anemia risk in hypertensive patients with 25OHD levels <75 nmol/l.

Our study has several strengths. First, this is a randomized, placebo-controlled trial. In addition, compared with previous interventional studies we were able to include a large number of patients. Next, we included only patients with relatively low circulating 25OHD levels. Moreover, the daily vitamin D dose was sufficient to increase in-study 25OHD levels on average clearly above 75 nmol/l. This value is recommended by many endocrinologists and vitamin D researchers as the lower target level for various clinical outcomes [33,34]. Our study also has some limitations. First, the prevalence of anemic patients at baseline was low. Second, the study duration of 8 weeks was relatively short, given that the half-life of red blood cells in circulation is 3 months. Third, we have no data on circulating levels of $1,25(OH)_2D$, which is the active, hormonal form of vitamin D.

Generally, evidence is increasing that $1,25(OH)_2D$ can stimulate erythropoiesis in red blood cell precursor cells by increasing EPO sensitivity. Furthermore, $1,25(OH)_2D$ can up-regulate proliferation of hematopoietic progenitor cells [35,36]. Nevertheless, our findings with plain vitamin D in patients with preserved kidney function do not support the beneficial impact on Hb levels of earlier studies with activated vitamin D in CKD patients [16,17]. It is, however, noteworthy that in CKD patients the prevalence of anemia is high and circulating $1,25(OH)_2D$ levels are low. Moreover, a recent meta-analysis of randomized controlled trials has demonstrated that after administration of vitamin D or activated vitamin D, the increase in circulating $1,25(OH)_2D$ tends to be much higher in CKD patients than in non-CKD patients [37]. In our study, a vitamin D-induced increase in circulating $1,25(OH)_2D$ levels may have been blunted by the suppressed PTH levels, and this may have contributed - at least in part - to the null effect on Hb levels.

In the present study, the prevalence of deficient 25OHD levels was low. Moreover, it was a surprising finding that all 13 anemic patients had initial 25OHD levels >50 nmol/l. Generally, epidemiological evidence suggests that circulating 25OHD levels are inversely associated with Hb levels and anemia risk [4,6]. A recent meta-analysis of retrospective observational studies showed that, compared with individuals with adequate 25OHD levels, low 25OHD levels were associated with an odds ratio for anemia of 2.25 (95% CI: 1.47-3.44) [38]. The cut-off for low 25OHD levels was 50 nmol/l in 5 out of the 7 included studies and 75 nmol/l in the remaining 2 studies. Only individuals without chronic diseases were included in that meta-analysis. However, two large studies in patients with cardiovascular disease [4,6] indicate that Hb levels are only significantly affected if circulating 25OHD levels are in the deficiency range. Also of

note is that observational studies have shown circulating $1,25(OH)_2D$ to be a better predictor of anemia than circulating 25OHD [4,5,39]. Altogether, data indicate that especially anemic patients with deficient 25OHD levels and low circulating $1,25(OH)_2D$ levels may benefit from improved vitamin D status.

In conclusion, our data demonstrate that a daily vitamin D supplement of 2800 IU for 8 weeks does not increase Hb levels in non-anemic hypertensive patients with 25OHD levels <75 nmol/l. Future studies in this field should focus on anemic individuals with deficient 25OHD levels.

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CHAPTER FOUR

The effect of vitamin D supplementation on anemia risk and hemoglobin levels in patients with end-stage heart failure: a randomized clinical trial

Prepared for Submission.

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Abstract

Background and Aims: Low 25-Hydroxyvitamin D (25OHD) levels and anemia are both prevalent in heart failure (HF). We aimed to investigate whether vitamin D supplementation can reduce anemia risk by improving hemoglobin (Hb) levels in end-stage HF.

Methods: EVITA (Effect of Vitamin D on Mortality in Heart Failure) was a randomized, placebo-controlled clinical trial in patients with initial 25OHD levels <75 nmol/l. Participants received either 4,000 IU vitamin D_3 daily or a matching placebo for 36 months. The present investigation was a post-hoc analysis in the per-protocol population (vitamin D group: n=85; placebo group: n=87) that used an analysis of covariance, while adjusting for baseline differences.

Results: Of the study cohort, 11.6% were anemic (Hb <12.5 g/dL) and 42.4% had deficient 25OHD levels (<30 nmol/l). Until study termination, anemia status increased by 12.9% in the vitamin D group and 10.3% in the placebo group, changes that were not influenced by vitamin D treatment (P=0.720). Moreover, there was no treatment effect on Hb levels (-0.04 g/dl [95% CI: -0.53 to 0.45 g/dL]; P=0.865). Results were unaffected by kidney function. Vitamin D treatment was however associated with a decrease in mean corpuscular hemoglobin concentration (-0.7 g/L [95%CI: -1.2 to -0.3 g/L]; P<0.001) and an increase in mean platelet volume (0.5 fL [95%CI: 0.2 to 0.9 fL]; P=0.007]) and plateletcrit (0.010% [95%CI: 0.00 to 0.020%]; P=0.046).

Conclusion: Our data indicate that a daily vitamin D supplement of 4,000 IU cannot be recommended for improving Hb levels or anemia risk in end-stage HF.

Introduction

Both, anemia (Hemoglobin [Hb] <12.5 g/dL) and vitamin D deficiency (25-hydroxyvitamin D [25OHD] values <30 nmol/l) are prevalent in patients with heart failure (HF) [1-3]. The estimated prevalence of anemia in HF varies from 12% to 67% [4,5]. This wide range is, at least in part, due to the use of inconsistent definitions of anemia and the selection of different cohorts (e.g. new-onset HF, end-stage HF) [6]. The prevalence of 25OHD levels <25 nmol/l varies between 28% and 66.7% [7,8]. Compared with age-matched healthy controls, patients with HF also have lower concentrations of the active vitamin D hormone 1,25-dihydroxyvitamin D (1,25$[OH]_2$D) [9].

Epidemiological evidence suggests that both aforementioned vitamin D metabolites are inversely associated with Hb levels and anemia risk in patients with cardiovascular disease [10-12]. including patients with HF [2]. There is also evidence from observational studies that the risk of anemia is stronger associated with low circulating 1,25$(OH)_2$D levels than with deficient circulating 25OHD levels [2,11-13]. Nevertheless, results of interventional studies with vitamin D have been inconsistent: Early interventional studies with small numbers of patients and/or no control-group [14-17] support the assumption of beneficial vitamin D effects on erythropoiesis and anemia control. However, results of more recent randomized controlled trials (RCTs) are mixed [18-21]. While two studies showed no effect of vitamin D on anemia control [19,20] two other studies showed significant decreases in the required dose of erythropoietin (EPO) and erythropoiesis stimulating agents (ESA) in the vitamin D group, respectively [18,21].

