Dissertation

Vitamin D, clinical outcomes and regulation of metabolism

submitted by

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Declaration

I hereby declare that this thesis is my own original work and that I have fully acknowledged by name all of those individuals and organizations that have contributed to the research for this thesis. Due acknowledgement has been made in the text to all other material used. Throughout this thesis and in all related publications I followed the "Standards of Good Scientific Practice and Ombuds Committee at the Medical University of Graz".

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Disclosures

Numerous people contributed to the data presented in this work.

Barbara Obermayer-Pietsch and Julia Münzker supervised this work.

Stan Ursem contributed to data analysis and data interpretation for sub-study1 and sub-study 3. In addition Martin Keppel, Verena Schwetz, Christian Trummer, Marlene Pandis, Martin R Grübler, Nicolas Verheyen, Marcus Kleber, Graciela Delgado, Angela Moissl, Winfried März, Andreas Tomaschitz, Stefan Pilz and Barbara Obermayer-Pietsch were involved in data interpretation and study proceedings in all three sub-studies in this work, while Annemieke Heijboer contributed to sub-studies 1 and 2. Benjamin Dieplinger and Valentin Borzan contributed to data interpretation and study proceeding of sub-study 2.

Cornelia Missbrenner and Julia Brunner contributed to sample management. Andrea Wöls assisted with laboratory measurements of sST2 for sub-study 2. Dr Ingrid Gergei assisted with heart failure patient data analysis of the LURIC study for sub-study 2. Andrea Groselj-Strele and Katharina Eberhard offered advice on statistical analyses.

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Abbreviations

Δ1,25$(OH)_2$D	change from baseline for 1,25$(OH)_2$D in the vitamin D treated group
Δ24,25$(OH)_2D_3$	change from baseline for 24,25$(OH)_2D_3$ in the vitamin D treated group
Δ25$(OH)D_3$	change from baseline for 25$(OH)D_3$ in the vitamin D treated group
1,25$(OH)_2$D	1,25-dihydroxyvitamin D
1,25D_3-MARRS	1,25D_3-membrane associated, rapid response steroid-binding
24,25$(OH)_2D_3$	24,25-dihydroxyvitamin D_3
25$(OH)D_3$	25-hydroxyvitamin D_3
ADMA	asymmetric dimethylarginine
bALP	bone-specific alkaline phosphatase
Bioavailable 25$(OH)D_3$	biologically available 25-hydroxyvitamin D_3
BMI	body mass index
CD4	cluster of differentiation 4
CD8	cluster of differentiation 8
CYP24A1	25-hydroxyvitamin D-24-hydroxylase
CTX	carboxy-terminal collagen crosslinks
CVD	cardiovascular disease
CYP27B1	25-Hydroxyvitamin D_3 1-alpha-hydroxylase

DBP	D-binding protein
eGFR	estimated glomerular filration rate
FGF 23	fibroblast growth factor 23
free 25(OH)D_3	free 25-hydroxyvitamin D_3
HDL cholesterol	high-density lipoprotein cholesterol
HF	heart failure
IL-33	interleukin 33
IL-6	interleukin 6
LDL cholesterol	low-density lipoprotein cholesterol
n-oxPTH	non-oxidized parathyroid hormone
NT-proBNP	N-terminal prohormone of brain natriuretic peptide
office diastolic BP	office diastolic blood pressure
office systolic BP	office systolic blood pressure
oxPTH	oxidized parathyroid hormone
P1NP	total procollagen type 1 N-terminal propeptide
PRIND	prolonged reversible ischemic neurologic deficit
PTH1R	parathyroid hormone 1 receptor
RCT	randomized controlled trial
ST2	suppression of tumorigenicity 2
sST2	soluble suppression of tumorigenicity 2
T2DM	type 2 diabetes mellitus
TIA	transient ischemic attack

tPTH	total parathyroid hormone
UVB	ultraviolet B light
VDR	vitamin D receptor
VMR	vitamin D metabolite ratio

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Abstract (Deutsch)

Vitamin D ist ein pleiotropes Steroidhormon und reguliert die Expression einer Vielzahl von Genen, u.a. auch für den Vitamin-D-Stoffwechsel Vitamin D-Spiegel zeigen bekannt saisonale Muster. Ein Mangel an Vitamin D wurde mit skelettalen sowie auch mit nicht-skelettalen Gesundheitszuständen wie Herz-Kreislauf-Erkrankungen in Verbindung gebracht, deren Steuerung noch wenig bekannt ist. Wir untersuchten drei dieser Aspekte hinsichtlich des Vitamin-D-Katabolismus und der Verwendung von 24,25-Dihydroxyvitamin D_3 (24,25$(OH)_2D_3$) sowie der Vitamin-D-Metaboliten-Ratio (VMR), der Saisonalität eines kardiovaskulären Biomarkers, des löslichen ST2 (sST2) und hinsichtlich der Auswirkungen von Vitamin D auf nicht oxidiertes PTH (n-oxPTH).

Anhand der in den „Styrian Vitamin D/Hypertension“- und „Ludwigshafen Risk and Cardiovascular Health“- Studien erhobenen Daten und Proben haben wir unsere Hypothesen bewertet. In der ersten Teilstudie stellten wir fest, dass VMR und 24,25$(OH)_2D_3$ nach der Behandlung mit Vitamin D anstiegen. Diese Parameter sind jedoch nicht zur Vorhersage von 25(OH)D-Spiegeln nach Supplementation geeignet. In der zweiten Teilstudie stellten wir fest, dass systemische sST2-Spiegel nicht durch eine Vitamin-D-Supplementierung beeinflusst wurden. In einer separaten Kohorte konnten wir zusätzlich nachweisen, dass sich dabei die sST2-Konzentrationen größtenteils nicht änderten und keine jahreszeitlichen Schwankungen unterlagen. In der dritten Teilstudie beobachteten wir eine Verringerung von Gesamt-PTH (tPTH) und n-oxPTH nach einer Vitamin-D-Supplementierung. Unsere Daten zeigen, dass die Messung von n-oxPTH zumindest bei PatientInnen, die zu oxidativem Stress neigen, eine bessere Alternative zu tPTH darstellen könnte.

Zusammenfassend lässt sich festhalten, dass Vitamin-D-Interventionsstudien in den letzten Jahren zwar überwiegend negative Ergebnisse gezeigt haben, aber dennoch nützliche Instrumente zur Aufdeckung vielversprechender und potenziell klinisch wichtiger Ergebnisse sind, wie es unsere Ergebnisse zu 24,25$(OH)_2D_3$, dem VMR, sST2 und n-oxPTH zeigen.

Abstract (English)

Vitamin D is a pleiotropic steroid hormone and regulates the expression of a wide variety of genes. Vitamin D metabolism is tightly regulated and serum levels show seasonal patterns. Vitamin D deficiency has been linked to skeletal as well as non-skeletal health conditions such as cardiovascular disease. Many aspects of governing the non-skeletal actions of vitamin D and even its metabolism are still poorly understood. We investigated three of these aspects regarding vitamin D catabolism and the usefulness of 24,25-dihydroxyvitamin D_3 (24,25$(OH)_2D_3$) and the vitamin D metabolite ratio (VMR), regarding the seasonality of a cardiovascular biomarker soluble ST2 (sST2) as well as regarding the effects of vitamin D on non-oxidized PTH.

We used the data and samples gathered in the Styrian Vitamin D in Hypertension Trial and the Ludwigshafen Risk and Cardiovascular Health Study to assess our hypotheses. In the first sub-study we found that VMR and 24,25$(OH)_2D_3$ increased after vitamin D treatment, although, these parameters can't be used to predict the changes in 25(OH)D levels after vitamin D treatment. In the second sub-study we found that systemic sST2 levels weren't affected by vitamin D supplementation and in a separate cohort we also demonstrated that sST2 concentrations didn't change alongside 25(OH)D concentrations and remained mostly constant without significant fluctuations throughout the whole year. In the third sub-study we observed a reduction in both total PHT (tPTH) and n-oxPTH after vitamin D supplementation and our data indicate that measuring n-oxPTH might be a better alternative to tPTH, at least in the case of patients prone to oxidative stress.

In conclusion, although vitamin D RCTs have shown mostly negative results in recent years, we found they can still be useful tools for uncovering promising and potentially clinically important findings as indicated by our findings regarding 24,25$(OH)_2D_3$, the VMR, sST2 and n-oxPTH.

1 Introduction

Vitamin D has emerged as one of the central players in hormone biology, seasonality as well as in a number of diseases and their regulations. To shed new light on some of these complex interactions, we dedicate several parts of this thesis to address these challenges.

1.1 Vitamin D biology

Vitamin D is a steroid hormone which is manly produced in the skin, when it is exposed to ultraviolet B (UVB; spectrum between 280-320 nm) radiation in sunlight which induces the conversion 7-dehydrocholesterol produced in the skin into vitamin D3 (cholecalciferol) (Christakos et al., 2016). The vitamin can also be acquired through ingestion of foods such as egg yolks, cod liver oil, fatty fish or mushrooms exposed to UVB radiation, although they traditionally play only a minor role in nutrition. Supplementation or ingestion of fortified foods are also potential sources of vitamin D. The human body is capable of storing and releasing vitamin D and its metabolites from adipose tissue (Christakos et al., 2016; Rooney et al., 2017; Martinaityte et al., 2017). A rough estimate predicts that around 80 % of the vitamin D supply in the human body is accounted for by UVB driven cutaneous production and around 20 % from food and supplements. This can vary considerably between individuals depending on the season, latitude, sun exposure habits, ethnicity and genetics, nutrition, microbiome as well as supplement intake (Holick, 2017; Christakos et al., 2016; Macdonald et al., 2011; Saternus et al., 2015; Waterhouse et al., 2018).

In order to have biological effects, vitamin D needs to be further metabolized (Christakos et al., 2016). The first step in this process is 25-hydroxylation of vitamin D in the liver by 25-hydroxylase enzymes. The rate of hydroxylation is mostly dependant on substrate concentration and continues until a steady state at higher 25(OH)D concentrations is reached (Christakos et al., 2016). Owing to the

fact that it has a longer half-life (2-3 weeks) than other vitamin D metabolites it is considered to be the best overall indicator of vitamin D status. When transported in the bloodstream, the majority of 25(OH)D (85-90 %) is bound to DBP, while 10-15 % is bound to albumin and about 1 % of serum 25(OH)D is free (unbound) (Gallagher and Bikle, 2017). The 25-hydoxylated form of vitamin D must undergo another enzymatic conversion to be physiologically active. This hydroxylation step is carried out mainly in the kidney (but also almost all extrarenal tissues) by renal 1-alpha-hydroxylase (CYP27B1), which converts 25(OH)D to $1,25(OH)_2D$, the active hormonal form of vitamin D. The conversion to 1,25(OH)2D is tightly regulated by phosphate metabolism and calcium concentrations, with PTH stimulating the enzyme and FGF-23 inhibiting it (Christakos et al., 2016; Gallagher and Bikle, 2017; Jorde and Grimnes, 2018). In a feedback loop, $1,25(OH)_2D$ and to a lesser extent 25(OH)D supress PTH secretion. Taken together, vitamin D deficiency/insufficiency leads to increased PTH levels to sustain calcium homeostasis. Treating vitamin D deficiency/insufficiency with supplementation will therefore supress PTH secretion (Ritter et al., 2006). Additionally, individual genetics might also affect the levels of 25(OH)D and $1,25(OH)_2D$ (Engelman et al., 2008).

$1,25(OH)_2D$ functions as a steroid hormone and exerts its effects by binding to the vitamin D receptor (VDR), a receptor expressed in almost all human tissues. Upon binding, VDR usually forms heterodimers with retinoid X receptors and then the hormone-receptor complexes translocate to the cell nucleus where it interacts with vitamin D response elements on the DNA and thereby regulates the expression levels of a wide variety of genes (Christakos et al., 2016; Gallagher and Bikle, 2017; Jorde and Grimnes, 2018). Vitamin D can also bind to plasma membrane bound VDRs and with the $1,25D_3$-MARRS (Membrane Associated Rapid Response Steroid-binding) receptor thereby activating rapid responses in target cells (Maddaloni et al., 2018).

1.1.1 Regulation of 25(OH)D and $1,25(OH)_2D$ levels

25(OH)D and $1,25(OH)_2D$ levels are regulated mainly by activity of the enzyme CYP27B1 and the enzyme 25-hydroxyvitamin D-24-hydroxylase (CYP24A1). CYP24A1 is located at the mitochondrial inner membrane, mainly in the kidney

(Jones, Prosser and Kaufmann, 2012). The enzyme hydroxylates both metabolites at C-24, although 1,25$(OH)_2$D has been proposed as the preferred substrate for CYP24A1 (Shinki et al., 1992). This enzymatic reaction thus forms 24,25$(OH)_2$D and 1,24,25$(OH)_3$D, respectively. Through these conversions CP24A1 reduces the 1,25$(OH)_2$D levels in the circulation and also reduces the availability of 25(OH)D for 1-hydroxylation by CYP27B1. 24-hydroxylated metabolites are targeted for excretion. Interestingly, the enzyme is present in all cells expressing the VDR and might therefore also be involved in regulating the local, not just the circulating 1,25$(OH)_2$D and 25(OH)D levels. This local regulation probably provides appropriate cellular responses (Christakos et al., 2016).

The expression and activity of the enzymes CYP27B1 and CYP24A1 in the kidney are tightly controlled. The main signal for inducing a higher production of 1,25$(OH)_2$D are elevated levels of PTH that are a result of hypocalcemia (Bikle, 2014). 1,25$(OH)_2$D also affects its own production through inhibition of CYP27B1 (Brenza and DeLuca, 2000). CYP24A1 on the other hand is stimulated by 1,25$(OH)_2$D and inhibited by low calcium and PTH levels (Bikle, 2014).

1.1.2 24,25$(OH)_2$D and the vitamin D metabolite ratio (VMR)

24,25$(OH)_2$D has been suggested to also be a physiologically active metabolite of 25(OH)D. For example, St. Arnaud has proposed, the metabolite might have relevant roles in regulating bone development, repair and growth as well as in cartilage development and possibly even in embryogenesis (St-Arnaud and Glorieux, 1998; St-Arnaud, 1999). Although, it is still questionable if and how 24,25$(OH)_2$D exerts its physiological activity and if the proposed receptor for the metabolite even exists.

Nonetheless, 24,25$(OH)_2$D has proven to be a very valuable parameter to be measured alongside 25(OH)D. For example, the ratio between 24,25$(OH)_2$D and 25(OH)D (the vitamin D metabolite ratio – VMR) is an indicator of CYP24A1 activity and vitamin D catabolism (Wagner et al., 2011; Cashman et al., 2015). The ratio is therefore very useful to help identify a rare genetic disorder, idiopathic infantile hypercalcemia. The patients with this disorder have severely elevated

calcium levels and low PTH levels. This occurs because of a lower capability to catabolize 1,25$(OH)_2$D and 25(OH)D due to an inactivating mutation in the gene that codes for CYP24A1 (Cashman et al., 2015; Schlingmann et al., 2011; Molin et al., 2015).

The VMR was also shown to be potentially useful for assessing vitamin D status, for example to better predict vitamin D deficiency. Recent studies show a possible use of VMR in predicting changes in 25(OH)D after vitamin D supplementation, although the results are so far inconclusive (Wagner et al., 2011; Cashman et al., 2015; Binkley et al., 2017).

CYP24A1 could be in part accountable for differences in serum 25(OH)D levels between individuals after vitamin D supplementation. Therefore, in theory, the dosing of vitamin D treatment could be individualized by using the VMR. Studies have although mostly focused on 25(OH)D at the start of vitamin D supplementation, as well as BMI, age, ethnicity and genetic background, for predicting the responses to vitamin D treatment (Mazahery and von Hurst, 2015). On the other hand, studies involving 24,25$(OH)_2$D and the VMR in this regard are scarce.

1.2 Vitamin D deficiency

Vitamin D insufficiency and deficiency are worldwide health problems (Holick, 2017). 25(OH)D levels above 30 ng/mL (approx. 75 nmol/L) are deemed as sufficient, levels between 20 (approx. 50 nmol/L) and 30 ng/mL are classified as insufficient and levels below 20 ng/ml are classified as deficient (Fischer, Thacher and Pettifor, 2008). The main view on the physiology of vitamin D have for long been only its effects in regulation of calcium and phosphorus homeostasis and thereby proper bone mineralization (Mirhosseini, Rainsbury and Kimball, 2018). More recently, vitamin D deficiency and insufficiency have also been linked to non-skeletal health conditions, for example cancer, diabetes and cardiovascular disease (CVD) (Thomas et al., 2012; Wang et al., 2008; Melamed et al., 2008).

