RESEARCH

Are soluble ST2 levels influenced by vitamin D and/or the seasons?

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Abstract

Objective: Cardiovascular disease manifestation and several associated surrogate markers, such as vitamin D, have shown substantial seasonal variation. A promising cardiovascular biomarker, soluble ST2 (sST2), has not been investigated in this regard – we therefore determined if systemic levels of sST2 are affected by seasonality and/or vitamin D in order to investigate their clinical interrelation and usability.

Design: sST2 levels were measured in two cohorts involving hypertensive patients at cardiovascular risk, the Styrian Vitamin D Hypertension Trial (study A; RCT design, 8 weeks 2800 IU cholecalciferol daily) and the Ludwigshafen Risk and Cardiovascular Health Study (LURIC; study B; cross-sectional design).

Methods: The effects of a vitamin D intervention on sST2 levels were determined in study A using ANCOVA, while seasonality of sST2 levels was determined in study B using ANOVA.

Results: The concentrations of sST2 remained unchanged by a vitamin D intervention in study A, with a mean treatment effect (95% confidence interval) of 0.1 (−0.6 to 0.8) ng/mL; $P = 0.761$), despite a rise in 25(OH)D (11.3 (9.2–13.5) ng/mL; $P < 0.001$) compared to placebo. In study B, seasonal variations were present in 25(OH)D levels in men and women with or without heart failure ($P < 0.001$ for all subgroups), while sST2 levels remained unaffected by the seasons in all subgroups.

Conclusions: Our study provides the first evidence that systemic sST2 levels are not interrelated with vitamin D levels or influenced by the seasons in subjects at cardiovascular risk.

Key Words

▶ soluble ST2
▶ seasonality
▶ vitamin D
▶ biomarker
▶ cardiovascular disease

Introduction

A broad range of cardiovascular disease (CVD) has been observed to be influenced by seasonal variation. Various parameters, ranging from temperature changes to vitamin D deficiency, have been shown to play a potential role in this complex phenomenon (1). Indeed, according to population-based and epidemiological studies, cardiovascular events are more common in winter, which coincides not only with lower temperatures and with the

peak of several cardiovascular risk factors as well as their biomarker surrogates but also the seasonal prevalence of vitamin D deficiency (1, 2, 3).

Soluble ST2 (sST2) is the truncated, soluble form of the ST2 receptor found in the circulation and functions as a decoy receptor, binding IL-33 and thereby inhibiting the effects of IL-33/ST2 signalling (4). This protein has been shown to be a promising prognostic cardiovascular biomarker (5). Elevated levels of sST2 have been associated with poor prognosis in myocardial infarction (6) and heart failure (HF) (7), with the risk of acute aortic dissection (8) as well as with subclinical brain injury and incident stroke (9).

Hypertension is one of the strongest risk factors for almost all cardiovascular diseases, including HF (10). Furthermore, coronary artery disease is known as a major risk factor for developing HF and its progression (11). sST2 has been most extensively researched in relation to HF, but several studies have also shown potential for sST2 being a prognostic or diagnostic biomarker for disease deterioration in hypertensives or other patients at cardiovascular risk (8, 12, 13, 14, 15).

Recently, a study in HF patients identified possible negative associations of serum sST2 with 25-hydroxy-vitamin D (25(OH)D) and 1,25-dihydroxyvitamin D $(1,25(OH)_2D)$ serum levels and positive associations with parathyroid hormone (PTH). It also showed that 25(OH)D, $1,25(OH)_2D$ and NT-proBNP were independent determinants of sST2 in a multiple regression analysis (16). Moreover, studies on primary human cells have shown that sST2 is predominantly secreted by lung epithelial cells and cardiac myocytes (17), while several other cell types, such as venous and arterial endothelial cells (18), as well as CD4 and CD8 lymphocytes (19), produce measurable amounts of the protein. Interestingly, all of these cells express the $1,25(OH)_2D$-producing enzyme CYP27B1, as well as the vitamin D receptor (20, 21, 22). Furthermore, a study on primary human lung epithelial cells and CD4 lymphocytes showed an increase in secretion of sST2 when treated with $1,25(OH)_2D$, while the lung epithelial cells also responded to treatment with 25(OH)D with an increase in sST2 secretion (19).

It is currently unknown if vitamin D and/or seasonality affect the systemic levels of sST2 and therefore influence its usefulness as a biomarker or its clinical relevance. We hypothesized that vitamin D supplementation might alter the serum concentrations of sST2 and investigated a vitamin D intervention in hypertensive patients enrolled in the randomized, placebo-controlled Styrian Vitamin D Hypertension Trial. In addition, we investigated sST2 concentrations measured cross-sectionally in hypertensive patients of the LURIC study for their potential changes due to the common seasonal variation of the measured 25(OH)D levels.