In HF, anemia is an independent risk factor of morbidity [22] and mortality [23]. Treatment of anemia can improve patient outcome [24]. Therefore, the present study aimed at investigating the effect of a daily vitamin D_3 supplement on Hb levels and anemia risk in patients with end-stage HF and 25OHD levels <75 nmol/l.

Methods

Study Design and Participants

The present investigation is a secondary analysis of the EVITA (Effect of Vitamin D on Mortality in Heart Failure) trial. This study is a single-center, randomized, placebo-controlled, clinical trial, performed at the Clinic for Thoracic and Cardiovascular Surgery of the Heart- and Diabetes Center North Rhine Westphalia, Bad Oeynhausen, Germany. Main study results have already been published elsewhere. Briefly, between November 2010 and July 2013, 400 patients with HF (332 men and 68 women) were recruited. All patients were ambulatory and regularly seen at our outpatient clinic. Eligible study participants were adults aged ≥18 to 79 years with congestive HF and New York Heart Association functional class ≥II. Participants were randomly allocated to receive 4,000 IU (100 µg) cholecalciferol per day as oily drops (Vigantol® Oel, producer: Merck KGaA, Darmstadt, Germany) or a matching placebo (Miglyol Oel, producer: Merck KGaA, Darmstadt, Germany) for 36 months. During the study, participants remained on guideline-recommended medications. None of the study participants received erythropoietin (EPO). Out of the 400 patients, 177 completed the study as planned (Figure 1). In 172 patients, data on relevant parameters for this secondary analysis were available. The study was registered at EudraCT (number 2010-020793-42) and clinicaltrials.gov (NCT01326650). All study participants gave written informed consent. The study was approved by the Ethics Committee of the Medical Council of Westfalen-Lippe, Germany.

Endpoints

The primary outcome measures of the present investigation were change of the hematological parameter Hb and anemia risk. In line with earlier classifications [12,25-27]. Hb concentrations <12.5 g/dL were considered as anemic, which corresponds to the average threshold value of the World Health Organization's gender-based definition (<13 g/dL in men and <12.0 g/dL in women).

Biochemical Measurements

Fasting venous blood samples were collected on study visits between 8 and 11 AM under standardized conditions. Blood samples were either measured directly within four hours of blood collection or stored at -80°C until analysis. Circulating 25OHD (sum of 25[OH]D_2 and 25[OH]D_3) and 1,25$(OH)_2$D (sum of 1,25$[OH]_2D_2$ and 1,25$[OH]_2D_3$) levels were measured by the autoanalyzer Liaison (DiaSorin, Stillwater, MN, USA). The measuring range for 25OHD lies between 10 and 375 nmol/l. Values <10 nmol/l were considered 9.9 nmol/l. The limit of 1,25$(OH)_2$D quantitation is 12 pmol/l, and we considered values below this limit as 11 pmol/l.

C-reactive protein (CRP) and creatinine values were analyzed by the Architect Autoanalyzer (Abbott, Wiesbaden, Germany). Estimated glomerular filtration rate (eGFR) was calculated using the Modification of Diet in Renal Disease [28]. Blood Hb, hematocrit (Htc), erythrocytes, leukocytes, thrombocytes, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), red blood cell distribution width (RDW), mean platelet volume (MPV), and plateletcrit were measured by automated procedures using the Abbott CellDyn 3500 hematology analyzer (Abbott, Wiesbaden, Germany).

According to previous classification [25], we used the following cut-off values for classifying 25OHD: <30 nmol/l as deficient, 30-49.9 nmol/l as insufficient, and 50-74.9 as borderline.

Statistics

Categorical data are presented as percentages. Continuous data with a normal distribution are shown as means with standard deviation (SD) and data with a skewed distribution are shown as medians with interquartile range. Normal data distribution was checked by using the Kolmogorov-Smirnov test and was considered when probability values were >0.05. We used the chi-squared test, unpaired *t*-test or Mann-Whitney-U-Test or for baseline study group comparisons.

Change from baseline data is shown as mean with 95% confidence interval [CI]. Skewed variables were log(*e*) transformed before use in parametric statistical analyses. We used ANCOVA with baseline adjustments to test for differences in the outcome variables between the two study groups at follow-up (36 months). The McNemar test was used to assess differences in anemic status within groups. The Fisher´s exact test was used to assess differences in anemic status between groups. We considered P-values <0.05 as statistically significant. P-values are two-sided. Analyses were performed using SPSS version 21.0 (SPSS, Inc., Chicago, IL, USA).

Results

Baseline characteristics of the participants are shown in Table 1. Initial MCV was significantly lower in the vitamin D group than in the placebo group. Compared with placebo, slightly but significantly more patients in the vitamin D group suffered from coronary heart disease. No significant group differences in any other clinical, biochemical, or medical treatment parameter was present (Table 1). At baseline, 11.8% and 11.5% in the vitamin D group and placebo group, respectively, were anemic. Moreover, 47.1% and 37.9% in the vitamin D group and placebo group, respectively, had deficient 25OHD levels. In addition, 30.6% and 22.4% in the vitamin D group and 41.4% and 20.7% in the placebo group had insufficient and borderline 25OHD levels, respectively.

Table 2 shows the results of vitamin D treatment on biochemical parameters. There was a significant treatment effect on circulating 25OHD levels. The increment was 55.3 nmo/l (95% CI: 43.5 to 67.1 nmol/l; $P<0.001$). The corresponding values concerning the effect on circulating $1,25(OH)_2D$ were +17.1 pmol/l (95% CI: 6.2 to 27.9 pmol/l; $P=0.004$). There was however no significant treatment effect on Hb levels. In detail, in-study Hb levels decreased on average by -0.5 g/dL (95% CI: -0.9 to -0.2) g/dL and -0.5 g/dL (95% CI: -0.9 to -0.1) g/dL, in the vitamin D and placebo group, respectively. The mean treatment effect was -0.04 g/dL (95% CI: -0.53 to 0.45 g/dL; $P=0.865$). Anemia status increased by 12.9% in the vitamin D group ($P=0.007$) and 10.3% in the placebo group ($P=0.049$; Figure 2A). No difference in anemic status was determined between the two study groups at study termination ($P=0.720$; Figure 2B). Compared with placebo, there was a significant increase in MPV and plateletcrit and a significant decrease in MCHC in the vitamin D group (Table 2). However, mean values were still within the respective reference range for adults (reference ranges: MPV 7.4-10.4 fl; plateletcrit 0.162-0.346 %; MCHC 31-37 g/dL). In the subgroup of patients with chronic kidney disease (CKD) stage 3 or 4 (n=49), 18.4% and 34.7% were anemic at baseline and study termination, respectively. Again, no difference in anemic status was determined between the two study groups at study termination (vitamin D group: 38.5% vs. placebo group: 30.4%; $P=0.764$). Moreover, vitamin D had no effect on Hb levels (±0.0 g/dL [95% CI: -1.0 to 1.0 g/dL]; Supplemental Table 1).