1.2.1 Vitamin D deficiency and cardiovascular disease

Most of the data on the connection between low serum vitamin D with CVD risk factors including dyslipidemia, inflammation and high blood pressure originate from observational studies (Fraser, Williams and Lawlor, 2010; Kunutsor et al., 2014; SCRAGG, SOWERS and BELL, 2007; Kendrick et al., 2009; Agrawal and Yin, 2014). Nonetheless, it was found that for CVD all relevant Hill's criteria for causality are fulfilled, thereby suggesting that low levels of 25(OH)D are an independent risk factor for CVD (Weyland et al., 2014). Recently, a meta-analysis suggested that supplementation with vitamin D might protect against CVD by improving the associated risk factors, including elevated PTH and blood pressure as well as inflammation and dyslipidemia (Mirhosseini, Rainsbury and Kimball, 2018).

1.3 Seasonality of CVD

A number of cardiovascular diseases have been reported to be influenced by seasonal variation and several parameters, including temperature changes, vitamin D deficiency etc., potentially play a role in the complex pathophysiology of these diseases (Stewart et al., 2017). Population-based and epidemiological studies have shown that cardiovascular events are more common during wintertime. Then, temperatures are lowest, cardiovascular risk factors and their corresponding biomarker surrogates are at their peak, and also seasonal vitamin D deficiency is most prevalent (Stewart et al., 2017; Norman and Powell, 2014; Marti-Soler et al., 2014). For example, dissection of aortic aneurysms, myocardial infarction, stroke, heart failure etc., have been shown to be more prevalent in winter (Gallerani et al., 2011). Risk factors such as blood pressure and CVD surrogate biomarkers levels of aldosterone, NT-proBNP and others, have also been shown to follow a seasonal pattern (Stewart et al., 2017; Radke and Izzo, 2010; Khezri et al., 2017). Although a great number of cardiovascular parameters have been investigated in this regard, soluble suppression of tumorigenicity 2 (sST2), a new prognostic cardiovascular biomarker (Pascual-Figal et al., 2016), was not paid attention to until now.

1.4 Soluble ST2 (sST2)

The ST2 receptor (also known as Interleukin 1 receptor-like 1 – IL-1RL1) and sST2, the shorter, secreted, soluble form of the ST2 receptor that lacks the transmembrane and cytoplasmic domains, are both encoded by the Il1rl1 gene and are members of the Toll-like/Interleukin 1 receptor superfamily. They both bind interleukin-33 (IL-33), a cytokine that works as a traditional cytokine and also a nuclear regulator of transcription (Griesenauer and Paczesny, 2017). ST2 and IL-33 together comprise a signalling system that has been shown to reduce atherosclerosis (Miller et al., 2010) and pressure-induced cardiac fibrosis and hypertrophy (Sanada et al., 2007). It might also be protective in obesity and possibly also in T2DM (Martínez-Martínez et al., 2013; Miller et al., 2010).

On the other hand, sST2 works as a decoy receptor, binding IL-33, preventing it from binding to the ST2 receptor and thereby inhibiting the effects of IL-33/ST2 signalling (Sanada et al., 2007). High levels of sST2 are linked with worse outcomes in aortic dissection (Wang et al., 2018), heart failure (HF) (Weinberg et al., 2003), subclinical brain injury and incident stroke (Andersson et al., 2015) as well as myocardial infarction (Shimpo et al., 2004). sST2 has also been shown to be involved in worsening of atherosclerosis (Miller et al., 2008) and several studies have found elevated levels in subjects with CVD, who also had diabetes (Sabatine et al., 2008; Dhillon et al., 2011; Fousteris et al., 2011). In addition, elevated levels of sST2 have also been observed in subjects with diabetes mellitus type 2 (T2DM) when compared to heathy subjects or subjects with diabetes (Lin et al., 2016).

A study in heart failure patients has recently found potential negative associations of sST2 with vitamin D metabolites 25(OH)D and $1,25(OH)_2D$ as well as positive associations with PTH. In the same study, a multiple regression analysis was used to show that N-terminal pro-B-type natriuretic peptide (NT-proBNP), 25(OH)D and $1,25(OH)_2D$ were independent determinants of sST2 (Gruson et al., 2016). Besides, a number of studies have determined the primary source of sST2 in humans and they have shown that sST2 is predominantly secreted by primary human cardiac myocytes and lung epithelial cells (Mildner et al., 2010). Other cell types, for example arterial and venous endothelial cells (Bartunek et al., 2008), as

well as cluster of differentiation 4 (CD4) and cluster of differentiation 8 (CD8) lymphocytes (Pfeffer et al., 2015), secrete measurable quantities of the protein. Intriguingly, all of these cell types have the capability to produce 1,25$(OH)_2$D with the enzyme 25-Hydroxyvitamin D3 1-alpha-hydroxylase (CYP27B1) and also express VDR (Zehnder et al., 1999; Razzaque, 2011; Kongsbak et al., 2013). In addition, the secretion of sST2 by primary human CD4 lymphocytes as well as lung epithelial cells was elevated after treatment with 1,25$(OH)_2$D. Interestingly, the sST2 secretion by lung epithelial cells was also augmented in response to treatment with 25(OH)D (Pfeffer et al., 2015).

1.5 Non-oxidized PTH (n-oxPTH)

As vitamin D regulation is strongly associated with parathyroid hormone (PTH), the interrelation of both hormones in the physiology of the human body is of particular interest to this research area.

Next to vitamin D, also PTH is crucial for maintaining homeostasis of serum calcium. PTH increases serum calcium concentrations by stimulating phosphate excretion, restricting calcium excretion, supporting conversion of 25(OH)D to 1,25$(OH)_2$D and by largely promoting bone resorption. The hormone binds to parathyroid hormone 1 receptor (PTH1R), a family B G-protein-coupled receptor, which is expressed primarily in bone, cartilage, vasculature and kidney (Cheloha et al., 2015).

Interestingly, the molecule of this protein hormone is prone to oxidation at methionine residues 8 and 18 (Zull, Smith and Wiltshire, 1990). Oxidation of these methionine residues results in an inability of the bound hormone to activate the PTH-1 receptor, thereby preventing the hormone to exert its biological effects (Zull, Smith and Wiltshire, 1990; Vogt, 1995; GALCERAN et al., 1984; Horiuchi, 2009). Recently, it has been shown that oxidation of the PTH molecule takes place only in vivo and does not increase after the blood samples have been withdrawn (Ursem et al., 2018). The present PTH assays are able to measure the full-length hormone of 84 amino acids, with a very low to no cross reactivity of its fragments, which is a great improvement over previous versions of the assay (Souberbielle,

Roth and Fouque, 2010). The values measured with use of these assays therefore reflect the function of PTH more accurately, which improves the interpretation of the measured levels, especially in PTH-related disorders. Nevertheless, in addition to fragmentation, also posttranslational oxidation of PTH can affect the measurements, because oxPTH, the biologically inactive form of PTH, cross-reacts in commonly used immunoassays (GALCERAN et al., 1984; Horiuchi, 2009).

Recently, a new PTH method has been developed that allows measurements of n-oxPTH exclusively. The method is based on an affinity column that effectively binds oxPTH, which leaves only n-oxPTH in the eluate. Commonly used PTH immunoassays can then be used to measure n-oxPTH levels in the eluate (Hocher et al., 2012). The method measuring only n-oxPTH was proposed to be favourable to the more commonly used methods, which measure total PTH (tPTH, meaning both oxidized and non-oxidized forms of PTH), because it might better reflect the hormonal function of PTH. The rationale behind is that only n-oxPTH is capable of activating the PTH-1 receptor and that oxidised PTH might rather indicate oxidative stress related pathologies (Tepel et al., 2013). For example, Tepel et al. observed increased survival in haemodialysis patients, who had higher concentrations of n-oxPTH. On the contrary, tPTH was associated with higher mortality in healthy individuals as well as chronic kidney disease (CKD) patients (Tepel et al., 2013; Tentori et al., 2008; Hagström et al., 2009). A recent study also observed that tPTH was associated with all-cause mortality in CKD, while n-oxPTH was not (Seiler-Mussler et al., 2018). These conflicting results therefore provide further support for the rationale that n-oxPTH might reflect the functional hormonal status of subjects more accurately and that further studies should be carried out to determine its viability for use in a clinical setting.

1.6 Aims

Taking into account the presented background information, the aims of our three sub-studies were the following:

In part 1, we focussed on determining if **baseline 24,25(OH)$_2$D$_3$ and VMR measurements are advantageous over baseline 25(OH)D measurements when used for predicting vitamin D-related metabolite levels after vitamin D supplementation**. Our hypothesis was that measurements of baseline VMR would be advantageous over baseline 25(OH)D measurements for this purpose.

In part 2, our main interest was focussed on **sST2 and its interrelation with vitamin D and seasonality**. Based on previous studies, we predicted that sST2 levels would be affected by a vitamin D intervention. Since information on the effects of vitamin D and/or seasonality on levels of systemic sST2 were not available or were inconclusive, we set out to investigate if a) vitamin D has an effect on sST2 levels in a randomized controlled trial (RCT) setting (sub-study 2). In addition, we predicted that b) **sST2 would follow a seasonal pattern, potentially in parallel to 25(OH)D**. Therefore, we investigated, if systemic sST2 levels fluctuate among seasons in a separate cohort with a cross-sectional study design (study 2).

In part 3, we investigated if the **effect of vitamin D supplementation on a) n-oxPTH** differed in comparison to tPTH in the setting of an RCT study (sub-study 3). We also determined b) the **relationships of n-oxPTH and tPTH with bone formation and resorption markers, parameters of mineral metabolism and markers of lipid metabolism**.

2 Materials and methods

2.1 Study cohorts

2.1.1 Styrian Vitamin D in Hypertension Trial (study 1)

2.1.1.1 Study design

The “Styrian Vitamin D Hypertension Trial” was designed as a double-blind, placebo-controlled (1:1), single-centre, randomized controlled trial that took place at the outpatient clinic of the Division of Endocrinology and Diabetology, Medical University of Graz, Austria. It was registered at www.clinicaltrialsregister.eu under the EudraCT number: 2009-018125-70 and at clinicaltrials.gov under the ClinicalTrials.gov identifier: NCT02136771. The study protocol was approved by the Ethics Committee of the Medical University of Graz, Austria, and all participants gave written informed consent.

Three sub-studies, referred to as sub-study 1, sub-study 2 and sub-study 3, were carried out on data and blood samples from study 1. Where necessary, the details regarding the sub-study in question are described in its own subchapter. Sub-study 1 is centred on the relation of 24,25$(OH)_2D_3$, VMR as well as vitamin D metabolism, sub-study 2 on the role of vitamin D in regulation of sST2 levels and sub-study 3 on the role of vitamin D in n-oxPTH as well as PTH metabolism.

2.1.1.2 Study subjects

200 subjects were included in the original study, of these 104 were male and 94 female. All study participants were 18 years of age or older and were diagnosed with arterial hypertension and a baseline serum 25(OH)D concentration below 30 ng/mL (multiply by 2.496 to convert ng/mL to nmol/L). The study participants were recruited between June 2011 and August 2014. For further details about the study and its results please refer to the publication of the original study (Pilz et al., 2015).

2.1.1.2.1 Sub-study 1

For this post-hoc cross-sectional investigation, we included 106 of the subjects originally included in the study, from which sufficient material for $25(OH)D_3$ and $24,25(OH)_2D_3$ mass spectrometry measurements was available at both study visits.

2.1.1.2.2 Sub-study 2

This investigation included 185 study participants, where sST2 measurements were available before and after vitamin D or placebo treatment.

2.1.1.2.3 Sub-study 3

In this post-hoc investigation, only subjects with sufficient material for n-ox PTH measurements were used, 108 subjects in total.

2.1.1.3 Intervention

The vitamin D intervention lasted for 8 weeks, consisting of 2800 IU cholecalciferol in the form of 7 oily drops per day (Oleovit D3, Fersenius Kabi Austria, Graz, Austria) or a matching placebo. The study participants were randomly allocated to the control group and intervention group, with 100 participants allocated to each of the groups. The randomization was performed using a web-based randomization software (Randomizer). The investigators and authors who enrolled participants, collected data, and assigned the intervention were masked to participant allocation. A more detailed description has been published previously (Pilz et al., 2015).

2.1.1.4 Measurements

The study participants were instructed to fast overnight before arriving for their study visit. Before blood samples were taken between 7:00 and 11:00 in the morning, the study participants were seated for at least 10 minutes at both study visits. The blood samples were either measured on-site by routine laboratory procedures or were stored at -80 °C until analysis. 25(OH)D levels were measured by means of a chemiluminescence assay (IDS-iSYS 25-hydroxyvitamin D S assay;

Immunodiagnostic Systems Ltd., Boldon, UK) on an IDS-iSYS multidiscipline automated analyser. The intra-assay and inter-assay Coefficient of Variation (CV) were 6.2% and 11.6%, respectively. sST2 measurements were performed in serum with the Human St2/IL-33 R Quantikine ELISA Kit (R&D Systems, United Kingdom) with intra-assay CVs of 4.5 to 5.6% and inter-assay CVs 6.3 to 7.1%, while the lower limit of detection was 5.1 pg/mL according to the manufacturer. For osteocalcin measurements the IDS-iSYS N-Mid Osteocalcin Assay (Immunodiagnostic Systems Ltd. (IDS Ltd.), United Kingdom) was used. Within-run CVs were 1.8-3.8%, total CVs were 3.4-9.2%, while the level of detection was at 0.27 ng/mL. Insulin was measured in serum samples using a solid phase two-site enzyme immunoassay (Insulin ELISA EIA-1825; DRG Instruments GmbH, Marburg, Germany). This assay had intra-assay CVs of 3.2% to 3.4% and inter-assay CVs of 2.9% to 3.6%. For the measurement of PTH, a sandwich ElectroChemiLuminescence Immunoassay (ECLIA) on an Elecsys 2010 (Roche Diagnostics, Mannheim, Germany) with an intra-assay CV of 1.5% to 2.7% and inter-assay CV of 3.0% to 6.5% was used. NT-proBNP measurements were performed using an ElectroChemiLuminescence immunoassay (Roche Diagnostics, Germany) on an autoanalyser (Elecsys 2010) with an intra-assay CV of 1.8% to 2.7 % and inter-assay CV of 2.3% to 3.2%. Vitamin D binding protein (DBP) was measured using the Quantikine Human Vitamin D Binding Protein immunoassay (R&D Systems, Inc., Minneapolis, U.S.A.), with an intra-assay CV of < 5.1 % and inter-assay CV of < 7.4 %. CTX was measured using the electrochemiluminescence immunoassay (Elecsys, Roche Diagnostics, Germany), with intra-assay CVs of 2.0 % and inter-assay CVs of 4.2 %. Concentrations of bone-specific alkaline phosphatase (bALP) were determined by a spectrophotometric immunoassay (IDS-ISYS Ostase BAP; Immunodiagnostic Systems Ltd. [IDS Ltd.], Boldon, Tyne & Wear, UK). The inter-assay CV was 5.2 %. Procollagen type 1 amino-terminal propeptide (P1NP) was measured using an automated electrochemiluminescence immunoassay (Elecsys, Roche Diagnostics). The inter-assay CV was 2.7%. Fibroblast growth factor 23 (FGF23) was measured using a multi-matrix ELISA (FGF23 (C-terminal) ELISA; Biomedica Medizinprodukte GmbH & CO KG, Vienna, Austria), with intra-assay CV of ≤ 12 % and inter-assay CV of ≤ 10 %. Total cholesterol, high density lipoprotein (HDL) and triglycerides were measured using an enzymatic colorimetric assay (Elecsys,

Roche Diagnostics). The Friedewald equation was used to calculate low density lipoprotein (LDL). Pulse wave velocity measurements have been described previously, while other parameters have been determined by routine laboratory procedures (Pilz et al., 2015).

2.1.1.4.1 Additional measurements for sub-study 1

EDTA plasma samples which were obtained after an overnight fast and were centrifuged and stored at −80 °C were used for this analysis. 25(OH)D_3 and 24,25$(OH)_2D_3$ were assessed at the Endocrine Laboratory of the VU University Medical Center by isotope dilution liquid chromatography-tandem mass spectrometry, as described previously (Dirks et al., 2019). The limit of quantitation for 25(OH)D_3 assay was 1.2 nmol/L, while the intra-assay CV was 3 % and the inter-assay CV 6 %. 24,25$(OH)_2D_3$ measurements had a limit of quantitation of 0.1 nmol/L and the intra- and inter-assay coefficients of variation (CV) were 5% and 9%, respectively.