Materials and methods

This investigation was carried out in two cohorts involving hypertensive patients at cardiovascular risk, the Styrian Vitamin D Hypertension Trial and the Ludwigshafen Risk and Cardiovascular Health Study (LURIC). Both cohorts are described separately in the following section.

Styrian vitamin D hypertension trial (study A)

Study design

The first part of the present investigation was a *post hoc* analysis of the 'Styrian Vitamin D Hypertension Trial' (23). This intervention trial was designed as a double-blind, placebo-controlled (1:1), single-centre study and took place at the outpatient clinic of the Division of Endocrinology and Diabetology, Medical University of Graz, Austria. The trial was registered at www. clinicaltrialsregister.eu (EudraCT number 2009-018125-70) and at clinicaltrials.gov (ClinicalTrials.gov identifier NCT02136771). The Ethics Committee of the Medical University of Graz, Austria, approved the study protocol and all participants gave written informed consent.

Study population

Participants were eligible to participate in the study at an age of 18 years or older with diagnosed arterial hypertension and a 25(OH)D baseline serum concentration below 30 ng/mL. The main exclusion criteria were published previously (23). The sample size of at least 86 study participants per group was determined assuming a standardized effect size of 0.5, for a two-sided alternative hypothesis with an α of 0.05 and a power of 90%. Further details can be found in the publication of the original study (23). Recruitment took place from June 2011 to August 2014 and 200 Caucasian study participants (106 men and 94 women) were included. The present investigation presents 185 study participants, where sST2 measurements were available before and after vitamin D or placebo treatment.

Intervention

Study participants included in the study were randomly allocated to receive 2800 IU cholecalciferol in the form of seven oily drops per day (Oleovit D3 drops, Fersenius Kabi Austria, Graz, Austria) or a matching placebo for 8 weeks. Hundred participants were allocated to the control group and 100 to the intervention group.

Outcome measure

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

Measurements

Blood samples were taken between 07:00 and 11:00 h in the morning after an overnight fast at both study visits. The samples were either measured by routine on-site laboratory procedures or stored at −80°C until analysis. Measurements of serum 25(OH)D were performed 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 with an intra-assay and inter-assay coefficient of variation (CV) of 6.2 and 11.6%, respectively. sST2 was measured in serum with the Human ST2/IL-33 R Quantikine ELISA Kit (R&D Systems). The intra-assay CVs were 4.5–5.6% and inter-assay CVs were 6.3–7.1%, while the lower limit of detection was 5.1 pg/mL according to the manufacturer. PTH was measured using a sandwich ElectroChemiLuminescence Immunoassay (ECLIA) on an Elecsys 2010 immunoassay analyser (Roche Diagnostics), with inter-assay CVs 5.7–6.3%. Measurements of other parameters have been described previously (23).

LURIC study (study B)

Study design

The second part of the present investigation was carried out as *post hoc* analysis of the LURIC study, 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 and in addition, to evaluate the predictive value of potential markers on long-term outcomes. The objectives of the study, recruitment procedures and characteristics have been described in more detail previously (24). The study protocol was approved by the Ethics Committee at the Landesärztekammer Rheinland-Pfalz (Mainz, Germany) in accordance with the Declaration of Helsinki, and written informed consent was obtained from each participant.

Study population

No fixed sample size estimates were made for the LURIC study design at the time of conception because of its exploratory nature. Nonetheless, a sample size of >3000 was determined based on a power calculation with an α of 0.05 and a power of 80% as well as 90% and, in addition, because it was anticipated that also subsets of the entire population will be investigated. Please refer to (24) for further details. Overall, 3316 patients were enrolled in the LURIC study. Of these, 1403 hypertensive patients with available sST2, 25OHD, 1,25(OH)$_2$D and PTH levels as well as NYHA and HF classification were included in the present analysis. The baseline examination protocols were described in detail previously (24). Arterial hypertension, defined as mean systolic and diastolic BP out of five measurements >140/90 mmHg, was determined by brachial artery pressure value measurements with an automated oscillometric device (Omron MX4, Omron Health Care GmbH, Hamburg, Germany) after the patient had rested in the supine position for at least 10 min. In addition, at least three consecutive systolic and diastolic BP measurements were taken with a minimum interval of 30 s. Only measurements conforming to the reproducibility criteria were entered into the database.

Outcome measure

The main aim of the present *post hoc* analysis was to determine if there is an effect of seasonality on the levels of sST2, while also determining possible associations of vitamin D-related parameters with sST2.