Table 1: Baseline characteristics of the study groups

Characteristics	Vitamin D 100 µg (*n*=85)	Placebo (*n*=87)	*P*-Value
Females (%)	12.9	21.8	0.124
Age (years)	56 (49-61)	54 (47-59)	0.088
Anemic subjects[1] (%)	11.8	11.5	0.956
BMI (kg/m^2)	28.4 (25.0-31.4)	28.1 (25.7-32.2)	0.593
NYHA (%)			
Class II	77.6	71.3	0.337
Class III	22.4	28.7	0.337
LVEF (%)	30.0 (23.5-35.0)	28.0 (24.0-35.0)	0.960
LVED (mm)	66.0 (59.0-73.0)	66.0 (60.0-75.0)	0.973
Primary Disease (%)			
Dilated cardiomyopathy	38.8	50.6	0.121
Coronary heart disease	57.6	42.5	0.047
Others	3.5	6.9	0.321
Comorbidities (%)			
Diabetes	28.2	16.1	0.055
Arterial hypertension	25.9	29.9	0.558
Renal insufficiency	10.6	8.0	0.566
Drug therapy (%)			
Loop diuretic	82.4	86.2	0.487
Thiazide diuretic	25.9	31.0	0.454
Aldosterone antagonist	81.2	86.2	0.372
ACE- inhibitor	72.9	71.3	0.806
AT II blocker	29.4	36.8	0.305
Beta-blocker	96.5	97.7	0.631
Digitalis	31.8	40.2	0.248
Antiarrhythmic drug	24.7	28.7	0.551
Lipid-lowering drug	57.6	54.0	0.632
Calcium channel blocker	3.5	3.4	0.977
Vitamin D supplement	0.0	0.0	>0.999
Vitamin D metabolites			
25OHD (nmol/l)	30.7 (22.0-46.6)	34.4 (26.7-45.9)	0.374
1,25(OH)$_2$D (pmol/l)[2]	81.6 (60.8-100.9)	87.7 (65.2-104.8)	0.183
Hematological parameters			
Hemoglobin (g/dL)	14.2±1.6	14.2±1.5	0.966
Hematocrit (%)	42.2±4.9	42.2±4.2	0.932
Leukocytes (10^9/L)	8.1 (6.8-9.6)	7.7 (6.2-9.6)	0.369
Erythrocytes (10^{12}/L)	4.7 (4.4-5.0)	4.6 (4.3-4.9)	0.415
MCV (µm^3)	90.6 (88.1-93.4)	92.9 (90.1-95.6)	0.005

MCH (pg Hb/red blood cell)	30.7±2.0	31.2±2.5	0.101
MCHC (g/L)	33.6 (33.0-34.8)	33.9 (33.0-34.7)	0.870
Thrombocytes (10^9/L)	211 (185-255)	212 (190-249)	0.932
RDW (%)	12.7 (11.9-13.5)	12.5 (11.9-13.2)	0.319
MPV (fl)	8.5 (7.5-9.3)	8.0 (7.3-9.3)	0.296
Plateletcrit (%)	0.183 (0.155-0.218)	0.184 (0.153-0.210)	0.473
Additional parameters			
eGFR (mL/min/1.73 m^2)	71.7±22.5	75.8±23.4	0.237
CRP (mg/dL)[3]	0.24 (0.10-0.69)	0.21 (0.09-0.40)	0.406

[1]Hemoglobin <12.5 g/dL

[2]data are based on 161 patients

[3]data are based on 155 patients

COPD: chronic obstructive pulmonary disease; CRP: C-reactive protein; eGFR: estimated globular filtration rate; LVEF; left ventricular ejection fraction; LVED: left ventricular end-diastolic diameter; MPV: mean platelet volume; RDW: erythrocytes distribution width

Table 2: Results of vitamin D treatment on biochemical parameters in patients with chronic heart failure