2.1.1.4.2 Additional measurements for sub-study 3

The 25(OH)D_3 levels measured for sub-study 1 were also used in sub-study 3. An oxPTH affinity column (A1112; Immundiagnostik AG, Bensheim, Germany) was used to measure n-oxPTH concentrations. These affinity columns contain slurry with antibodies that specifically bind the oxidized form of PTH. After the columns are centrifuged, only the non-oxidized form of PTH can be found in the eluate (Hocher et al., 2012). 300 µL EDTA plasma was filled into the columns and they were incubated end-over-end at room temperature for 1 h. Afterwards, n-oxPTH was measured in the eluate by using a second generation PTH immunoassay (Elecsys, Roche Diagnostics). The inter-assay CV < 2 pmol/L was 10 % and > 2 pmol/L 2.4 %, calculated taking into account the combined measurement including the n-oxPTH columns.

2.1.1.5 Study objectives

2.1.1.5.1 Sub-study 1

In this sub-study, we aimed to determine the associations of baseline vitamin D-related parameters with the changes from baseline in $25(OH)D_3$, $1,25(OH)_2D$ and $24,25(OH)_2D_3$. Additionally, we aimed to determine the effects of vitamin D treatment on the vitamin D-related parameters and ratios between them.

2.1.1.5.2 Sub-study 2

The primary outcome measure in this sub-study was the between group-difference in serum sST2 concentrations at study end, adjusted for baseline values.

2.1.1.5.3 Sub-study 3

In this sub-study, we aimed to investigate the effects of vitamin D supplementation on tPTH and n-oxPTH concentrations between groups, adjusted for baseline values. We also studied the associations between n-oxPTH and tPTH concentrations, bone turnover markers as well as parameters of calcium and phosphate homeostasis.

2.1.2 LURIC (study 2)

2.1.2.1 Study design

The LURIC study was a prospective study which aimed to determine biochemical and genetic risk factors for coronary artery disease (CAD) in a hospital-based cohort of Caucasian individuals, who were referred for coronary angiography. In addition, it also aimed to evaluate the predictive value of potential markers on long-term outcomes. The details about the objectives of the study, recruitment procedures, and characteristics have been described previously (Winkelmann et al., 2001). The Ethics Committee at the Landesärztekammer Rheinland-Pfalz (Mainz, Germany) approved the study protocol in accordance with the Declaration of Helsinki. Each study participant provided written informed consent.

2.1.2.2 Study subjects

3316 patients took part in the LURIC study and, of these, 2338 hypertensive patients with available sST2, 25OHD, 1,25(OH)2D, and PTH levels were included in this sub-study. For a detailed description of baseline examination protocols please refer to (Winkelmann et al., 2001). Arterial hypertension was defined as five measurements of mean systolic and diastolic BP > 140/90 mm Hg. Brachial artery pressure value measurements with an automated oscillometric device (Omron MX4, Omron Health Care GmbH, Hamburg, Germany) were performed after the patient had rested in the supine position for at least 10 min. At least three consecutive systolic and diastolic BP measurements were performed, with a minimum of a 30 second interval between measurements. Only measurements which conformed to the reproducibility criteria were entered into the database.

2.1.2.3 Measurements

Drawing of venous blood samples took place between 8:00 and 10:00 in the morning after an overnight fast and before the patients' scheduled coronary angiography. Routine laboratory parameters were measured immediately on a daily basis as previously described. (Winkelmann et al., 2001) The blood samples assigned for storage at - 80 °C until further analysis, were first centrifuged at 3000g for 10 min and then frozen. All sample processing was performed within 30 min after venepuncture. EDTA plasma samples were used to measure sST2 approximately 12 years after the recruitment period of the LURIC study was closed. All of the sST2 measurements were performed in 1 batch for all patient samples on a fully automated BEP® 2000 instrument (Siemens Healthcare Diagnostics) with the Presage™ ST2 sandwich immunoassay assay (Critical Diagnostics). The CVs were 4.0 %, as reported previously (Dieplinger et al., 2009). Once per week, 25(OH)D concentrations were measured in serum samples using a commercial radioimmunoassay (DiaSorin SA, Antony, France) with an intra-assay CV of 8.6 % and an inter-assay CV of 9.2%. In addition, 25(OH)D concentrations were measured previously in a set of 100 randomly selected samples by liquid chromatography tandem mass spectrometry with isotopic labelled internal standard. These measurements were highly significantly

correlated with the 25(OH)D levels measured by RIA (r = 0.875; P < 0.001) (Tomaschitz et al., 2010). 1,25(OH)2D concentrations were measured in serum samples by RIA (Nichols Institute Diagnostika GmbH, Bad Nauheim, Germany) on a Berthold LB2014 multicrystal counter.

2.1.2.4 Study objective

Our main aim of this sub-study was to determine if seasonal variability affects sST2 levels. We also aimed to determine possible associations of vitamin D-related parameters with sST2.

2.2 Statistical analysis

Continuous data following a normal distribution are shown as means with standard deviations (SD). Variables with skewed distributions are given as medians with interquartile ranges. Categorical variables are reported as percentages of observations. Unpaired Student's t-tests, Mann-Whitney-U-tests or chi-squared tests were used to compare groups at baseline or assess the differences between groups. When appropriate, variables were log or log(e) transformed, if skewed, before performing further analysis using parametrical statistical tests. All variables were analysed according to the intention-to-treat principle without data imputation, e.g. in the Styrian Vitamin D Hypertension Trial only participants, of which successful measurements of the respective outcome variable were available both at baseline and follow-up, were included in the analyses. In the cases where outliers were detected in the analyses by the software (defined for ANCOVA as cases with standardized residuals greater than ±3 standard deviations, while for ANOVA they were defined as cases with values higher or lower than 1.5*IQR (interquartile range)), the corresponding values were removed and the analysis repeated to determine their potential effect on the analysis. The results of the analysis where the outlier was present are reported if the outlier had no significant effect on the analysis. A p-value of < 0.05 was considered statistically significant in all analyses. All statistical analyses were performed using SPSS version 22 (SPSS, Chicago, IL, USA).

The statistical tests implemented in the specific sub-studies are described in the following sub-chapters.

2.2.1 Study 1

2.2.1.1 Sub-study 1

$\Delta 25(OH)D_3$, $\Delta 1,25(OH)_2D$ and $\Delta 24,25(OH)_2D_3$ depict the changes from baseline for $25(OH)D_3$, $1,25(OH)_2D$ and $24,25(OH)_2D_3$, respectively, in the vitamin D treated group. They were calculated as the difference between the measured values at the final study visit and the measured values at baseline. We calculated VMR as the ratio between $24,25(OH)_2D_3$ and $25(OH)D_3$.

To determine the strength of associations between vitamin D related parameters and $\Delta 25(OH)D_3$, $\Delta 24,25(OH)_2D_3$ as well as $\Delta 1,25(OH)_2D$ we used Pearson's correlation analysis. We used partial correlation analysis to determine the correlation coefficients adjusted for gender, age, BMI, PTH, eGFR, serum phosphate and serum calcium. We used Bonferroni correction to account for multiple testing.

An extreme outlier (25(OH)D > 4xSD at baseline) was removed before conducting Pearson's correlation analyses, because of its significant effects on the analyses. This outlier had no significant effect on the ANCOVA analysis, therefore it was not removed from these analyses.

2.2.1.2 Sub-study 2

Associations of parameters with sST2 were determined with Spearman's correlation analyses and Bonferroni correction was used to account for multiple comparisons. This approach was also used for determining associations between parameters from study 2.

Testing for differences in sST2 and 25OHD between the placebo and the treatment group at the follow-up visit, was performed by analysis of covariance (ANCOVA) with adjustments for baseline values.

2.2.1.3 Sub-study 3

The Mann-Whitney U-test was used to compare the percentage differences between the decreases in n-oxPTH with the decreases in tPTH. The relationship between baseline parameters, n-oxPTH and tPTH was assessed by Pearson's correlation analysis, while Spearman's correlation analysis was used for skewed variables that could not be brought to a normal distribution. Additionally, the difference between the parameters measured at the final study visit and the same parameter at the baseline visit, for n-oxPTH, tPTH, n-oxPTH/tPTH ratio, $25(OH)D_3$, Calcium, Phosphate, BAP, FGF23, ADMA, LDL and HDL, in the vitamin D-treated group was calculated. These calculated changes from baseline are identifiable by the 'Δ' symbol. Spearman's correlation coefficient was used to assess the correlation between the changes from baseline in n-oxPTH, tPTH, n-oxPTH/tPTH ratio and the other aforementioned calculated differences from baseline. Also, Bonferroni corrected values were reported. Differences in the outcome variables (n-oxPTH, tPTH, the n-oxPTH/tPTH ratio, $25(OH)D_3$, biologically available $25(OH)D_3$, $1,25OH_2D$, plasma calcium and 24-hour calcium excretion, ADMA) were determined by analysis of covariance (ANCOVA) adjusted for baseline values. If values were more than 3SD from the mean they were treated as outliers, meaning they were removed and the analysis repeated. This is additionally marked in the results section. Equations adapted from Vermeulen et al. were used to calculate biologically available $25(OH)D_3$ (Vermeulen, Verdonck and Kaufman, 1999). We refer to the supplement of Powe et al. for the formula (Powe et al., 2011). The percentage changes of parameters in the vitamin D treated group were calculated by dividing the change from baseline after treatment by its respective baseline value.

2.2.2 Study 2

Seasonal variability and calculations to determine which monthly mean values of 25(OH)D and sST2 were significantly different to the peak monthly mean, were assessed by ANOVA followed by the Bonferroni post-hoc test. Welch ANOVA followed by the Games-Howell post-hoc test was used when the assumption of

homogeneity of variances was not met. Group comparisons with adjustment for age and eGFR were performed by ANCOVA.

3 Results

The results are presented in 3 parts according to the hypotheses. In part 1, the results from sub-study 1, in part 2 the results from sub-study 2 and study 2, and finally in part 3 the results from sub-study 3 will be presented.

3.1 Part 1: Sub-study 1

The results presented in part 1 originate from the analysis of data from sub-study 1.

The baseline characteristics of study participants of sub-study 1 can be found in Table 1. We found no differences between the placebo and vitamin D treated groups at baseline.

Table 1: Baseline characteristics of the sub-study 1 cohort.

Parameter	All (n = 106)	Placebo (n = 54)	Vitamin D (n = 52)	p-value
Age (years)	62.0 (51.3 to 68.7)	64.8 (50.8 to 70.2)	59.6 (52.4 to 66.6)	0.318
Body mass index (kg/m^2)	30.0 ± 5.4	29.7 ± 5.9	30.3 ± 4.9	0.562
Gender (% female)	57	57	56	0.865
$24,25(OH)_2D_3$ (nmol/L)	3.5 ± 1.6	3.4 ± 1.5	3.6 ± 1.5	0.419
$25(OH)D_3$ (nmol/L)	48 ± 18	46 ± 19	49 ± 18	0.401

VMR ((nmol/L)/(nmol/L))	0.073 ± 0.017	0.072 ± 0.018	0.073 ± 0.017	0.768
PTH (pmol/L)	5.5 (4.1 to 6.7)	5.5 (4.0 to 6.7)	5.3 (4.1 to 6.7)	0.779
1,25(OH)$_2$D (pmol/L)	126 ± 53	118 ± 52	133 ± 52	0.142
Serum phosphate (mmol/L)	0.94 ± 0.17	0.96 ± 0.17	0.92 ± 0.16	0.282
Serum calcium (mmol/L)	2.26 (2.21 to 2.33)	2.26 (2.21 to 2.34)	2.26 (2.20 to 2.33)	0.773
eGFR (mL/min/1.73m^2)	72 ± 17	69 ± 16	74 ± 18	0.152
24h urinary calcium excretion (mmol/24h)	3.30 (1.90 to 5.00)	2.95 (1.83 to 4.78)	3.70 (2.10 to 6.30)	0.222
Calculated free 25(OH)D$_3$ (pmol/L)	15 (9 to 21)	12 (8 to 21)	17 (11 to 20)	0.153
Vitamin D binding protein (µg/mL)	247.1 ± 109.5	254.8 ± 110.6	239.3 ± 109.0	0.772
Calculated bioavailable 25(OH)D$_3$ (nmol/L)	5.9 (3.9 to 8.2)	5.2 (3.2 to 8.5)	6.6 (4.1 to 8.0)	0.149
1,25(OH)$_2$D /25(OH)D$_3$ ((nmol/L)/(nmol/L))	0.0023 (0.0019 to 0.0036)	0.0027 (0.0018 to 0.0039)	0.0028 (0.0021 to 0.0035)	0.753

25(OH)D_3 – 25-hydroxyvitamin D, 1,25(OH)2D – 1,25-dihydroxyvitamin D, 24,25(OH)$_2D_3$ – 24,25-dihydroxyvitamin D, VMR – vitamin D metabolite ratio, eGFR – estimated glomerular filtration rate.

The treatment effects we calculated after vitamin D treatment are gathered in table 2. Significant treatment effects could be observed for all included parameters. For 25(OH)D_3, 1,25(OH)$_2$D, 24,25(OH)$_2D_3$, VMR, calculated free 25(OH)D_3, calculated bioavailable 25(OH)D_3, the 1,25(OH)$_2$D/25(OH)D_3 ratio and the 1,25(OH)$_2$D /24,25(OH)$_2D_3$ ratio the treatment effects were 32 nmol/L (95% CI: 26 to 39; $p < 0.001$), 26 pmol/L (9 to 42; $p = 0.003$), 3.3 nmol/L (2.7 to 3.9; $p < 0.001$), 0.015 (nmol/L)/(nmol/L) (0.010 to 0.020; $p < 0.001$), 12 pmol/L (6 to 18; $p < 0.001$), 4.66 nmol/L (2.63 to 6.68; $p < 0.001$), -0.0010 (nmol/L)/(nmol/L) (-0.0013 to -0.0006; $p < 0.001$) and -0.020 (nmol/L)/(nmol/L) (-0.026 to -0.015; $p < 0.001$), respectively.

Table 2: ANCOVA analysis for the effect of vitamin D or placebo treatment on vitamin D related parameters.

Parameter	Group	Baseline	Follow-up	Treatment effect (95% confidence interval)	p-value
25(OH)D_3 (nmol/L)	Placebo, N=54	46 ± 19	45 ± 20	32 (26 to 39)	< 0.001
	Vitamin D, N=52	49 ± 18	79 ± 19		
1,25$(OH)_2$D (pmol/L)	Placebo, N=52	118 ± 52	114 ± 39	26 (9 to 42)	0.003
	Vitamin D, N=52	133 ± 52	150 ± 63		
24,25$(OH)_2D_3$ (nmol/L)	Placebo, N=54	3.4 ± 1.5	3.3 ± 1.8	3.3 (2.7 to 3.9)	< 0.001
	Vitamin D, N=52	3.6 ± 1.6	6.8 ± 1.7		
VMR ((nmol/L)/(nmol/L))	Placebo, N=54	0.072 ± 0.018	0.071 ± 0.017	0.015 (0.010 to 0.020)	< 0.001
	Vitamin D, N=52	0.073 ± 0.017	0.087 ± 0.018		
Calculated free 25(OH)D_3 (nmol/L)	Placebo, N=53	12 (8 to 21)	12 (8 to 18)	12 (6 to 18)	< 0.001
	Vitamin D, N=51	17 (11 to 20)	21 (17 to 31)		
Calculated bioavailable 25(OH)D_3 (nmol/L)	Placebo, N=53	5.22 (3.15 to 8.51)	4.99 (2.95 to 6.83)	4.66 (2.63 to 6.68)	< 0.001
	Vitamin D, N=51	6.60 (4.10 to 8.02)	8.69 (6.58 to 12.51)		
	Placebo, N=52	0.0027 (0.0018 to 0.0039)	0.0026 (0.0019 to 0.0036)		< 0.001

$1,25(OH)_2D/25(OH)D_3$ ((nmol/L)/(nmol/L))*	Vitamin D, N=52	0.0028 (0.0021 to 0.0035)	0.0019 (0.0014 to 0.0026)	-0.0010 (-0.0013 to -0.0006	
$1,25(OH)_2D/24,25(OH)_2D_3$ ((nmol/L)/(nmol/L))*	Placebo, N=52	0.036 (0.024 to 0.051)	0.037 (0.026 to 0.052)	-0.020 (-0.026 to -0.015)	< 0.001
	Vitamin D, N=52	0.035 (0.026 to 0.050)	0.022 (0.016 to 0.028)		

$25(OH)D_3$ – 25-hydroxyvitamin D, $1,25(OH)_2D$ – 1,25-dihydroxyvitamin D, $24,25(OH)_2D_3$ – 24,25-dihydroxyvitamin D, VMR – vitamin D metabolite ratio. The parameters that were log transformed before being used in the analysis are marked with "".*

The associations between vitamin D related parameters and the changes from baseline in $25(OH)D_3$, $1,25(OH)_2D$ and $24,25(OH)_2D_3$ in the vitamin D treated group are shown in Table 3. The parameters included in the study didn´t show any significant associations with $\Delta 25(OH)D_3$ or $\Delta 1,25(OH)_2D$ after Bonferroni correction. On the other hand, $\Delta 24,25(OH)_2D_3$ was significantly correlated with baseline $25(OH)D_3$, $24,25(OH)_2D_3$, calculated free $25(OH)D_3$ and calculated bioavailable $25(OH)D_3$ ($r=-0.562$, $p<0.001$; $r=-0.476$, $p=0.003$; $r=-0.382$, $p=0.048$ and $r=-0.393$, $p=0.032$, respectively). No associations were observed with other parameters.