Characteristics	Vitamin D group (n=85)			Placebo group (n=87)			Treatment effect	P-value
	Baseline	Follow-up (36 months)	Mean change from baseline[1]	Baseline	Follow-up (36 months)	Mean change from baseline[1]		
Vitamin D metabolites								
25OHD (nmol/l)	30.7 (22.0-46.6)	92.1 (65.6-128.0)	65.6 (55.0 to 76.3)	34.4 (26.7-45.9)	40.2 (30.5-57.9)	9.9 (4.3 to 15.5)	55.3 (43.5 to 67.1)	<0.001
$1,25(OH)_2D$ (pmol/l)	81.6 (60.8-100.9)	90.0 (68.3-121.8)	12.3 (3.1 to 21.4)	87.7 (65.2-104.8)	76.1 (60.5-105.1)	-7.8 (-15.5 to 0.0)	17.1 (6.2 to 27.9)	0.004
Hematological parameters								
Hemoglobin (g/dL)	14.2±1.6	13.7±1.8	-0.5 (-0.9 to -0.2)	14.2±1.5	13.8±1.9	-0.5 (-0.9 to -0.1)	-0.04 (-0.53 to 0.45)	0.865
Hematocrit (%)	42.2±4.9	41.6±5.7	-0.6 (-1.7 to 0.4)	42.2±4.2	40.9±5.6	-1.3 (-2.5 to -0.1)	0.7 (-0.8 to 2.2)	0.358
Leukocytes (10^9/L)	8.1 (6.8-9.6)	8.2 (6.4-9.4)	-0.3 (-0.7 to 0.1)	7.7 (6.2-9.6)	7.2 (5.7-9.2)	-0.3 (-0.8 to 0.2)	0.2 (-0.4 to 0.7)	0.596
Erythrocytes (10^{12}/L)	4.7 (4.4-5.0)	4.7 (4.3-5.1)	0.0 (-0.1 to 0.1)	4.6 (4.3-4.9)	4.6 (4.1-5.0)	-0.5 (-1.5 to 0.5)	0.15 (-0.04 to 0.33)	0.103
MCV (μm^3)	90.6 (88.1-93.4)	88.7 (84.8-94.0)	-1.6 (-3.0 to -0.2)	92.9 (90.1-95.6)	90.6 (87.1-93.1)	-1.9 (-3.6 to 0.1)	0.5 (-2.4 to 1.4)	0.329
MCH (pg Hb/red blood cell)	30.7±2.0	29.4±2.7	-1.3 (-1.7 to 0.8)	31.2±2.5	30.3±2.2	-0.9 (-1.4 to 0.4)	0.6 (-1.3 to 0.0)	0.051
MCHC (g/L)	33.6 (33.0-34.8)	32.9 (31.9-34.9)	-0.8 (-1.2 to -0.4)	33.9 (33.0-34.7)	33.7 (32.8-34.5)	-0.0 (-0.4 to 0.4)	-0.7 (-1.2 to -0.3)	0.001
Thrombocytes (10^9/L)	211 (185-255)	201 (168-242)	-13 (-23 to -3)	212 (190-249)	210 (176-243)	-12 (-22 to -2)	-0.7 (-13.9 to 12.5)	0.864
RDW (%)	12.7 (11.9-13.5)	13.8 (12.5-15.0)	1.0 (0.6 to 1.4)	12.5 (11.9-13.2)	13.0 (12.4-14.5)	0.8 (0.4 to 1.2)	0.2 (-0.3 to 0.8)	0.371
MPV (fl)	8.5 (7.5-9.3)	8.1 (7.3-9.3)	-0.2 (-0.5 to 0.0)	8.0 (7.3-9.3)	7.5 (6.8-8.3)	-0.7 (-1.0 to -0.4)	0.5 (0.2 to 0.9)	0.007
Plateletcrit (%)	0.183 (0.155-0.218)	0.163 (0.144-0.196)	-0.019 (-0.027 to -0.011)	0.184 (0.153-0.210)	0.161 (0.134-0.183)	-0.026 (-0.034 to -0.018)	0.010 (0.00 to 0.020)	0.046
Additional parameters								
eGFR (mL/min/1.73 m^2)	71.7±22.5	61.9±25.3	-9.7 (-13.7 to 5.8)	75.8±23.4	69.9±26.0	-5.9 (-10.0 to -1.8)	-4.7 (-10.2 to 0.8)	0.094
CRP (mg/dL)	0.24 (0.10-0.69)	0.27 (0.14-0.73)	0.04 (-0.12 to 0.19)	0.21 (0.09-0.40)	0.23 (0.11-0.57)	0.02 (0.0 to 0.03)	-0.08 (-0.29 to 0.13)	0.856

[1]Change from baseline data is shown as means and 95% confidence interval

$1,25(OH)_2D$: 1,25-dihydroxyvitamin D; 25OHD: 25-hydroxyvitamin D; CRP: C-reactive protein; eGFR: estimated globular filtration rate; MCH: mean corpuscular hemoglobin; MCHC: mean corpuscular hemoglobin concentration; MCV: mean corpuscular volume; MPV: mean platelet volume; RDW: erythrocytes distribution width

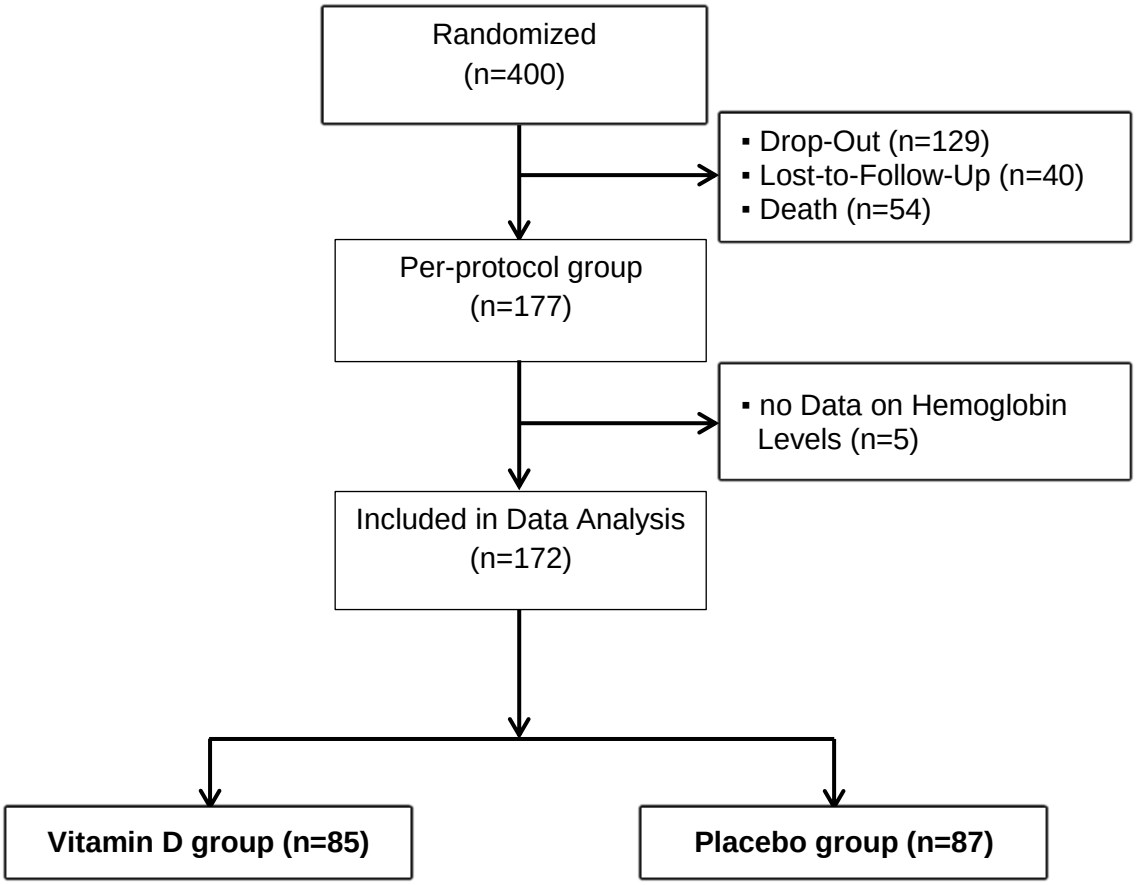

Figure 1: Participant flow chart of this secondary analysis of the EVITA Trial.

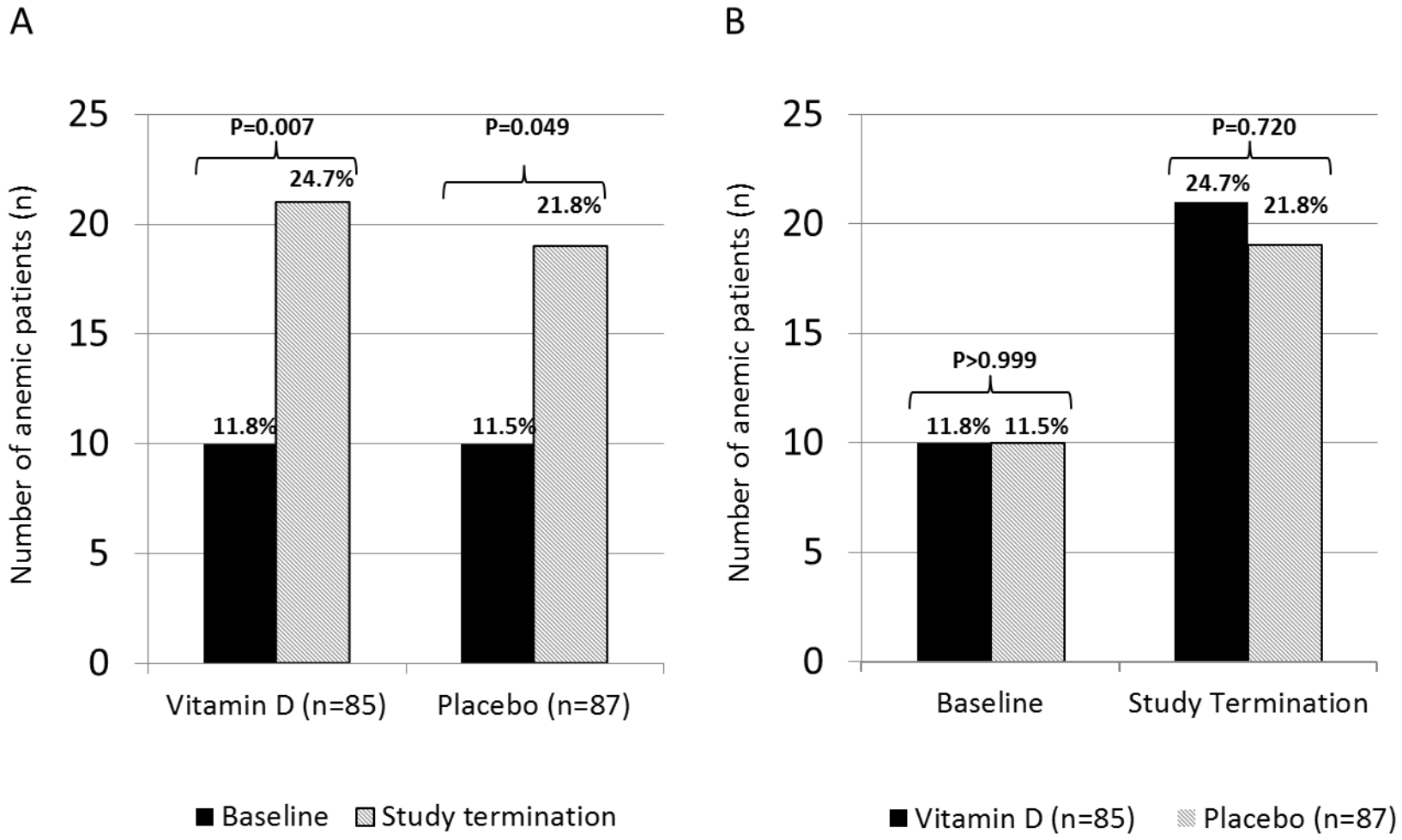

Figure 2: **Anemia prevalence in 172 participants of the EVITA Trial at baseline and study termination;** A: by study group; P-values indicate within study group results; B: by follow-up; P-values indicate between study group results

Table S1: Results of vitamin D treatment on biochemical parameters in patients with chronic heart failure and estimated glomerular filtration rate values <60 mL/min/1.73 m²