Table 3: Correlations of baseline vitamin D related parameters with the changes from baseline of 25(OH)D3, 1,25(OH)2D and 24,25(OH)2D3 after vitamin D supplementation.

Baseline parameters		$\Delta 25(OH)D_3$	$\Delta 1,25(OH)_2D$	$\Delta 24,25(OH)_2D_3$
$25(OH)D_3$	Pearson correlation coefficient	-0.388	-0.142	-0.562
	p-value	0.007	0.322	< 0.001
	Bonferroni adjusted p-value	0.056	1.000	< 0.001
$1,25(OH)_2D$	Pearson correlation coefficient	-0.287	-0.260	-0.272
	p-value	0.041	0.065	0.053
	Bonferroni adjusted p-value	0.328	0.520	0.424

$24,25(OH)_2D_3$	Pearson correlation coefficient	-0.374	-0.122	-0.476
	p-value	0.007	0.392	< 0.001
	Bonferroni adjusted p-value	0.056	1.000	0.003
VMR	Pearson correlation coefficient	-0.109	-0.027	-0.015
	p-value	0.448	0.850	0.916
	Bonferroni adjusted p-value	1.000	1.000	1.000
Calculated free $25(OH)D_3$ *	Pearson correlation coefficient	-0.373	-0.281	-0.382
	p-value	0.007	0.046	0.006
	Bonferroni adjusted p-value	0.056	0.368	0.048
Calculated bioavailable $25(OH)D_3$ *	Pearson correlation coefficient	-0.375	-0.280	-0.393
	p-value	0.007	0.047	0.004
	Bonferroni adjusted p-value	0.056	0.376	0.032
$1,25(OH)_2D/25(OH)D_3$ *	Pearson correlation coefficient	-0.004	-0.058	0.176

	p-value	0.980	0.687	0.216
	Bonferroni adjusted p-value	1.000	1.000	1.000
$1,25(OH)_2D$ /$24,25(OH)_2D_3$ *	Pearson correlation coefficient	0.053	-0.028	0.181
	p-value	0.711	0.843	0.204
	Bonferroni adjusted p-value	1.000	1.000	1.000

*$25(OH)D_3$ – 25-hydroxyvitamin D, $1,25(OH)_2D$ – 1,25-dihydroxyvitamin D, $24,25(OH)_2D_3$ – 24,25-dihydroxyvitamin D, VMR – vitamin D metabolite ratio, $\Delta 25(OH)D_3$ – change from baseline for $25(OH)D_3$ in the vitamin D treated group, $\Delta 1,25(OH)_2D$ – change from baseline for $1,25(OH)_2D$ in the vitamin D treated group, $\Delta 24,25(OH)_2D_3$ – change from baseline for $24,25(OH)_2D_3$ in the vitamin D treated group. *Log transformed parameters; One extreme outlier was removed (25(OH)D > 4xSD at baseline) because of its significant effect on all of the analyses.*

We observed similar results in the correlation analyses after adjustment for gender, age, BMI, PTH, eGFR, serum phosphate and serum calcium. Also, in these analyses none of the vitamin D-related parameters were significantly correlated with $\Delta 25(OH)D_3$ or $\Delta 1,25(OH)_2D$ after Bonferroni correction. We did, however, find significant associations of $\Delta 24,25(OH)_2D$ with $25(OH)D_3$ (-0.657, p = 0.008). Table 4 depicts the results of these analyses.

Table 4: Correlations of baseline vitamin D related parameters adjusted for gender, age, BMI, PTH, eGFR, serum phosphate and serum calcium, with the

changes from baseline of 25(OH)D, 1,25(OH)2D and 24,25(OH)2D after vitamin D supplementation.

Baseline parameters		$\Delta 25(OH)D_3$	$\Delta 1,25(OH)_2D$	$\Delta 24,25(OH)_2D_3$
25(OH)D3	Pearson correlation coefficient	-0.508	-0.277	-0.657
	p-value	0.013	0.201	0.001
	Bonferroni adjusted p-value	0.104	1.000	0.008
$1,25(OH)_2D$	Pearson correlation coefficient	-0.350	-0.171	-0.430
	p-value	0.102	0.435	0.040
	Bonferroni adjusted p-value	0.816	1.000	0.320
$24,25(OH)_2D$	Pearson correlation coefficient	-0.490	-0.129	-0.597
	p-value	0.018	0.559	0.003
	Bonferroni adjusted p-value	0.440	1.000	0.096
VMR	Pearson correlation coefficient	-0.064	0.137	-0.516
	p-value	0.773	0.534	0.012
	Bonferroni adjusted p-value	1.000	1.000	0.096
Calculated free $25(OH)D_3$ *	Pearson correlation coefficient	-0.451	-0.363	-0.399
	p-value	0.031	0.089	0.059
	Bonferroni adjusted p-value	0.248	0.712	0.472

Calculated bioavailable $25(OH)D_3$ *	Pearson correlation coefficient	-0.451	-0.363	-0.404
	p-value	0.031	0.089	0.056
	Bonferroni adjusted p-value	0.248	0.712	0.448
$1,25(OH)_2D/25(OH)D_3$	Pearson correlation coefficient	0.122	0.272	0.218
	p-value	0.578	0.209	0.318
	Bonferroni adjusted p-value	1.000	1.000	1.000
$1,25(OH)_2D$ $/24,25(OH)_2D_3$ *	Pearson correlation coefficient	0.126	0.136	0.211
	p-value	0.565	0.536	0.333
	Bonferroni adjusted p-value	1.000	1.000	1.000

*$25(OH)D_3$ – 25-hydroxyvitamin D, $1,25(OH)_2D$ – 1,25-dihydroxyvitamin D, $24,25(OH)_2D_3$ – 24,25-dihydroxyvitamin D, VMR – vitamin D metabolite ratio, $\Delta 25(OH)D_3$ – change from baseline for $25(OH)D_3$ in the vitamin D treated group, $\Delta 1,25(OH)_2D$ – change from baseline for $1,25(OH)_2D$ in the vitamin D treated group, $\Delta 24,25(OH)_2D_3$ – change from baseline for $24,25(OH)_2D_3$ in the vitamin D treated group. *Log transformed parameters; One extreme outlier was removed (25(OH)D > 4xSD at baseline) because of its significant effect on all of the analyses.*

3.2 Part 2: Sub-study 2 and study 2

Part 2 presents the results obtained by analysing data from sub-study 2 and study 2.

Our first aim was to assess if a vitamin D intervention affects sST2 levels. This analysis was performed on data from sub-study 2 and the baseline characteristics are depicted in Table 5 (top; sub-study 2). At baseline, significant differences were present between men and women for serum calcium, sST2, eGFR and serum phosphate. No differences were observed for the parameters included in the study between the placebo and treatment groups (data not shown).

Table 5: Baseline characteristics in sub-study 2 (top) and general characteristics in study 2 (bottom) according to gender.

Sub-study 2	All (n = 185)	Men (n = 98)	Women (n = 87)	p-value
Age (years)	62.4 (52.9 to 68.1)	60.9 (52.9 to 68.1)	63.7 (55.5 to 68.8)	0.177
BMI (kg/m^2)	29.7 (27.1 to 32.8)	29.9 (27.0 to 32.6)	29.3 (27.1 to 33.2)	0.568
Gender (% female)	47	n.a.	n.a.	n.a.
sST2 (ng/mL)	13.3 (10.0 to 17.5)	16.2 (11.6 to 21.2)	11.6 (9.0 to 15.3)	< 0.001
25(OH)D (nmol/L)	54.7 (42.7 to 64.2)	53.4 (37.9 to 64.6)	56.7 (43.9 to 63.9)	0.412
PTH (pg/mL)	49.1 (39.6 to 62.9)	48.7 (37.9 to 60.8)	51.4 (41.6 to 64.0)	0.181
$1,25(OH)_2D$ (pmol/L)	115.9 (93.4 to 158.7)	143.8 (97.8 to 161.2)	107.0 (82.9 to 157.3)	0.053
Serum calcium (mmol/L)	2.3 (2.2 to 2.3)	2.3 (2.2 to 2.3)	2.3 (2.2 to 2.4)	0.033
Serum phosphate (mmol/L)	2.9 ± 0.5	2.7 ± 0.5	3.1 ± 0.5	< 0.001
eGFR ($mL/min/1.73m^2$)	82.7 ± 17.7	84.7 ± 16.6	80.5 ± 18.7	0.107
Active or previous smoker (%)	53	69	36	< 0.001

Type 2 diabetes mellitus (%)	37	44	29	0.033
Previous myocardial infarctions (%)	7	10	4	0.073
Heart failure (%)	0	0	0	/
C-reactive protein (mg/L)	1.8 (0.9-3.5)	1.8 (0.9-3.4)	1.9 (0.8-3.6)	0.714
Study 2	All (n = 1403)	Men (n = 994)	Women (n = 409)	p-value
Age (years)	65.6 (58.6 to 71.8)	64.9 (57.8 to 71.0)	67.7 (61.6 to 73.3)	< 0.001
BMI (kg/m^2)	27.3 (25.0 to 30.1)	27.4 (25.3 to 30.1)	26.9 (24.4 to 30.2)	0.008
Gender (% female)	35	/	/	/
sST2 (U/mL)	19.2 (15.4 to 24.6)	19.8 (16.0 to 25.4)	17.3 (14.4 to 22.6)	< 0.001
25(OH)D (nmol/L)	38.7 (25.0 to 57.2)	41.4 (28.2 to 58.7)	31.4 (20.1 to 51.4)	< 0.001
PTH (pg/mL)	30.0 (22.0 to 41.0)	29.0 (22.0 to 39.0)	32.0 (24.0 to 44.0)	0.001
$1,25(OH)_2D$ (pmol/L)	80.9 (63.1 to 106.3)	83.0 (65.6 to 108.8)	50.6 (56.9 to 100.6)	< 0.001
Serum calcium (mmol/L)	2.3 (2.3 to 2.4)	2.3 (2.3 to 2.4)	2.3 (2.3 to 2.4)	0.683
Serum phosphate (mg/dL)	3.5 (3.1 to 3.9)	3.4 (3.1 to 3.7)	3.7 (3.3 to 4.1)	< 0.001
eGFR ($mL/min/1.73m^2$)	83.6 (69.2 to 97.1)	83.6 (69.2 to 97.1)	83.6 (68.5 to 98.6)	0.824
IL-6 (ng/L)	3.3 (1.9 to 6.1)	3.4 (1.9 to 6.3)	3.3 (2.0 to 5.7)	0.332

Active or previous smoker (%)	64	76	33	< 0.001
Type 2 diabetes mellitus (%)	36	37	35	0.367
Previous myocardial infarctions (%)	43	47	33	< 0.001
Previous stroke, PRIND and/or TIA (%)	11	11	12	0.585
Heart failure (%)	48	46	54	0.065
C-reactive protein (mg/L)	3.7 (1.9 to 6.1)	3.4 (1.4 to 9.1)	3.9 (1.6 to 9.1)	0.160

BMI – body mass index; sST2 – soluble ST2; eGFR – estimated glomerular filtration rate; 25(OH)D – 25-hydroxyvitamin D; PTH – parathyroid hormone; 1,25(OH)D – 1,25-dihydroxyvitamin D; PRIND – prolonged reversible ischemic neurologic deficit; TIA – transient ischemic attack. Adapted from (Francic et al., 2019).

In the whole cohort, we found no significant associations of baseline sST2 with 25(OH)D, PTH or 1,25(OH)$_2$D neither in women (r_s = -0.191, p = 0.077; r_s = -0.186, p = 0.084; r_s = -0.187, p = 0.084; respectively) nor men (r_s = -0.033, p = 0.746; r_s = 0.062, p = 0.543; r_s = -0.124, p=0.229; respectively). There were also no correlations present in a sub-cohort where baseline 25(OH)D levels were below 50 nmol/L (20 ng/mL; or below 37.4 nmol/L (15 ng/mL); data not shown) (Table 6).

We observed a rise in 25(OH)D with a mean treatment effect (95% confidence interval [CI]) of 28.2 (23.0 to 33.7) nmol/L (p < 0.001) after a vitamin D intervention when compared to placebo. In addition, we found a decrease in PTH (-5.9 (-9.4 to -2.2) pg/mL; p = 0.002) and an increase in 1,25(OH)$_2$D (22.8 (12.2 to 34.9) pmol/L; p < 0.001) (Table 5). The levels of sST2 were unchanged in the whole cohort (0.1 (-0.6 to 0.8) ng/mL; p = 761) as well as in a sub-cohort where baseline 25(OH)D levels were below 50 nmol/L (20 ng/mL) (0.4 (-2.1 to 2.1) ng/mL; p = 0.639) or < 37.4 nmol/L (15 ng/mL; data not shown). (Table 7)

Because the concentrations of sST2 were different in men and women, we determined if the vitamin D intervention had different effects on sST2 levels in both genders. We found no significant treatment effects on sST2 concentrations neither in women (-0.02 (-1.2 to 1.2) ng/mL; p = 0.977) nor men (0.3 (-1.3 to 1.9) ng/mL; p = 0.753) (Table 7).

Table 6: Baseline Spearman's correlations of the parameters from sub-study 2 and study 2 with sST2 in all subjects included in the study as well as in subjects with 25(OH)D below 50 nmol/L (20 ng/mL).

	sST2 (in all included subjects)				sST2 (in subjects with 25(OH)D below 20 ng/ml)			
Sub-study 2	Men (N=96)		Women (N=87)		Men (N=37)		Women (N=32)	
	Spearmans's rho	p-value	Spearmans's rho	p-value	Spearmans's rho	p-value	Spearmans's rho	p-value
25(OH)D	-0.033	0.746	-0.191	0.077	0.080	0.633	-0.190	0.299
$1,25(OH)_2D$	-0.124	0.229	-0.187	0.084	0.021	0.904	-0.313	0.081
PTH	0.062	0.543	-0.186	0.084	0.042	0.800	-0.191	0.296
Study 2	Men (N=994)		Women (N=409)		Men (N=633)		Women (N=301)	
	Spearmans's rho	p-value	Spearmans's rho	p-value	Spearmans's rho	p-value	Spearmans's rho	p-value
25(OH)D	-0.106*	0.001	-0.055	0.271	-0.106*	0.001	-0.055	0.271
$1,25(OH)_2D$	-0.031	0.323	-0.026	0.603	-0.031	0.323	-0.026	0.603
PTH	0.084	0.008	-0.008	0.879	0.084	0.008	-0.008	0.879

*sST2 – soluble ST2; 25(OH)D – 25-hydroxyvitamin D; PTH – parathyroid hormone; 1,25(OH)D – 1,25-dihydroxyvitamin D. * Correlation coefficients significant also after Bonferroni correction. Adapted from (Francic et al., 2019).*

Table 7: ANCOVA analysis for the effect of vitamin D or placebo treatment on parameters from sub-study 2.

Parameter	Group	Baseline	Follow-up	Treatment effect (95 % confidence interval)	p-value
25(OH)D	Vitamin D	57.4 (47.4 to 65.6)	88.4 (40.7 to 71.1)	28.2 (23.0 to 33.7)	< 0.001
	Placebo	52.9 (37.9 to 63.4)	57,4 (75.8 to 105.1)		
$1,25(OH)_2D$*	Vitamin D	116.9 (96.3 to 158.7)	141.1 (115.3 to 173.0)	22.8 (12.2 to 34.9)	< 0.001
	Placebo	111.4 (88.4 to 158.5)	116.64 (88.4 to 148.8)		
PTH*	Vitamin D	49.0 (39.6 to 61.5)	45.5 (37.8 to 54.3)	-5.9 (-9.4 to -2.2)	0.002
	Placebo	51.4 (39.2 to 63.9)	50.6 (38.6 to 66.1)		
sST2*	Vitamin D	13.3 (10.4 to 17.3	13.8 (10.6 to 17.1)	0.1 (-0.6 to 0.8)	0.761
	Placebo	13.5 (9.4 to 18.3)	12.8 (9.2 to 17.4)		
sST2 in subjects	Vitamin D	15.5 (9.1 to 18.3)	14.7 (9.9 to 19.4)	0.4 (-1.3 to 2.1)	0.639

with baseline 25(OH)D below 50 nmol/L (20 ng/mL)*	Placebo	15.9 (10.1 to 18.7)	13.7 (10.4 to 18.0)		
sST2 in men*	Vitamin D	16.5 (13.1 to 19.2)	15.1 (12.9 to 20.2)	0.3 (-1.3 to 1.9)	0.753
	Placebo	16.0 (10.7 to 23.0)	15.4 (10.4 to 22.4)		
sST2 in women*	Vitamin D	12.3 (9.1 to 13.7)	11.6 (8.9 to 13.8)	-0.02 (-1.2 to 1.2)	0.977
	Placebo	11.4 (8.8 to 16.2)	11.9 (9.0 to 13.7)		

*sST2 – soluble ST2; 25(OH)D – 25-hydroxyvitamin D; PTH – parathyroid hormone; 1,25(OH)D – 1,25-dihydroxyvitamin D. * Analyses where outliers were present but didn't significantly affect the results. Adapted from (Francic et al., 2019).*

Our second aim was to determine if seasonal variation is present in sST2 levels. The data from study 2 were used for this analysis and general characteristics of study 2 cohort can be found in Table 5 (bottom; study 2). Significant differences in sST2, age, 25(OH)D, $1,25(OH)_2D$, PTH, BMI, serum phosphate, eGFR, active or previous smoker percentage and previous myocardial infarction percentage, between genders were detected.