Characteristics	Vitamin D group (n=26)			Placebo group (n=23)			Treatment effect	P-value
	Baseline	Follow-up (36 months)	Mean change from baseline[1]	Baseline	Follow-up (36 months)	Mean change from baseline[1]		
Anemic patients (%)	19.2	38.5	-	17.4	30.4	-	-	-
Vitamin D metabolites								
25OHD (nmol/l)	31.5 (22.9-54.2)	79.9 (62.4-148.3)	70.5 (45.1 to 95.9)	34.7 (25.7-52.4)	38.7 (30.5-63.4)	11.8 (-1.3 to25.0)	58.6 (29.3 to 88.0)	<0.001
$1,25(OH)_2D$ (pmol/l)	74.4±41.2	86.5±27.9	12.0 (-3.7 to 27.8)	90.3±36.6	68.9±38.6	-19.2 (-36.2 to -2.1)	21.7 (3.3 to 40.1)	0.022
Hematological parameters								
Hemoglobin (g/dL)	13.7±1.8	13.0±1.8	-0.8 (-1.5 to 0.1)	13.8±1.7	13.0±2.1	-0.8 (-1.8 to 0.2)	0.0 (-1.0 to 1.0)	0.997
Hematocrit (%)	40.6±5.5	39.9±5.8	-0.7 (-2.9 to 1.5)	40.6±5.1	39.1±5.9	-1.5 (-4.5 to 1.5)	0.8 (-2.4 to 3.9)	0.615
Leukocytes (10^9/L)	8.3 (7.6-9.7)	8.3 (6.9-9.2)	-0.5 (-1.3 to 0.3)	8.8 (6.3-10.6)	7.9 (5.8-9.2)	-0.8 (-1.6 to 0.1)	0.4 (-0.5 to 1.3)	0.396
Erythrocytes (10^{12}/L)	4.5 (3.9-4.9)	4.5 (4.1-4.9)	0.0 (-0.2 to 0.3)	4.4 (4.0-4.9)	4.2 (3.8-4.6)	-2.0 (-5.8 to 1.9)	0.3 (0.0 to 0.7)	0.064
MCV (μm^3)	90.6±5.2	88.5±8.5	-2.1 (-5.2 to 1.0)	93.7±6.1	92.3±5.0	-1.4 (-3.8 to1.1)	-2.1 (-5.9 to 1.7)	0.275
MCH (pg Hb/red blood cell)	31.1 (29.1-32.1)	29.4 (26.4-31.1)	-1.9 (-2.9 to -0.9)	32.5 (30.5-33.0)	30.5 (28.8-31.7)	-1.2 (-2.3 to 0.1)	-1.3 (-2.7 to 0.1)	0.060
MCHC (g/L)	33.9±1.0	32.5±1.4	-1.3 (-2.1 to 0.6)	34.0±1.3	33.2±1.5	-0.8 (-1.6 to 0.0)	-0.7 (-1.5 to 0.2)	0.126
Thrombocytes (10^9/L)	195 (182-244)	187 (164-215)	-12 (-29 to 6)	221 (195-254)	213 (170-272)	-15 (-38 to 8)	-6 (-33 to 20)	0.789
RDW (%)	13.4±1.2	15.2±1.9	1.7 (-1.0 to 2.5)	13.2±1.5	14.6±2.4	1.4 (-0.4 to 2.3)	0.5 (-0.7 to 1.6)	0.428
MPV (fl)	8.7±1.2	8.7±1.6	0.0 (-0.7 to 0.7)	8.2±1.5	7.5±1.0	-0.7 (-1.3 to -0.1)	1.1 (0.3 to 1.8)	0.008
Plateletcrit (%)	0.188±0.042	0.170±0.035	0.019 (-0.036 to -0.002)	0.193±0.057	0.165±0.044	-0.031 (-0.051 to -0.011)	0.009 (-0.010 to 0.029)	0.325
Additional parameters								
eGFR (mL/min/1.73 m^2)	46.0 (39.3-52.0)	37.5 (29.8-48.0)	-5.4 (-11.3 to 0.6)	48.0 (42.0-56.0)	45.0 (32.0-64.0)	0.4 (-7.2 to 7.9)	-6.3 (-15.5 to 2.9)	0.195
CRP (mg/dL)	0.29 (0.15-0.86)	0.33 (0.20-0.96)	-0.12 (-0.45 to 0.21)	0.29 (0.18-0.53)	0.41 (0.21-1.20)	0.34 (-0.14 to 0.82)	-0.4 (-0.9 to 0.0)	0.225

[1]Change from baseline data is shown as means and 95% confidence interval

$1,25(OH)_2D$: 1,25-dihydroxyvitamin D; 25OHD: 25-hydroxyvitamin D; CRP: C-reactive protein; eGFR: estimated globular filtration rate; MCH: mean corpuscular hemoglobin; MCHC: mean corpuscular hemoglobin concentration; MCV: mean corpuscular volume; MPV: mean platelet volume; RDW: erythrocytes distribution width

Discussion

The present investigation indicates that a daily vitamin D supplement of 4,000 IU for 36 months does not improve Hb values in patients with end-stage HF. Moreover, the incidence of anemia increased in both groups to a similar extent. Comparable results were obtained in the subgroup of patients with initial eGFR values <60 mL/min/1.73 m^2.

Our data do not support results of a recent meta-analysis, reporting an inverse association of circulating 25OHD with anemia risk [29]. However, our data are in line with results of three other RCTs in subjects with concurrent iron-deficiency anemia, ethnic minorities living in Norway, and hypertensive patients [19,20,25]. In these earlier studies, duration ranged from 8 to 16 weeks and vitamin D doses ranged from 400 IU to 2,800 IU daily or 600,000 IU once intramuscularly. Although in another small, placebo-controlled trial [21] 650,000 IU vitamin D (50,000/weekly over a 4-months period) significantly decreased the required dose of EPO, no correlation between Hb levels and 25OHD concentrations could be shown. Moreover, it remains unclear why in that RCT 20 out of the 64 study subjects were excluded from data analysis. The required ESA dose was also reduced in a small RCT in children with CKD stage 5, who received vitamin D_2 or placebo in conjunction with oral alfacalcidol (0.25 µg/capsule) for 12 weeks [18]. Unfortunately, no data on circulating $1,25(OH)_2D$ levels were presented. $1,25(OH)_2D$ has been shown to stimulate erythroid precursor proliferation via increased erythropoietin sensitivity [30]. Some earlier observational studies reported that compared with the reference category of circulating $1,25(OH)_2D$ levels >70 pmol/l, the multivariable-adjusted odds ratio for anemia was 2.35 to 4.08 in the categories of circulating $1,25(OH)_2D$ levels <40 pmol/l [2,11,12]. In the present RCT, the treatment effect on circulating $1,25(OH)_2D$ was on average +17.1 pmol/l. The increment is in line with a recent meta-analysis reporting that vitamin D supplements increase circulating $1,25(OH)_2D$ on average by +18.8 pmol/l (95% CI: 9.2 to 28.4 pmol/l) [31], but was probably too small to achieve significant effects on anemia risk. It might also be that in the present RCT the average initial $1,25(OH)_2D$ level of 81.6 pmol/l was already above the threshold for stimulating erythropoiesis. It is also noteworthy that anemia risk is associated with the severity of the HF [4,23,32,33], Therefore, there may simply be no causal relationship between vitamin D status and anemia in end-stage HF. This assumption is further supported by the fact that even in the subgroup of patients with eGFR values <60 mL/min/1.73 m^2, where EPO production and circulating $1,25(OH)_2D$ levels are reduced, vitamin D treatment had no effect on Hb levels and anemia risk.

Vitamin D treatment increased MPV and plateletcrit and decreased MCHC values significantly. High MPV and plateletcrit values are associated with acute myocardial infarction [34] and long term mortality in patients non-ST-segment elevation myocardial infarction [35]. Since median

values of both MCHC and plateletcrit were still in the reference range, we conclude that the vitamin D-mediated treatment effects are not clinical relevant.

Our study has several strengths, but also some limitations. Strengths are the study design, the large number of study participants, the study duration of 36 months, and the measurement of the active hormonal form of vitamin D, $1,25(OH)_2D$, in addition to circulating 25OHD levels. One limitation is that we have no data on nutritional risk factor of anemia such as folate or vitamin B12 deficiency. Another limitation is that the study population was restricted to Caucasian ethnicity.

In summary, the present study was unable to show significant effects of a daily vitamin D supplement of 4,000 IU for 36 months on Hb values and anemia risk in patients with end-stage HF. Our data challenge the clinical relevance of vitamin D supplementation to increase Hb levels.

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GENERAL DISCUSSION

The aim of this thesis was to investigate the associations between vitamin D metabolites and anemia risk in patients with CVD and to examine if these associations are causal by analyzing data of two RCTs. The first cross-sectional study (CHAPTER ONE) investigated the associations between the vitamin D metabolites 25OHD and $1,25(OH)_2D$ with preoperative anemia (Hb <12.5 g/dL) in 3,615 patients scheduled for cardiac surgery. In the second cross-sectional study (CHAPTER TWO) the same associations were investigated in 3,299 patients referred for coronary angiography. In two secondary analyses of RCTs, we examined if these associations were causal: The first interventional study (CHAPTER THREE) investigated whether a vitamin D_3 supplementation of 2,800 IU daily for eight weeks can improve Hb levels and reduce anemia risk in 200 hypertensive patients compared to a matching placebo. The second investigation (CHAPTER FOUR) examined the impact of a vitamin D_3 supplementation of 4,000 IU daily versus placebo for 36 months on Hb levels and therefore anemia risk in 172 heart failure patients. In both interventional trials, patients had initial 25OHD levels below 75 nmol/l.

Both cross-sectional studies demonstrate an independent inverse association between vitamin D status and anemia risk in patients with CVD (CHAPTER ONE and CHAPTER TWO). In cardiac surgical patients and patients referred to coronary angiography with deficient 25OHD levels (<30 nmol/l) mean Hb levels were 0.5 g/dL and 0.6 g/dL lower than in patients with adequate levels (50-125 nmol/l), respectively. Regarding $1,25(OH)_2D$, mean Hb concentrations were 1.2 g/dL and 1.3 g/dL lower in the lowest $1,25(OH)_2D$ category (<40 pmol/l) compared to the highest $1,25(OH)_2D$ category (>70 pmol/l). In both studies, multivariable adjusted logistic regression analyses showed that the ORs for anemia of the lowest categories of 25OHD (<30 nmol/l) and $1,25(OH)_2D$ (<40 pmol/l) were significantly higher compared with patients who had adequate 25OHD levels (50-125 nmol/l) and $1,25(OH)_2D$ values in the highest category (>70 pmol/l). In both studies, these associations were stronger for $1,25(OH)_2D$ than 25OHD and anemia risk was highest in patients with dual deficiency.

Our results regarding the association between 25OHD status and anemia risk are in line with findings from observational studies conducted in various groups of individuals [1-13]. These studies included patients with CVD, e.g. cardiac surgical patients [14] and patients with heart failure [15], also demonstrating an independent association of anemia risk with low 25OHD concentrations. In accordance with these data, a recent meta-analysis of observational studies showed that vitamin D deficiency (<75 nmol/l or 50 nmol/l) was associated with an increased incidence of anemia (OR=2.25 [1.47-3.44]), compared with individuals with adequate vitamin D status [16]. In contrast to the broad availability of data regarding the association between 25OHD and anemia risk, the association of $1,25(OH)_2D$ with anemia risk has rarely been studied. The few available studies have been conducted in patients with heart failure, older Australian men and patients with CKD. In regard to the study also examining heart failure

patients, results are in line with our study, showing that the association was stronger for $1,25(OH)_2D$ concentrations than for circulating 25OHD concentrations [15]. In a study examining 1,666 older men, only $1,25(OH)_2D$ levels, but not 25OHD levels, were significantly associated with Hb levels [17]. This difference may be, at least to some extent, due to the smaller number of individuals studied by Hirani et al. and the lower statistical power to achieve significant results. In contrast, in patients with CKD, the association between 25OHD and anemia was found to be stronger than between $1,25(OH)_2D$ and anemia [8].

However, although statistical adjustments were performed to preclude some sources of bias, the observational study design of CHAPTER ONE and CHAPTER TWO does not allow a confirmation of a causal relationship between vitamin D and anemia. Therefore, two secondary analyses of RCTs were performed to examine if vitamin D deficiency is a factor in the etiology of anemia in patients with CVD.

Both investigations (CHAPTER THREE and CHAPTER FOUR) indicate that compared to placebo, a daily vitamin D supplement does not improve Hb values in patients with CVD. In hypertensive patients (CHAPTER THREE), a daily vitamin D supplementation of 2,800 IU for eight weeks resulted in a mean treatment effect on Hb levels of 0.04 (-0.14 to 0.22) g/dL (P=0.661). Thus, vitamin D treatment did not influence anemic status significantly. In the vitamin D group, 7.5% of patients were anemic at baseline and study termination (>0.999). In addition, vitamin D had no effect on other hematological parameters. Since patients with deficient vitamin D status (<30 nmol/l) had the lowest Hb levels and the highest risk of being anemic in CHAPTER ONE and CHAPTER TWO, a subgroup analysis in the group of patients with initial 25OHD levels <30 nmol/l was conducted. Again, there was no vitamin D effect on Hb levels. However, with eight weeks, the study duration was relatively short, given that it usually takes approximately three months to reach a steady state in circulating 25OHD levels [18] and that the half-life of red blood cells in circulation is also three months. In addition, prevalence of anemic patients and patients with 25OHD levels <30 nmol/l at baseline was low and no measurement of $1,25(OH)_2D$ was conducted. Since both observational studies included in this thesis showed that the association with anemia risk was stronger for $1,25(OH)_2D$ concentrations than for circulating 25OHD concentrations and, among others, $1,25(OH)_2D$ has been shown to stimulate erythroid precursor proliferation by increasing EPO sensitivity (INTRODUCTION) , this measurement is of great interest.