Significant associations of sST2 levels with 25(OH)D were present in men (r_s = -0.106, p=0.001; significant after Bonferroni correction). None were found with PTH and $1,25(OH)_2D$ (r_s = 0.084, p=0.008; r_s = -0.031, p = 0.0323; respectively, both not significant after Bonferroni correction). In women, no significant correlations were

found (r_s = -0.055, p = 0.271; r_s = -0.008, p = 0.879; r_s=-0.026, p=0.603; for 25(OH)D, PTH and 1,25$(OH)_2$D respectively). We found similar associations in the sub-cohort with baseline 25(OH)D levels below 50 nmol/L (20 ng/mL; and < 37.4 nmol/L (15 ng/mL); data not shown). These results are summarized in Table 6.

Table 8 shows the parameter values of subjects subgrouped according to gender and the meteorological definition of the seasons. We found monthly variations in 25(OH)D levels in women and also in men, with and without heart failure (p < 0.001 for all subgroups). sST2 levels on the other hand were affected by the seasons only in men with HF (p = 0.44) but not in men without HF (p = 0.895) or women with and without HF (p = 512 and p = 0.948; respectively). After we adjusted both parameters for age, BMI, eGFR, T2DM status, IL-6 levels, any previous myocardial infarctions, smoking status, any previous strokes, transient ischemic attacks and/or prolonged reversible ischemic neurologic deficits, we found that the seasonal variances remained significant in men and women with and without HF for 25(OH)D (p < 0.001 for all subgroups). For sST2 the variances were not significant in any of the subgroups (for men without HF, p = 0.803 and with HF, p = 0.073; for women without HF, p = 0.512 and with HF, p = 0.948).

Table 8: Parameter values of subjects subgrouped according to gender and the meteorological definition of the seasons.

	Spring		Summer		Autumn		Winter	
LURIC study	Men (N=195)	Women (N=78)	Men (N=243)	Women (N=91)	Men (N=343)	Women (N=148)	Men (N=213)	Women (N=92)
Age (years)	64.3 (58.3 to 70.8)	68.1 (61.2 to 74.4)	63.1 (56.1 to 70.2)	67.3 (59.7 to 72.6)	66.4 (58.4 to 71.6)	67.2 (61.7 to 73.4)	65.2 (58.4 to 71.8)	68.0 (62.4 to 72.7)
BMI (kg/m^2)	27.0 (24.9 to 30.1)	26.0 (24.1 to 31.3)	27.8 (25.7 to 30.3)	27.2 (24.5 to 29.8)	27.4 (25.2 to 29.8)	26.5 (24.1 to 30.0)	27.4 (25.2 to 30.5)	27.4 (24.7 to 30.1)
sST2 (U/mL)	20.2 (15.8 to 25.6)	18.6 (13.1 to 25.2)	19.8 (16.2 to 25.9)	16.9 (14.1 to 21.7)	19.7 (15.9 to 25.4)	17.1 (14.4 to 17.1)	19.9 (16.4 to 26.1)	17.5 (15.1 to 22.6)
Vitamin D (nmol/L)	30.2 (20.5 to 39.2)	13.0 (16.2 to 31.4)	53.6 (39.2 to 69.9)	46.4 (29.7 to 63.1)	46.4 (34.9 to 62.7)	34.4 (35.9 to 56.4)	34.7 (20.5 to 48.9)	23.2 (16.9 to 34.9)

PTH (pg/mL)	32.0 (22.0 to 42.0)	31.5 (24.0 to 46.3)	29.0 (22.0 to 37.0)	30.0 (21.0 to 42.0)	29.0 (22.0 to 39.0)	33.0 (23.3 to 44.0)	29.0 (21.0 to 39.0)	31.0 (25.0 to 46.8)
1,25$(OH)_2$D (pmol/L)	68.9 (53.9 to 95.6)	63.8 (49.2 to 91.1)	85.9 (71.2 to 105.8)	80.4 (61.7 to 101.6)	64.6 (72.1 to 121.3)	79.7 (62.4 to 109.3)	79.9 (57.7 to 106.8)	72.5 (54.7 to 93.6)
Serum calcium (mmol/L)	2.3 (2.3 to 2.4)	2.3 (2.2 to 2.4)	2.3 (2.3 to 2.4)	2.3 (2.2 to 2.4)	2.3 (2.3 to 2.4)	2.3 (2.3 to 2.4)	2.3 (2.3 to 2.4)	2.3 (2.3 to 2.4)
Serum phosphate (mg/dL)	3.4 (3.1 to 3.7)	3.8 (3.4 to 4.2)	3.3 (3.0 to 3.7)	3.8 (3.3 to 4.1)	3.4 (3.0 to 3.7)	3.6 (3.3 to 4.0)	3.4 (3.0 to 3.8)	3.7 (3.4 to 4.0)
eGFR (CKD-EPI) (mL/min/1.73m^2)	83.6 (67.7 to 97.1)	78.6 (61.6 to 93.4)	83.6 (67.0 to 97.1)	83.6 (74.0 to 98.6)	82.6 (70.0 to 95.7)	85.8 (70.2 to 101.5)	85.8 (72.0 to 100.0)	83.6 (67.2 to 96.8)
C-reactive protein (mg/L)	3.6 (1.4 to 9.9)	5.1 (2.1 to 9.8)	4.1 (1.7 to 9.2)	4.5 (1.9 to 8.8)	3.7 (1.4 to 9.9)	3.7 (1.6 to 9.3)	2.8 (1.1 to 6.5)	3.3 (1.3 to 7.1)
IL-6 (ng/L)	3.3 (1.7 to 6.6)	3.1 (1.6 to 6.1)	3.7 (2.1 to 6.2)	3.6 (2.1 to 5.4)	3.6 (2.1 to 6.8)	3.2 (2.2 to 5.8)	2.9 (1.7 to 5.3)	3.0 (1.5 to 5.8)

Active or previous smoker (%)	74	37	92	31	74	35	74	29
Type 2 diabetes mellitus (%)	38	39	36	33	35	33	41	35
Previous myocardial infarctions (%)	46	37	49	34	47	32	47	27
Previous stroke, PRIND and/or TIA (%)	9	15	13	12	13	12	8	10
Heart failure (%)	50	59	48	57	46	50	41	42

BMI – body mass index; sST2 – soluble ST2; eGFR – estimated glomerular filtration rate; 25(OH)D – 25-hydroxyvitamin D; PTH – parathyroid hormone; 1,25(OH)D – 1,25-dihydroxyvitamin D; PRIND – prolonged reversible ischemic neurologic deficit; TIA – transient ischemic attack. Adapted from (Francic et al., 2019).

Median sST2 and 25(OH)D concentrations with 95 % CI are depicted in Figure 1. We observed peak concentrations of 25(OH)D in August in subjects of both genders with HF (median 57.9 nmol/L (23.2 ng/mL) for men with HF and 51.9 nmol/L (20.8 ng/mL) for women with HF), while in subjects without HF they were in July for men (median 60.9 nmol/L (24.4 ng/mL)) and in August for women (median 67.4 nmol/L (27.0 ng/mL)). In subjects with HF the nadir was observed in January in men (median 20.2 nmol/L (8.1 ng/mL)) and in February in women (median 16.5 nmol/L (6.6 ng/mL)), while in men and women without HF the lowest values were observed in March (median 27.2 nmol/L (10.9 ng/mL) and 17.7 nmol/L (7.1 ng/mL), respectively).

Peak sST2 concentrations were observed in May in men with HF (median 24.7 U/mL) and in February in women with HF (median 22.6 U/mL). In subgroups without HF the highest levels were observed in January for men (median 20.2 U/mL) and in March for women (median 19.2 U/mL). The nadir was in June in men with HF (median 19.4 U/mL) and in August in women with HF (median 20.0 U/mL). In subjects without HF, the lowest levels were observed in May in men (median 17.7 U/mL) and in April in women (median 14.5 U/mL).

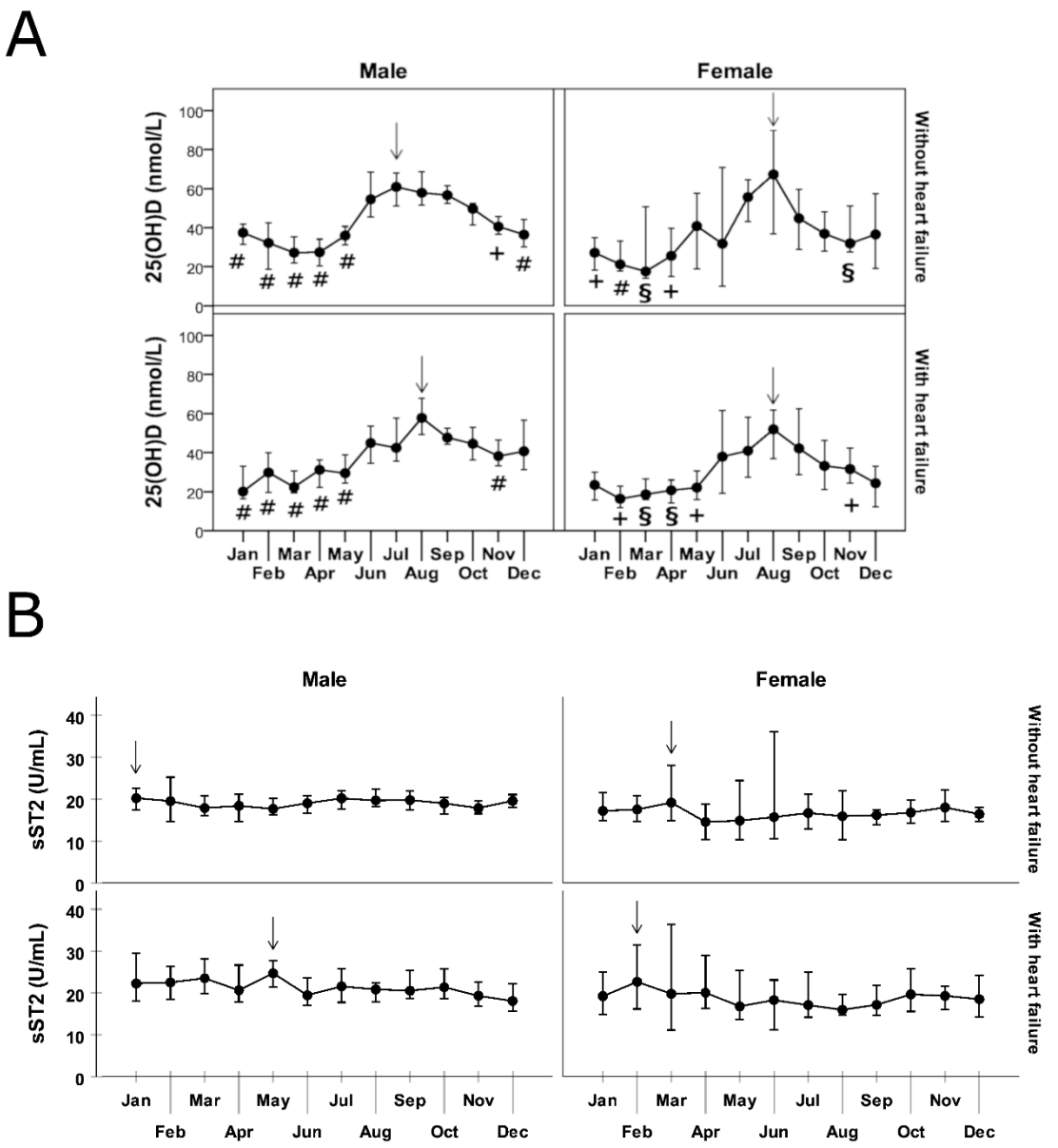

Figure 1: Seasonal variation in 25(OH)D (A) and sST2 (B) concentrations (monthly median values with 95 % CI) in men and women further subgrouped into subjects with and without heart failure in study B. Significant differences from the peak value (↓); §, $p<0.05$; +, $p<0.01$; #, $p<0.001$. The data were log transformed before being used in ANOVA analyses. Outliers were present in all ANOVA analyses but didn't significantly affect the results. Adapted from (Francic et al., 2019).

We also performed the same analyses with the measurements grouped into seasons according to the meteorological definition of the seasons. The results we

observed were very similar to the ones with calculations according to the months and can be seen in Figure 2.

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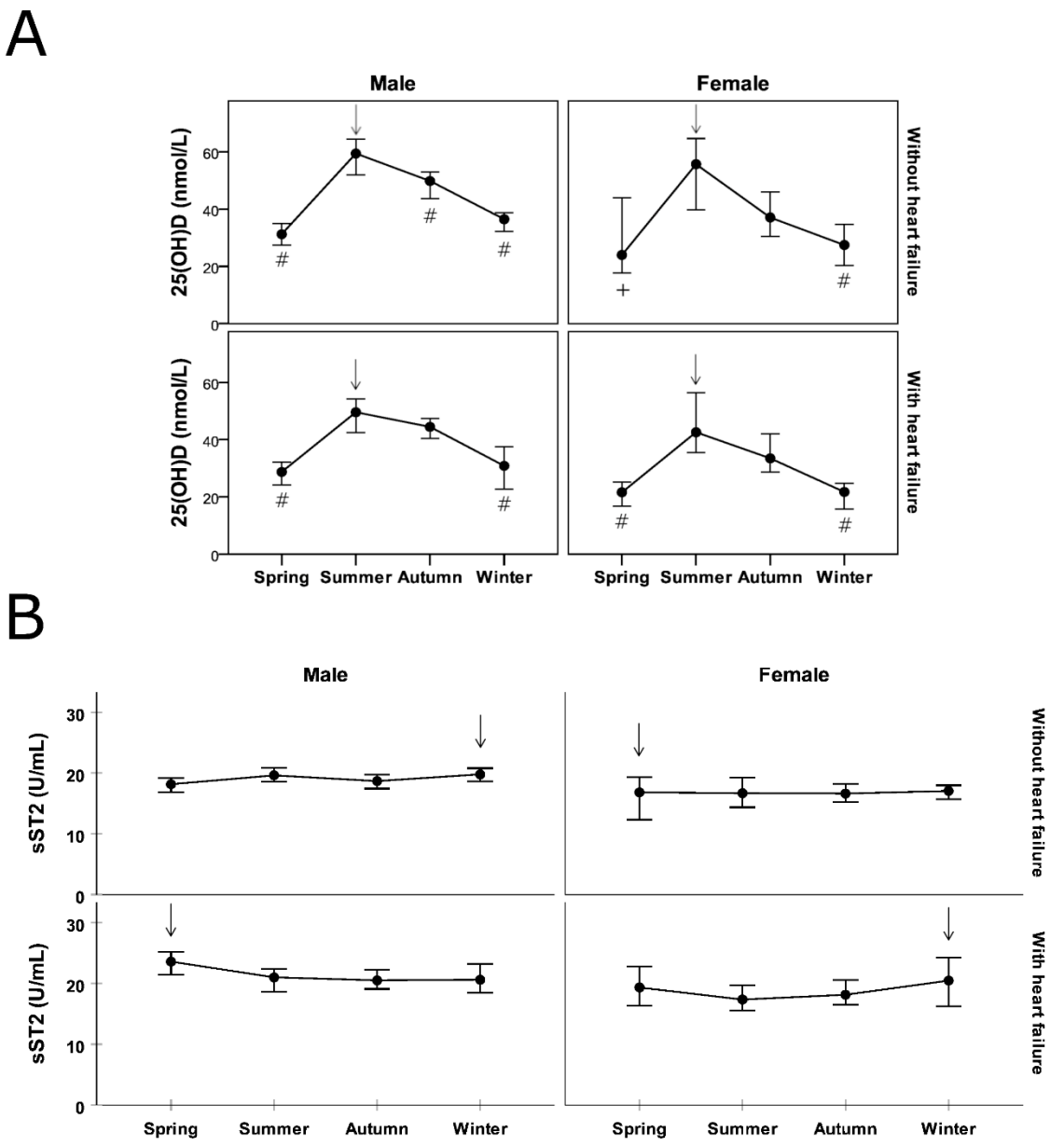

Figure 2: Seasonal variation in 25(OH)D (A) and sST2 (B) concentrations (seasonal median values with 95 % CI) in men and women further subgrouped into subjects with and without heart failure in study B. Significant differences from the peak value (↓); +, P<0.01; #, P<0.001. The data were log transformed before being used in ANOVA analyses. Outliers were present in several ANOVA analyses but didn't significantly affect the results. Adapted from (Francic et al., 2019).