Therefore, in CHAPTER FOUR, a post-hoc analysis in the per-protocol population of an RCT with a daily vitamin D supplement of 4,000 IU for a study duration of 36 months and a higher initial prevalence of anemia (11.6%) including $1,25(OH)_2D$ measurement was conducted. However, the investigation confirms the main result of CHAPTER THREE: A daily vitamin D supplement of 4,000 IU for 36 months does not improve Hb values. In fact, the incidence of anemia increased

in both groups from 11.6% to a similar extent, leading to an overall anemia prevalence of 23.3%. Therefore, mean treatment effect on Hb levels was -0.04 (-0.53 to 0.45) g/dL (P=0.865). Regarding circulating $1,25(OH)_2D$, the mean treatment effect was +17.1 (6.2 to 27.9) pmol/l (P=0.004), which is in line with a recent meta-analysis showing that vitamin D supplementation increase $1,25(OH)_2D$ levels on average by +18.8 (9.2 to 28.4) pmol/l [19]. However, the increase may have been too small to achieve significant effects on anemia risk. Although the reference range for circulating $1,25(OH)_2D$ levels is not yet well defined, the Gaussian distribution of apparently healthy individuals assume a reference interval of 38 to 134 pmol/l with a mean value of 90 pmol/l [19,20]. Possibly, the average initial $1,25(OH)_2D$ level of 81.6 pmol/l was already above the threshold for stimulating biological actions reducing anemia risk e.g stimulating erythropoiesis. Therefore, a subgroup analysis including only patients with eGFR values <60 mL/min/1.73 m^2 who have reduced EPO production and circulating $1,25(OH)_2D$ levels, was performed (CHAPTER FOUR, Supplemental Table 1). Again, vitamin D treatment had no effect on Hb levels and anemia risk.

Our results are in line with other recent RCTs. Madar et al. did not find a difference in change of Hb values between those receiving oral vitamin D_3 supplementation (400 or 1,000 IU/d combined) or those receiving placebo after 16 weeks in healthy ethnic minorities living in Norway [21]. In this cohort, 90% of the study subjects had initial 25OHD concentrations of <50 nmol/l. However, although the vitamin D doses used were sufficient to increase the 25OHD levels, it failed to increase the 25OHD levels above 50 nmol/l in 43% of the 1,000 IU supplementation group and in 62% of the 400 IU supplementation group [21]. In contrast, with 25OHD concentrations of 90.3 nmol/l and 92.1 nmol/l at study termination, adequate circulating levels (50 to 125 nmol/l) were reached in our study cohorts of hypertensive patients and patients with heart failure, respectively. Therefore the scenario that the given vitamin D dosages were too low to reach sufficient 25OHD concentrations can be ruled out. Another randomized, single-blinded, placebo-controlled 12-week trial in vitamin D deficient subjects with iron-deficiency also failed to show a significant difference in Hb values between groups after one intramuscularly dose of 600,000 IU vitamin D_3 [22]. Naini et al. showed that 650,000 IU vitamin D over a 4-months period (50,000/weekly) significantly decrease the required dose of EPO compared to placebo [23]. However, there was no correlation between the Hb levels and 25OHD concentrations. In children with CKD stage 5, oral vitamin D_2 treatment for 12 weeks significantly decreased the required dose of ESA [24].

Although recent RCTs, including CHAPTER THREE and FOUR, failed to demonstrate significant Hb-improving effects of vitamin D supplementation, first non-randomized interventional studies in patients with CKD demonstrated an increase in Hb levels after 12 months of intravenous $1,25(OH)_2D$ administration, resulting in improved control of anemia with reduced need for EPO

[25,26]. In a prospective study examining the effect of high-dose alfacalcidol (1α-hydroxyvitamin D) in anemic hemodialysis patients, Hb levels increased significantly by 2.0 g/dL and 1.8 g/dL at 12 and 18 months, respectively. Of note, initial Hb levels were 8.7 g/dL [27]. In the other two aforementioned studies, initial Hb values were 10.7 g/dL and 10.2 g/dL, respectively [25,26]. In our study cohorts, initial Hb values were 14.4 g/dL and 14.2 g/dL, respectively. Thus, with 6.5 % (CHAPTER THREE) and 11.6 % (CHAPTER FOUR), the prevalence of anemic patients at baseline was low. Possibly, the missing effects can be explained by the aforementioned Hb levels which are already within the reference range of 13.5-17.5 g/dL for men and 12-16 g/dL for women. Therefore, it may be a possible explanation that the initial Hb levels of both study cohorts were too high and the prevalence of anemic patients too low to achieve any improving effects on Hb levels. Nevertheless, these first interventional studies have some limitations since they included only small numbers of participants and had no control group. In these non-randomized studies the administered vitamin D were active metabolites. In contrast, we used vitamin D_3 supplements in both interventional studies.

The investigations of this thesis have several strengths, but also some limitations. Strengths are (1) the large number of included patients in all four studies, (2) the measurement of the active vitamin D metabolite, $1,25(OH)_2D$, in three out of the four studies and (3) the performance of multivariable-adjusted analyses including important confounders conducted in CHAPTER ONE and CHAPTER TWO. Regarding CHAPTER THREE and CHAPTER FOUR the strengths are (4) the randomized, placebo-controlled study design, the fact that (5) only patients with relatively low circulating 25OHD levels were included and (6) the daily vitamin D doses, which were sufficient to increase in-study 25OHD levels distinctly above 75 nmol/l on average. The included studies also have some limitations. First, the findings are restricted to selected study cohorts of Caucasian participants with CVD. Therefore, the results may not be generalizable to other study populations. Second, regarding the study design of CHAPTER THREE and CHAPTER FOUR, two secondary analyses of RCTs were conducted. Therefore, clinical studies investigating change in Hb levels as the primary endpoint are still warranted. Third, in none of the included studies data on nutritional risk factors such as folate or vitamin B12 deficiency as well as reticulocytes, EPO concentrations and use of ESA was available.

In conclusion, despite the broad range of encouraging observational studies including CHAPTER ONE and CHAPTER TWO of this thesis, results of recent RCTs including CHAPTER THREE and CHAPTER FOUR do not support a causal relationship between vitamin D supplementation and anemia risk. It may be that both, anemia and vitamin D deficiency, are markers of progressive health status rather than causal factors depending on each other. Overall, this thesis argues against clinically relevant Hb-improving effects of vitamin D_3 supplements in patients with CVD. However, given the study design, studies investigating the effect of vitamin D supplementation or administration on Hb levels in vitamin D deficient individuals as primary endpoint are still warranted. Furthermore, since there is a stronger independent association between $1,25(OH)_2D$ concentrations and anemia risk than between 25OHD levels and anemia risk and first interventional studies using $1,25(OH)_2D$ administration showed promising results, future studies should explore the administration of active vitamin D metabolites in anemic and vitamin D deficient patients in more detail.

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