We also prepared graph shape comparison graphs to be able to more accurately assess whether the seasonal patterns of 25(OH)D and sST2 are similar. As can be seen in Figure 3 the graph shapes and patterns are distinctly different for both subgroups in both genders between 25(OH)D and sST2.

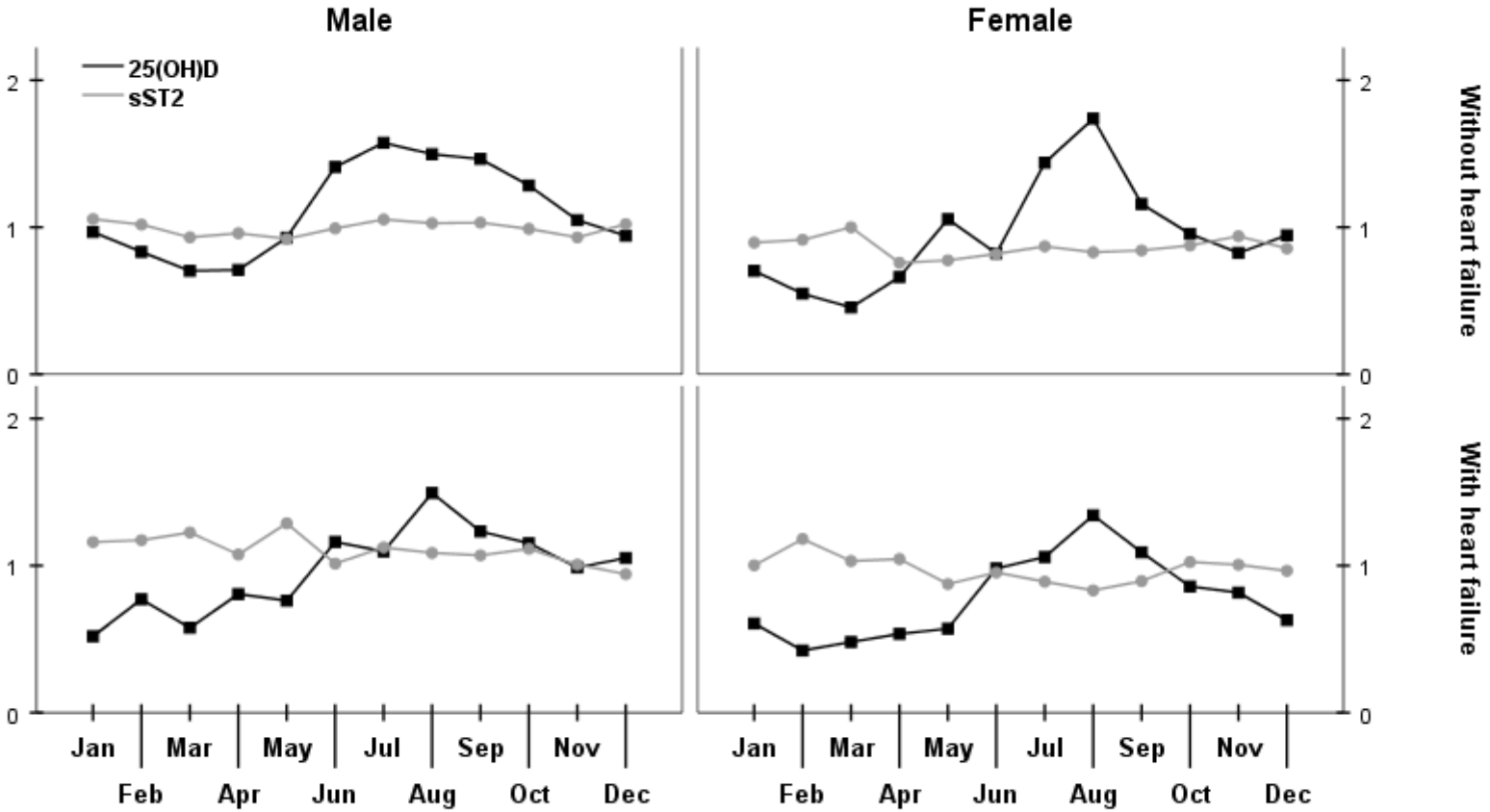

Figure 3: Graph shape comparisons of seasonal variation in 25(OH)D and sST2 concentrations (monthly medians, normalized to corresponding overall medians; 37.8 nmol/L (15.15 ng mL) for 25(OH)D and 19.18 U/mL for sST2) in men and women further subgrouped into subjects with and without heart failure in study B. Adapted from (Francic et al., 2019).

3.3 Part 3: Sub-study 3

Data from sub-study 3 was analysed to obtain the results for this part of the results section and the baseline characteristics of patients are depicted in Table 9. At baseline, we found no significant differences between the vitamin D and placebo treated groups.

Table 9: Baseline characteristics of participants in the vitamin D and placebo treated groups in sub-study 3.

Parameters	All	Vitamin D	Placebo	p-value
Age (years)	60.1 ± 11.9	59.5 ± 11.8	60.7 ± 12.1	0.384
Gender (% female)	60	29	31	0.863
% postmenopausal females	47	24	23	0.561
BMI (kg/m2)	30.0 ± 5.34	30.3 ± 4.85	29.7 ± 5.8	0.501
25(OH)D_3 (nmol/L)	46.0 (25.0 to 58.0)	46.0 (37.0 to 58.0)	45.5 (30.0 to 58.5)	0.538
1,25$(OH)_2$D (pmol/L)	115.4 (87.9 to 162.2)	116.4 (93.6 to 165.2)	101.8 (71.1 to 160.7)	0.153
Vitamin D Binding Protein (µg/mL)	249 ± 110	237 ± 108	258 ± 112	0.355
Bioavailable 25(OH)D_3 (nmol/L)	5.80 (3.76 to 8.13)	6.60 (4.10 to 8.03)	5.15 (3.20 to 8.47)	0.123
Serum calcium (mmol/L)	2.26 (2.21 to 2.33)	2.26 (2.21 to 2.33)	2.26 (2.21 to 2.33)	0.824
Serum phosphate (mmol/L)	0.94 (0.17)	0.92 ± 0.16	0.96 ± 0.17	0.199

24h urinary calcium excretion (mmol/24h)	3.34 (1.85 to 5.01)	3.63 (1.88 to 6.23)	2.96 (1.85 to 4.86)	0.325
N-oxPTH (pmol/L)	1.11 ± 0.28	1.13 ± 0.31	1.09 ± 0.26	0.340
tPTH (pmol/L)	5.3 (4.0 to 6.7)	5.2 (4.1 to 6.7)	5.5 (3.9 to 6.8)	0.710
bALP (µg/L)	16.8 (12.9 to 20.5)	16.8 (13.2 to 20.6)	17.2 (12.6 to 20.4)	0.962
CTX (ng/mL)	0.19 (0.11 to 0.33)	0.19 (0.13 to 0.34)	0.18 (0.10 to 0.28)	0.647
Osteocalcin (ng/mL)	13.0 (9.6 to 18.5)	12.6 (10.1 to 18.2)	14.1 (9.0 to 18.8)	0.859
P1NP (ng/mL)	38.0 (31.2 to 52.0)	37.9 (29.4 to 55.7)	38.0 (31.9 to 48.9)	0.879
eGFR (mL/min/1.73 m^2)	82.8 ± 18.5	85.0 ± 18.3	81.1 ± 18.7	0.336
FGF23 (pmol/L)	0.83 (0.59 to 1.24)	0.77 (0.54 to 1.17)	0.89 (0.69 to 2.02)	0.069
Office systolic BP* (mmHg)	144.6 ± 16.3	144.5 ± 17.1	144.8 ± 15.7	0.856
Office diastolic BP* (mmHg)	87.2 ± 10.5	87.2 ± 10.0	87.3 ± 10.9	0.981
Ratio n-oxPTH/tPTH	0.22 ± 0.07	0.22 ± 0.07	0.22 ± 0.06	0.850
Total cholesterol (mg/dl)	192 ± 40	198 ± 39	186 ± 40	0.104
HDL cholesterol (mg/dl)	55.5 (47 to 66)	54.0 (45 to 66)	56.0 (47 to 66)	0.612
LDL cholesterol (mg/dl)	112 ± 35	117 ± 33	107 ± 37	0.137
Triglycerides (mg/dl)	110 (69 to 153)	109 (69 to 152)	110 (69 to 162)	0.996

ADMA (µmol/L)	0.70 (0.63 to 0.78)	0.69 (0.63 to 0.78)	0.71 (0.64 to 0.79)	0.247

** Maximum of the two arms. BMI – body mass index; eGFR – estimated glomerular filtration rate; 25(OH)D_3 – 25(OH)-hydroxyvitamin D_3; 1,25(OH)D – 1,25-dihydroxyvitamin D; Bioavailable 25(OH)D3 - biologically available 25(OH)D_3 ; n-oxPTH – non-oxidized parathyroid hormone; tPTH – total parathyroid hormone; bALP – bone-specific alkaline phosphatase; CTX – carboxy-terminal collagen crosslinks; P1NP – total procollagen type 1 N-terminal propeptide; FGF 23 – fibroblast growth factor 23; office systolic BP – office systolic blood pressure; office diastolic BP – office diastolic blood pressure; HDL cholesterol – high-density lipoprotein cholesterol; LDL cholesterol – low-density lipoprotein cholesterol; ADMA – asymmetric dimethylarginine. Adapted from (Ursem et al., 2019).*

The cross cross-sectional analyses are presented first. The calculated Pearson correlation coefficients between tPTH, n-oxPTH, FGF23, a bone resorption marker (CTX), bone formation markers (osteocalcin, bALP, P1NP) as well as total cholesterol, HDL cholesterol, LDL cholesterol and triglycerides are depicted in Table 10. n-oxPTH and tPTH were significantly associated ($r = 0.555$; $p < 0.001$; Figure 4). Resorption and bone formation markers as well as FGF23 and osteocalcin didn't show significant correlations with either n-oxPTH or tPTH at baseline after Bonferroni adjustment. Also triglycerides, HDL cholesterol, LDL cholesterol and total cholesterol showed no significant association neither with n-oxPTH nor with tPTH after Bonferroni adjustment.

Table 10: Correlations between bone turnover parameters, parameters of lipid metabolism and n-oxPTH or tPTH in sub-study 3.

Parameters	n-oxPTH			Total PTH*		
	Pearson's r	p-value	Bonferroni adjusted p-value	Pearson's r	p-value	Bonferroni adjusted p-value

Total PTH*	0.555	<0.001	<0.001	n.a.	n.a.	n.a.
bALP*	0.144	0.148	1.000	0.024	0.813	1.000
CTX*	−0.028	0.783	1.000	−0.060	0.552	1.000
Osteocalcin*	0.237	0.014	0.252	0.108	0.268	1.000
P1NP*	0.169	0.088	1.000	−0.029	0.774	1.000
FGF23*	−0.016	0.869	1.000	0.010	0.916	1.000
Total cholesterol	−0.047	0.625	1.000	0.008	0.934	1.000
HDL cholesterol*	0.254	0.008	0.144	0.166	0.085	1.000
LDL cholesterol	−0.089	0.365	1.000	−0.021	0.831	1.000
Triglycerides*	−0.216	0.025	0.450	−0.042	0.666	1.000

** Log transformed parameters were used for the analysis. Total PTH – total parathyroid hormone; bALP – bone-specific alkaline phosphatase; CTX – carboxy-terminal collagen crosslinks; P1NP – total procollagen type 1 N-terminal propeptide; FGF 23 – fibroblast growth factor 23; HDL cholesterol – high-density lipoprotein cholesterol; LDL cholesterol – low-density lipoprotein cholesterol. Adapted from (Ursem et al., 2019).*

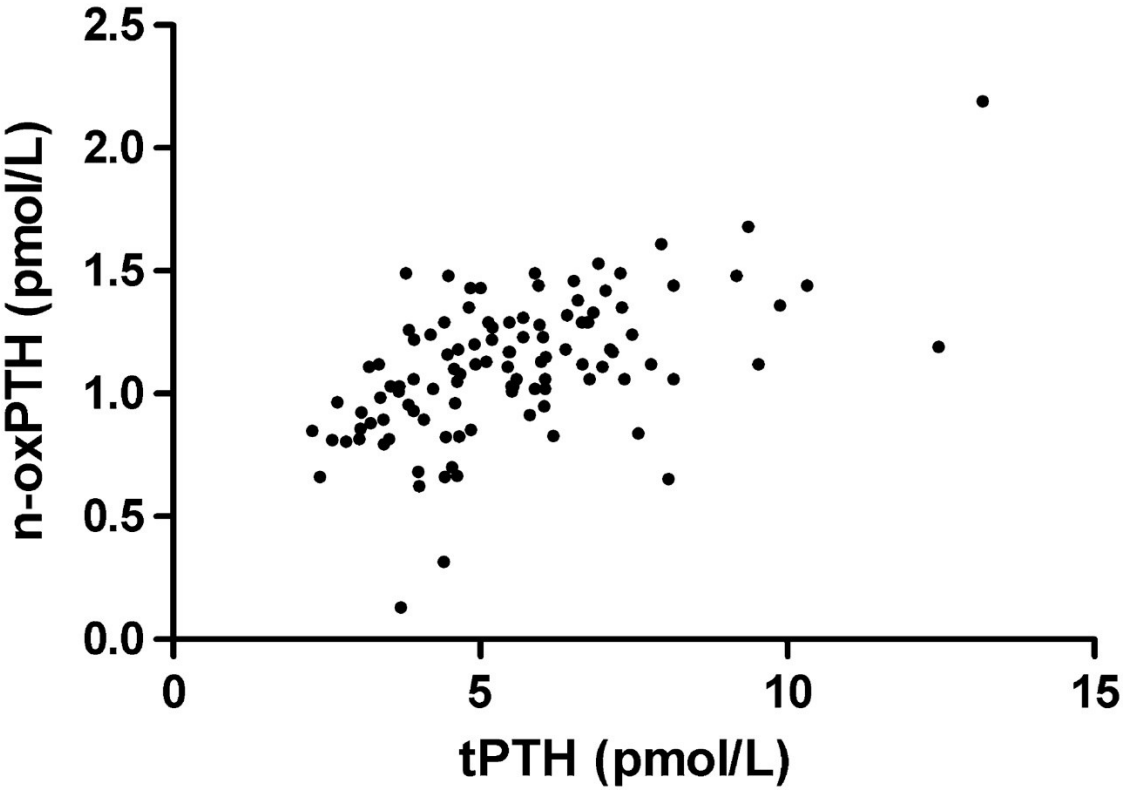

Figure 4: Scatterplot of tPTH and n-oxPTH at baseline (n = 108). Pearson's r = 0.555; P < 0.001. Adapted from (Ursem et al., 2019).

Table 11 depicts the associations between tPTH and n-oxPTH with vitamin D, mineral metabolism markers and ADMA. We found that after Bonferroni correction none of the parameters correlated significantly with tPTH or n-oxPTH.

Table 11: Correlations between parameters of calcium and phosphate homeostasis and n-oxPTH or tPTH in sub-study 3.

Parameters	n-oxPTH			Total PTH*		
	Pearson's r	p-value	Bonferroni adjusted p-value	Pearson's r	p-value	Bonferroni adjusted p-value
25(OH)D_3*	−0.069	0.477	1.000	−0.056	0.564	1.000
Bioavailable 25(OH)D_3*	−0.062	0.526	1.000	−0.111	0.258	1.000

1,25(OH)$_2$D*	0.179	0.065	1.000	0.215	0.026	0.572
eGFR	0.080	0.410	1.000	−0.063	0.519	1.000
Calcium	−0.053	0.588	1.000	0.008	0.935	1.000
Phosphate	−0.114	0.239	1.000	−0.213	0.027	0.594
UPCR	0.088	0.363	1.000	0.183	0.059	1.000
24-h urinary calcium†	0.063	0.542	1.000	−0.127	0.216	1.000
ADMA*	−0.104	0.285	1.000	0.017	0.861	1.000

*n-oxPTH – non-oxidized parathyroid hormone; total PTH – total parathyroid hormone; 25(OH)D$_3$ – 25(OH)-hydroxyvitamin D$_3$; 1,25(OH)D – 1,25-dihydroxyvitamin D; Bioavailable 25(OH)D3 - biologically available 25(OH)D3; eGFR – estimated glomerular filtration rate; UPCR - Urinary phosphate to creatinine ratio; ADMA – asymmetric dimethylarginine. *Log transformed; †Spearman's rho. Adapted from (Ursem et al., 2019)*

The results of the interventional analysis are presented second and the results are shown in Table 12. The vitamin D intervention significantly increased concentrations of 25(OH)D$_3$ (mean treatment effect [95 % CI], 32.4 [25.9 to 38.8] nmol/L; p <0.001). Biologically available 25(OH)D levels increased as well (3.91 [2.64 to 5.18] nmol/L; p <0.001). 1,25(OH)$_2$D levels also increased after the intervention (25.4 [9.8 to 42.9] pmol/L; p = 0.002). n-oxPTH and tPTH decreased (-0.08 [-0.01 to -0.15] pmol/L; p = 0.025 and -0.90 [-0.40 to -1.40] pmol/L; p <0.001, respectively). The decreases in n-oxPTH and tPTH (7 % and 9 %, respectively) were not significantly different to each other (p = 0.51). The n-oxPTH/tPTH ratio on the other hand increased after vitamin D treatment (0.022 [0.003 to 0.042]; P = 0.027). If expressed in percentage the change was 2.2 % (0.3 % to 4.2 %). Finally, plasma calcium concentrations, 24-hour urinary calcium excretion and ADMA were not affected.

Table 12: ANCOVA analysis for the effect of vitamin D or placebo treatment in sub-study 3.

Parameters	Group	Baseline (SD/IQR)	Follow-up (SD/IQR)	Treatment effect (95%CI)	p-value
25(OH)D_3 (nmol/L)	Vitamin D	49.0 ± 18.1	79.3 ± 19.1	32.4 (25.9 to 38.8)	< 0.001
	Placebo	46.0 ± 18.5	45.3 ± 19.7		
Total PTH (pmol/L) *	Vitamin D	5.19 (4.13 to 6.69)	4.64 (3.90 to 5.80)	-0.90 (-0.40 to -1.40)	< 0.001
	Placebo	5.46 (3.92 to 6.66)	5.33 (4.07 to 7.07)		
n-oxPTH (pmol/L)	Vitamin D	1.13 ± 0.31	1.06 ± 0.27	-0.08 (-0.01 to -0.15)	0.025
	Placebo	1.09 ± 0.26	1.10 ± 0.28		
Plasma calcium (mmol/L)	Vitamin D	2.28 ± 0.10	2.27 ± 0.09	0.02 (-0.01 to 0.05)	0.294
	Placebo	2.27 ± 0.11	2.25 ± 0.11		
24-hour urinary calcium excretion (mmol/24h) *	Vitamin D	3.60 (1.45 to 6.25)	3.80 (1.80 to 6.40)	0.66 (-0.07 to 1.39)	0.077
	Placebo	2.95 (1.90 to 5.40)	3.00 (1.50 to 4.60)		
1,25(OH)$_2$D (pmol/L)	Vitamin D	128.4 ± 52.2	144.5 ± 62.7	25.4 (9.8 to 42.9)	0.002
	Placebo	112.8 ± 52.2	108.7 ± 39.7		
Vitamin D Binding Protein (µg/mL)	Vitamin D	239 ± 108	323 ± 319	11.7 (-87.5 to 110.9)	0.816
	Placebo	258 ± 112	315 ± 173		
Bioavailable 25(OH)D_3 (nmol/L) *†	Vitamin D	6.60 (4.08 to 8.01)	8.64 (6.57 to 12.0)	3.91 (2.64 to 5.18)	< 0.001
	Placebo	5.22 (3.15 to 8.51)	4.98 (2.94 to 6.87)		
Ratio n-oxPTH/tPTH	Vitamin D	0.22 ± 0.07	0.23 ± 0.08	0.022 (0.003 to 0.042)	0.027
	Placebo	0.22 ± 0.06	0.20 ± 0.05		

ADMA*,† (µmol/L)	Vitamin D, *N* = 53	0.69 (0.63 to 0.77)	0.73 (0.65 to 0.81)	0.008 (−0.028 to 0.045)	0.759
	Placebo, *N* = 54	0.72 (0.65 to 0.79)	0.75 (0.67 to 0.80)		

n-oxPTH – non-oxidized parathyroid hormone; total PTH – total parathyroid hormone; 25(OH)D_3 – 25(OH)-hydroxyvitamin D_3; 1,25(OH)D – 1,25-dihydroxyvitamin D; Bioavailable 25(OH)D3 - biologically available 25(OH)D3; ADMA – asymmetric dimethylarginine.* Skewed variables for which transformed values were used in ANCOVA, but untransformed values are shown in the table; † 1 outlier was excluded; this had no effect on the significance level of the analysis. Adapted from (Ursem et al., 2019).

We also applied correlation analysis to determine if the changes from baseline in several parameters after vitamin D supplementation in the vitamin D-treated group were associated with ΔtPTH, Δn-oxPTH, the ratio Δn-oxPTH/tPTH and several other parameters. These results are depicted in table 13. The data show that Δ in serum phosphate was significantly and inversely correlated with Δn-oxPTH, but interestingly not with ΔtPTH (rs = −0.418; p = 0.002; Bonferroni adjusted p = 0.020 and rs = −0.314; p = 0.022; Bonferroni adjusted p = 0.264, respectively). ΔHDL was also significantly associated with Δn-oxPTH, but not ΔtPTH (rs = 0.499; p < 0.001; Bonferroni adjusted p = 0.002 and rs = 0.079; p = 0.576; Bonferroni adjusted p = 1.0, respectively). Interestingly, we found no correlations of the Δn-oxPTH/tPTH ratio with any of the tested parameters.

Table 13: Correlations for changes in n-oxPTH, PTH, n-oxPTH/tPTH ratio and changes in several parameters for the vitamin D group.

Parameters	Δ n-oxPTH			Δ Total PTH			Δ n-oxPTH/tPTH ratio		
	Spearman's ρ	p-value	Bonferroni adjusted p-value	Spearman's ρ	p-value	Bonferroni adjusted p-value	Spearman's ρ	p-value	Bonferroni adjusted p-value
Δ n-oxPTH	n.a.	n.a.	n.a.	0.448	0.001	0.010	0.241	0.082	0.988
Δ 25(OH)D_3	0.008	0.958	1.000	0.043	0.764	1.000	−0.016	0.910	1.000
Δ Calcium	−0.335	0.014	0.155	−0.312	0.023	0.275	0.051	0.718	1.000
Δ Phosphate	−0.418	0.002	0.020	−0.314	0.022	0.264	0.032	0.821	1.000
Δ BAP	0.117	0.417	1.000	0.010	0.944	1.000	0.076	0.599	1.000
Δ FGF23	−0.263	0.057	0.631	−0.141	0.315	1.000	−0.030	0.828	1.000
Δ ADMA	0.095	0.499	1.000	−0.016	0.908	1.000	−0.057	0.685	1.000
Δ LDL cholesterol	0.150	0.290	1.000	−0.039	0.782	1.000	0.088	0.533	1.000
Δ HDL cholesterol	0.499	<0.001	0.002	0.079	0.576	1.000	0.216	0.120	1.000
Δ Total cholesterol	0.194	0.165	1.000	−0.124	0.377	1.000	0.197	0.158	1.000

∆ Total PTH – calculated change from baseline in the vitamin D treated group for total parathyroid hormone; ∆ n-oxPTH – calculated change from baseline in the vitamin D treated group for non-oxidized parathyroid homrmone; ∆ n-oxPTH/tPTH ratio – calculated change from baseline in the vitamin D treated group for the n-oxPTH/tPTH ratio; ∆ 25(OH)D_3 – calculated change from baseline in the vitamin D treated group for 25(OH)-hydroxyvitamin D_3; ∆ Calcium – calculated change from baseline in the vitamin D treated group for calcium; ∆ Phosphate – calculated change from baseline in the vitamin D treated group for phosphate; ∆ BAP – calculated change from baseline in the vitamin D treated group for bone alkaline phosphatase; ∆ FGF23 – calculated change from baseline in the vitamin D treated group for fibroblast growth factor 23; ∆ ADMA – calculated change from baseline in the vitamin D treated group for assymetric dimetylarginine; ∆ LDL cholesterol – calculated change from baseline in the vitamin D treated group for low-density lipoprotein cholesterol; ∆ HDL cholesterol – calculated change from baseline in the vitamin D treated group for high-density lipoprotein cholesterol; ∆ Total cholesterol – calculated change from baseline in the vitamin D treated group for total cholesterol. Adapted from (Ursem et al., 2019).

4 Discussion

The discussion is divided into 3 parts. In part 1 the results involving the measurements of baseline 24,25$(OH)_2D_3$ as well as VMR, and their hypothesized advantageousness over baseline 25(OH)D measurements, when used for predicting vitamin D-related metabolite levels after vitamin D supplementation, are discussed. This is followed by the discussion of the effects of a vitamin D intervention as well as seasonality on sST2 in part 2 and finally, we discuss the effects of a vitamin D intervention on n-oxPTH in comparison to tPTH.

4.1 Part 1

Several recent studies (Wagner et al., 2011; Cashman et al., 2015) were pointing towards a possibility of using 24,25$(OH)_2D_3$ measurements and especially the calculated VMR to provide additional information for predicting responses to different vitamin D treatment protocols. Our observations in this vitamin D RCT in vitamin D insufficient patients with hypertension do not offer additional support for this hypothesis.

In contrast to these studies, there were no significant associations of Δ25$(OH)D_3$ with any of the included pre-vitamin-D-treatment parameters, including 24,25$(OH)_2D_3$ and VMR. Our observations are in line with the study by Binkley *et al*. (Binkley et al., 2017) where VMR also didn't offer the possibility to predict the increase in 25(OH)D after four months of vitamin D supplementation. Nonetheless, we detected trends for associations of Δ25$(OH)D_3$ with baseline 24,25$(OH)D_3$, 25$(OH)D_3$, bioavailable 25$(OH)D_3$ and free 25$(OH)D_3$. Even though the correlations are only borderline significant, the correlation strengths are particularly similar among these parameters. This therefore further demonstrates that, there is negligible additional value to use parameters other than baseline 25(OH)D levels to predict increases after vitamin D supplementation. The strengths and directions of the association coefficients in our study are similar to the values reported by Cashman et al (Cashman et al., 2015) and Wagner et al. (Wagner et al., 2011). However, the study by Binkley et al. (Binkley et al., 2017) reports a correlation

coefficient of -0.232. Although, the differences might stem from the design of their study when compared to ours. For example, the cohort consisted only of postmenopausal women, the cohort size was smaller, the dose of vitamin D treatment was smaller at 1800 IU and the duration was longer. It is important to point out that the association coefficients between Δ25(OH)D_3 and 25(OH)D_3 were negative in the present study and all aforementioned studies (Wagner et al., 2011; Binkley et al., 2017; Cashman et al., 2015). This suggests that the rise (change) in 25(OH)D_3 after the vitamin D intervention is smaller in individuals that have higher baseline 25(OH)D_3 levels.

We also studied the changes in other vitamin D related parameters after vitamin D treatment. Therefore, we determined if changes from baseline levels could be predicted for 1,25$(OH)_2$D and 24,25$(OH)_2D_3$ by using baseline parameters included in the study. There were no associations of the change from baseline in 1,25$(OH)_2$D with any included baseline parameter. This observation could be explained by the fact that 1,25$(OH)_2$D levels are mainly regulated by calcium levels (Mazahery and von Hurst, 2015). In contrast, the change form baseline in 24,25$(OH)_2D_3$ showed a significant association with baseline 25(OH)D_3, calculated bioavailable 25(OH)D_3, calculated free 25(OH)D_3 and. Also, in this case the significant correlation coefficients are negative. This suggests that the change in 24,25$(OH)_2D_3$ from baseline after vitamin D treatment is smaller when baseline 25(OH)D_3 and 24,25$(OH)_2D_3$ are higher. Based on these results, one may speculate that the catabolism of 25(OH)D rises more slowly at higher concentrations 25(OH)D. This might be a consequence of enzyme saturation.

Bioavailable and free 25(OH)D concentrations were previously shown to be independent determinants of bone mineral density (Li et al., 2017) and levels of bioavailable 25(OH)D_3 were associated with the risk of mortality in coronary artery disease patients (Yu et al., 2018), whereas total 25(OH)D, in both of these studies, was not. Furthermore, both metabolites were proposed to provide more accurate information on the vitamin D function than 25(OH)D measurements (Chun et al., 2014). Based on our data, their usefulness in providing more accurate predictions of the response after vitamin D treatment seems to be limited.

$1,25(OH)_2D$ is formed by converting 25(OH)D by CYP27B1 (1-alpha-hydroxylase). Therefore, the ratio between the two metabolites has been proposed to reflect CYP27B1 activity (Pasquali et al., 2015). As described above, similarly the ratio between $24,25(OH)_2D$ and 25(OH)D, i.e. the VMR reflects CYP24A1 activity (24-hydroxylase). Furthermore, the ratio between the metabolites $1,25(OH)_2D$ and $24,25(OH)_2D_3$ was proposed as part of a 3D model for analysing vitamin D metabolic pathways (Tang et al., 2019). Therefore we determined whether these ratios could be useful for predicting responses to vitamin D treatment. However, on the contrary, we found that neither of the ratios at baseline was significantly correlated to any of the changes from baseline in vitamin D related parameters. From this we deduce, that the ratios between vitamin D metabolites do not provide added value in this regard, at least in the cohort we studied.

An increase in VMR was revealed by the ANCOVA analysis. This calculation reflects the CYP24A1 activity and therefore points toward an increase in 25(OH)D catabolism. The decrease in the ratio between $1,25(OH)_2D$ and $25(OH)D_3$ suggests that the conversion of 25(OH)D to $1,25(OH)_2D$ was reduced. Therefore, this implies the physiological metabolic shift from anabolic to catabolic pathways in the presence of excessive vitamin D. This notion is further supported by a decrease in the ratio between $1,25(OH)_2D$ and $24,25(OH)_2D_3$.

This study has several limitations. First, we derived the results from post hoc analyses of study 1. Second, because we used a relatively specific study population demographic, namely vitamin D insufficient hypertensives, our findings might not be readily extrapolated to the general population. Furthermore, in study 1, the 25(OH)D concentrations for the inclusion criterion of vitamin D insufficiency were measured using a chemiluminescence assay. At present mass spectrometry-based methods are the gold standard (Tuckey, Cheng and Slominski, 2019). Nonetheless, we additionally measured 25(OH)D and 24,25(OH)D for the present study by using a dedicated LC-MS/MS method. Also of note, the subjects in study 1 were treated with vitamin D for a relatively short period of 8 weeks. In addition, severe vitamin D deficiency (25(OH)D levels below 10 ng/ml (25 nmol/L) (Kennel, Drake and Hurley, 2010) was detected only in a small number of subjects. Nevertheless, the successful vitamin D intervention and especially the RCT design are clear strengths of this study. Another strength is also the high number of

parameters measured with gold-standard methods. It is also important to point out that we, in contrast to the majority of previous exploratory studies on the topic of $24,25(OH)_2D_3$ and VMR, adjusted the p-values of associations with the Bonferroni method to reduce type I errors.

4.2 Part 2

A number of studies (Gruson et al., 2016; Mildner et al., 2010; Bartunek et al., 2008; Pfeffer et al., 2015; Zehnder et al., 1999; Razzaque, 2011; Kongsbak et al., 2013) were implying that sST2 levels might be regulated by vitamin D. Despite this solid background and an increase of both 25(OH)D as well as $1,25(OH)_2D$ levels and a decrease in PTH levels after the vitamin D intervention, we could not observe any effects on serum concentrations of sST2 in sub-study 2. One of the possibilities why we observed no effects of vitamin D might be the fact that the sST2 concentrations of study subjects in sub-study 2 were relatively low and that they in general also didn't suffer from severe forms of CVD. To illustrate, the effects of the vitamin D intervention might have been more distinct in subjects with elevated levels of sST2, such as in patients with myocardial infarction or HF (Shimpo et al., 2004; Weinberg et al., 2003). The change in sST2 secretion might have also been more pronounced locally in vitamin D target cell surrounding, as demonstrated in experiments with cultured primary human cells (Pfeffer et al., 2015), but we weren't able to detect them as changes in the systemic levels. Furthermore, the subjects in sub-study 2 were not severely vitamin D deficient but vitamin D insufficient, which could also have influenced the results observed. Taken together, we believe that the most plausible explanation is that sST2 secretion is unaffected by vitamin D treatment. This proposition would be in line with recent meta-analyses of vitamin D RCTs, which showed limited or no benefits on vitamin D interventions on CVD mortality, events and diverse surrogate cardiovascular parameters (Zittermann, 2018).

Our second aim in part 2 was to determine if sST2 levels adhere to a seasonal pattern that might be related to that of 25(OH)D. Therefore, if sST2 levels would be affected by changes in vitamin D concentrations, we would have expected to see a specific pattern of sST2 throughout the year, similar to the 25(OH)D

seasonal variability. It was also possible that sST2 would follow its own distinct yearly pattern, as was seen in the case of the NT-proBNP, a frequently used cardiovascular biomarker. Namely, the levels NT-proBNP were shown to exhibit periodical elevations in summer months in primary care patients, even though its seasonal pattern didn't parallel that of total cholesterol, hypertension, BMI or vitamin D (Khezri et al., 2017). Opposed to our hypotheses, we observed no changes in sST2 levels throughout the year in study 2, even though the concentrations of 25(OH)D exhibited the expected seasonal patterns in both genders and all subgroups (Klenk et al., 2013; Klingberg et al., 2015; Kasahara, Singh and Noymer, 2013). Despite finding significant differences in men with HF after using a statistical tests with correction for several parameters also including age and eGFR, we regard these differences as biologically irrelevant (for instance, the largest difference in adjusted log-transformed sST2 concentration means was between the months March and July, amounting to only 0.132 log(U/mL)). We mainly observed results of a similar nature when we sub-grouped the subjects of study 2 as specified by the meteorological definition of the seasons (Figure 2) and when performing a graph shape comparison (Figure 3). Because a separate cohort was used to obtain these results than those in the case of the vitamin D intervention experiments, the findings further underline that vitamin D itself seems to have limited or no noticeable impact on systemic concentrations of sST2. Furthermore, our findings support the assumption that sST2 levels probably don't change as a consequence of external influences originating from the environment, for example differences in ambient temperature proposed to be involved in the seasonality of CVD (Stewart et al., 2017).

We also found that the month, when the concentrations of 25(OH)D started to rise, differed between the genders and also seemed to have been influenced by the HF status of the subgroups. This rise was detected in June in men and in May in women without HF. An explanation for this discrepancy might be the potential different outdoor activity levels throughout the year between men and women in the study 2 population. The 25(OH)D levels started to rise in June in both genders in the subgroups with HF. This could point toward a similar degree of impact of HF on the subjects' outdoor activity levels. The higher sun exposure grades might have also contributed to higher median 25(OH)D levels we observed in men when

not stratifying for HF. Gender differences in 25(OH)D were also observed previously in patients undergoing coronary angiography, with a conclusion that this contrast between the genders might play an important role in the severity of the observed CAD (Verdoia et al., 2015).

We also evaluated the associations of sST2 with vitamin D-related parameters and found significant associations with 25(OH)D but only in men in study 2 and not in women nor any subgroup of sub-study 2. Why we didn't observe any associations with vitamin D related parameters in sub-study 2 might have been due to a lack of cardiovascular disease complications as severe as in study 2 and in a previously published study by Gruson et al. For this study they recruited a cohort of HF patients and found similar associations to ours in study 2. They found correlations not only with 25(OH)D, but also with PTH and 1,25(OH)2D, although they didn't stratify the cohort according to genders as we did (Gruson et al., 2016). The levels of 1,25(OH)2D were also substantially lower in the aforementioned study (24.0 pg/mL; 57.6 pmol/L) (Gruson et al., 2016), when compared to our studies in part 2 (115.9 pmol//L (48.3 pg/mL) in sub-study 2 and 84.1 pmol/L (33.7 pg/mL) in study 2), which is in line with previous observations in HF patients (Zittermann and Ernst, 2016). These differences between study cohorts could have at least in part be responsible for the absence of associations with 1,25(OH)2D in sub-study 2 and study 2. The observed dissimilarity of associations we found in sub-study 2 and study 2 might also be due to differences in cohort size between the studies. The difference in cohort sizes between both studies in part 2 might have also resulted in contrasting differences in baseline parameter concentrations between women and men.

The design of part 2 has some limitations, one of them is that the data originate from post-hoc analyses of sub-study 2 and study 2. Since the analyses were performed on vitamin D insufficient hypertensives in sub-study 2 and on patients referred for coronary angiography in study 2, it might not be possible to extrapolate the results to the general population. sST2 is not as established as a prognostic and diagnostic marker in hypertension and in CVD as it is in HF, even though the body of research is increasing. It is important to mention that the measurements of sST2 in sub-study 2 and study 2 were carried out with different methods. Nevertheless, we could observe the expected higher levels of sST2 in men

compared to women in measurements from both procedures (Dieplinger et al., 2009; Miller et al., 2012), thereby verifying the reliability our measurements. Another clear strength of part 2 design was the fact that sub-study 2 was designed as an RCT and that the incorporated vitamin D intervention was successful. Other strong points were the cohort size and duration of study 2 which allowed the data to be collected over the course of several years, as well as reliable parameter measurements in both sub-study 2 and study 2. Finally, because the results originate from two non-related cohorts and imply the same conclusion, the validity of our findings are further highlighted.

4.3 Part 3

In part 3 we set out to determine if a vitamin D intervention had an effect on n-oxPTH levels in an RCT. The study cohort consisted of vitamin D insufficient hypertensives with normal kidney function. We observed a significant decrease in n-oxPTH levels after the intervention supplementation. We also found a relatively weak association between tPTH and n-oxPTH at baseline (0.555; $p < 0.001$), which indicates that large inter-individual differences in the amount of oxidized PTH are present in our cohort.

Previous studies have indicated that N-oxPTH is the biologically active form of PTH and could therefore reflect its hormonal activity more accurately, especially because the oxidation of the hormone prevents its binding to the PTH-1 receptor (Hocher et al., 2013). As expected, after vitamin D intervention tPTH levels decreased and so did n-oxPTH levels. What we found particularly interesting, was the significant increase in the n-oxPTH/tPTH ratio after supplementation. From this result we can deduce that vitamin D intervention increased the non-oxidized fraction of PTH, from which follows that the oxidized proportion must have been reduced. Presently it’s still unclear if this finding has clinical relevance. In addition, the observed change in the n-oxPTH/tPTH ratio is particularly small, therefore we can’t draw vast conclusions. Nonetheless, we could hypothetically deduce from this finding that vitamin D treatment leads to a reduced level in the oxidation of PTH.

When interpreting this ratio. Ursem et al. suggest to apply caution. They have previously published that the aforementioned ratio can't accurately indicate the non-oxidized percentage of tPTH because at present no n-oxPTH standard is available. Of note, the calculated ratio also exhibited only a low level of reproducibility between several immunoassays (Ursem et al., 2018). Because of these facts, we aren't able to determine the exact non-oxidized amount of PTH. Instead, we were able to draw conclusions based on the differences in the non-oxidized proportion of PTH that resulted from the vitamin D intervention.

A number of studies reported that vitamin D has protective effects against oxidative stress. Whether or not vitamin D has direct or indirect effects in oxidative stress prevention remains to be elucidated. Nonetheless, protective effects were proven in vitro as well as studies in rats and in also humans (Uberti et al., 2014; Wiseman, 1993; Sardar, Chakraborty and Chatterjee, 1996; Tarcin et al., 2009). Our data suggest a possible influence of vitamin D supplementation on the oxidation of PTH. Nevertheless, in our study we found no significant treatment effect on a downstream marker of oxidative stress ADMA (Frijhoff et al., 2015; Grübler et al., 2018).

One of the main actions of PTH is the stimulation of bone resorption. This process is activated by PTH binding to the PTH-1 receptor expressed on osteoblasts. Once PTH activates its receptor, osteoblasts in turn increase the expression of RANK and inhibit osteoprotegerin secretion. This process stimulates osteoclast differentiation, but it is important to note that the level of PTH doesn't accurately reflect bone turnover. For example, based on bone histomorphometry measurements, PTH levels don't exhibit strong associations with bone turnover, with increasing PTH levels in CKD patients (Garrett et al., 2013). The mechanism behind this phenomenon might be end organ resistance, but another possibility is also oxidation of the PTH molecule (Vervloet, Brandenburg and CKD-MBD working group of ERA-EDTA, 2017). Interestingly, also the PTH-1 receptor can be oxidized. Ardura et al. studied this using a modified PTH (1-34) synthetic protein, where norleucine was used to replace both methionine residues located at positions 8 and 18 in the protein. With this method they stabilised the two oxidation prone residues. Thereafter, the cells expressing the PTH-1 receptor were exposed to H_2O_2 (hydrogen peroxide; an oxidant), which resulted in a reduced signalling in

all PTH-dependent pathways (Ardura et al., 2017). Based on this study it is plausible to predict that oxidation of both the PTH-1 receptor and the PTH molecule probably contribute to the pool of oxidised PTH which in turn shows limited correlations with bone histomorphometry in individuals with heightened oxidative stress levels, for example in the aforementioned CKD patients.

It would therefore be of interest to measure n-oxPTH levels when assessing bone turnover, especially in the aforementioned oxidation prone individuals. In addition, it may also be relevant to assess it in the case of PTH-based therapies. For example when treating hypoparathyroidism with recombinant PTH (1-84) or when using teriparatide (PTH[1-34]), a recombinant analogue of human 1-34 PTH, as an anabolic drug for treatment of osteoporosis. Endogenous PTH has a half-life of about 4 minutes, whereas teriparatide's is at about 60 minutes, which is favourable from a pharmacological perspective (Satterwhite et al., 2010). Additionally, it seems that teriparatide can be oxidised at least in vitro, as reported Al-Riyami et al. in their abstract with preliminary data (Al-Riyami et al., 2018). Although, the prolonged half-life might also expose the molecule to oxidative stress for longer and this could then translate into differences in inter-individual effectiveness of teriparatide, but this idea is still speculative and remains to be investigated.

PTH leads to a decreased rate in the reabsorption of phosphate, to a decreased rate in the excretion of calcium and to the stimulation of the renal 1-alpha-hydroxylase enzyme. In sub-study 3 the n-oxPTH and tPTH concentrations at baseline were not correlated with systemic phosphate levels. Of note, the change (Δ) in phosphate did not exhibit an association with ΔtPTH, but did exhibit a significant association with Δn-oxPTH (Table 11). The biological activity of n-oxPTH might be an explanation for the lack of a significant correlation between phosphate and tPTH, while a significant relationship between phosphate and n-oxPTH was present.

tPTH and n-oxPTH didn't associate significantly with any of the bone turnover makers included in the study. However, it is important to mention that sub-study 3 didn't have an optimal setting and wasn't the most appropriate model for studying these mechanisms. A study in patients with renal failure would have been a more appropriate setting, therefore further studies into this mechanism are still needed.

Despite growing knowledge about the interrelation between the parameters of lipid metabolism and PTH, the exact interrelation is still not completely understood. In primary and secondary hyperparathyroidism high PTH levels seem to be accompanied by changes in lipid metabolism parameters (Bolland et al., 2010; Valdemarsson, Lindblom and Bergenfelz, 1998). In secondary hyperparathyroidism, these changes could be a result of many interlinking factors, as for example in the case of nephrotic syndrome. In primary hyperparathyroidism, on the other hand, a causalrelationship could also underlie these changes, as improvements in primary hyperparathyroidism patients' lipid profiles are frequently noticed after parathyroidectomy; however, results from studies are still inconclusive (Valdemarsson, Lindblom and Bergenfelz, 1998; Ljunghall et al., 1978). We find the association between n-oxPTH and HDL cholesterol particularly interesting, because HDL cholesterol has been shown to possess anti-oxidative properties (Soran, Schofield and Durrington, 2015). Therefore, higher HDL cholesterol levels, which would mean a better anti-oxidant status, might be an explanation for the positive association with n-oxPTH. Future studies with a focus on oxidized and non-oxidized PTH might provide explanations for the inconsistencies in previous studies on lipid metabolism and PTH.

Sub-study 3 had some limitations. Post-hoc analysis was used in this study, which only offers a relatively small percentage of subjects with severe vitamin D deficiency in the study cohort is a limitation. Another was the rather short duration of 8 weeks of vitamin D supplementation. Additionally, the cohort was very specific because it consisted of hypertensive patients only. The difference in n-oxPTH we observed might be an underestimation because hypertension can also contribute to oxidative stress levels (Montezano and Touyz, 2012), which would increase the fraction of oxidized PTH and in turn lower n-oxPTH levels. Nevertheless, the RCT design, a successful vitamin D intervention and the broad range of parameters included in the study are clear strengths of sub-study 2 study.

5 Conclusion

In this PhD thesis, three different approaches to investigate on the pleiotropy of vitamin D were performed - a study on vitamin D metabolism and the usefulness of $24,25(OH)_2D_3$ and the VMR, as well as the first two studies focusing on determining the effects of vitamin D metabolism on sST2 and n-oxPTH.

In part 1 of the dissertation, we focused on determining if $24,25(OH)_2D_3$ and the VMR are of added value in predicting the levels of vitamin D in response to vitamin D treatment. We were also interested in the effects of vitamin D treatment on $24,25(OH)_2D_3$ levels and the VMR. Based on our results we can conclude that both VMR and $24,25(OH)_2D_3$ increased after vitamin D treatment. **These parameters, however, can't be used to predict the changes in 25(OH)D levels after vitamin D treatment,** at least in vitamin D insufficient hypertensive patients. We also found that baseline 25(OH)D levels can predict $24,25(OH)_2D_3$ levels after vitamin D treatment, this information, however, seems to be of limited use at present, but might still find its use after more studies have been conducted on this topic. The calculated ratios between vitamin D-related metabolites have also been found to be of limited use, since they couldn't predict changes in 25(OH)D. Although, these calculated ratios might still be useful in future studies as part of multidimensional models for more accurate prediction of an individual's vitamin D status. Since our results might be in part cohort specific, further studies are warranted determine if VMR, $24,25(OH)_2D_3$ and/or other vitamin D-related metabolite ratios should be measured routinely in the clinics for more accurate decision making. Additional studies could also shed more light on the usefulness of the studied parameters in clinical decision making, especially in specific maladies which are distinguished by abnormal vitamin D metabolism, for example in chronic kidney disease.

Part 2 was focused on determining the effects of vitamin D supplementation on sST2 levels and detecting possible effects of seasonality on sST2 concentrations. Although a number of studies including primary cell culture, animal and human studies pointed towards the possibility of sST2 being regulated by vitamin D, **we found that systemic sST2 levels weren't affected by vitamin D**

supplementation in a cohort of vitamin D insufficient hypertensive subjects. In a separate cohort of subjects referred to coronary angiography, **we demonstrated that sST2 concentrations didn't change alongside 25(OH)D concentrations and remained mostly constant without significant fluctuations throughout the whole year**. Since both analyses are derived from data from two non-related cohorts, we believe our data provides strong evidence that vitamin D levels are not interrelated with systemic sST2 concentrations. Although our analyses were carried out on cohorts including cardiovascular patients, studies in HF patients are warranted to provide definitive answers, because HF patients have higher levels of sST2 and the changes in response to vitamin D treatment might be more pronounced in these circumstances. Nonetheless, our findings in part 2 provide additional confidence for the usefulness of sST2 not just in research but in the clinical setting as well.

Part 3 was devoted to determining the effects of vitamin D treatment on the levels of n-oxPTH in vitamin D insufficient hypertensives. **We observed a reduction in both tPTH and n-oxPTH after vitamin D supplementation**. From a slight but significant reduction in the proportion of oxidized PTH we deducted that the vitamin D intervention affected the oxidation of PTH. We couldn't determine the underlying mechanisms for the observed changes as the study wasn't designed for such an investigation. Although our observations point in the direction that **measuring n-oxPTH might be a better alternative to tPTH, at least in the case of patients prone to oxidative stress**, further studies should be performed to determine if n-oxPTH is a clinically relevant parameter and to establish if oxidation does indeed play any role in PTH-based therapies.

To summarize, our studies show that

- VMR and 24,25$(OH)_2D_3$ can't be used to predict the changes in 25(OH)D levels after vitamin D treatment.

They also provide the first evidence of a

- lack of interrelation of sST2 with vitamin D and the seasons.

In addition, we showed an

- effect of vitamin D on n-oxPTH levels and thereby probably on oxidative stress levels.

Although vitamin D RCTs have shown mostly negative results in recent years, we found they can still be useful tools for uncovering promising and potentially clinically important findings.

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