Measurements

Venous blood samples were drawn after an overnight fast (between 08:00 and 10:00 h) before coronary angiography. Routine laboratory parameters were immediately measured on a daily basis as previously described (24). The remaining blood samples were centrifuged at 3000 g for 10 min, aliquoted, frozen and stored at 80°C until further analysis within 30 min after venepuncture. sST2 was determined in EDTA plasma samples in one batch for all patient samples approximately 12 years after the recruitment period of the LURIC study was closed. sST2 was measured on a fully automated BEP 2000 instrument

(Siemens Healthcare Diagnostics) with the Presage ST2 sandwich immunoassay assay (Critical Diagnostics) with CVs of 4.0%, as previously published (15). 25(OH)D levels were assayed weekly in serum samples using a commercial radioimmunoassay (DiaSorin SA) with intra-assay and inter-assay coefficients of variation of 8.6 and 9.2%, respectively. Further, 25(OH)D levels were previously determined in 100 randomly selected samples by liquid chromatography tandem mass spectrometry with isotopic labelled internal standard, which showed a highly significant correlation between the 25(OH)D levels obtained by RIA and liquid chromatography tandem mass spectrometry ($r=0.875$; $P<0.001$). PTH was measured using a sandwich ElectroChemiLuminescence Immunoassay (ECLIA) on an Elecsys 2010 immunoassay analyser (Roche Diagnostics), with intra- and inter-assay CVs 1.5–2.7% and 3.0–6.5%, respectively (25). $1,25(OH)_2D$ levels were analysed in serum samples by RIA (Nichols Institute Diagnostika GmbH, Bad Nauheim, Germany) on a Berthold LB2014 multicrystal counter. A high-sensitivity ELISA assay (R&D Systems Inc.) was used to measure the plasma levels of interleukin-6 (IL-6).

Data analyses

Continuous data following a normal distribution are reported as means with standard deviations (s.d.), while variables with a skewed distribution are shown as medians with interquartile ranges and categorical variables are shown as percentages of observations.

Groups at baseline were compared using unpaired Students *t*-tests, Mann–Whitney *U* tests or chi-squared tests. Skewed variables were log transformed when appropriate before being used in parametrical statistical analysis. Spearman's correlation analyses were used to test for associations of parameters with sST2. Bonferroni correction was used to account for multiple comparisons.

Analysis of covariance (ANCOVA) with adjustments for baseline values was used to test for differences in the outcome variable (sST2) and 25OHD between the placebo and the treatment group at the follow-up visit.

ANOVA followed by the Bonferroni *post hoc* test was used to determine seasonal variability and to calculate which monthly mean values of 25(OH)D and sST2 were significantly different to the peak monthly mean. When the assumption of homogeneity of variances was not met, Welch ANOVA followed by the Games–Howell *post hoc* test was used instead. ANCOVA was used for group comparisons with adjustment for age, eGFR, BMI, IL-6 levels, T2DM status, smoking status, any previous

myocardial infarctions, any previous strokes, transient ischaemic attacks and/or prolonged reversible ischaemic neurologic deficits.

If outliers were detected in the analyses by the software (for ANCOVA defined as cases with standardized residuals greater than ±3 s.d. and for ANOVA defined as cases with values higher or lower than 1.5*IQR (interquartile range)), they were removed and the analysis repeated to determine their potential effect on the analysis. If the outlier had no significant effect on the analysis, the results including the outlier are reported.

All variables from both studies were analysed without data imputation, for example in the Styrian Vitamin D Hypertension Trial, where only participants with both baseline and follow-up values of the respective outcome variable were included in the analyses.

A *P* value of <0.05 was considered statistically significant in all analyses. All statistical analyses were performed using SPSS version 22 software (SPSS).

Results

Firstly, we set out to assess if vitamin D treatment affects sST2 levels. The baseline characteristics of the study A cohort can be found in Table 1 (top; study A). We found significant differences in sST2, serum calcium and serum phosphate between men and women at baseline. At baseline, there were no differences between the placebo and treatment groups between the parameters included in the study (data not shown). We found no significant correlations of baseline sST2 with 25(OH)D, $1,25(OH)_2D$ or PTH neither in men ($r_s=-0.033$, $P=0.746$; $r_s=-0.124$, $P=0.229$; $r_s=0.062$, $P=0.543$; respectively) nor women ($r_s=-0.191$, $P=0.077$; $r_s=-0.187$, $P=0.084$; $r_s=-0.186$, $P=0.084$; respectively) in the whole cohort and also not in a sub-cohort of study participants with baseline 25(OH)D levels below 20 ng/mL (or <15 ng/mL; data not shown) (Table 2; study A). sST2 correlated significantly also with eGFR in both men and women ($r_s=-0.222$, $P=0.028$ and $r_s=-0.230$, $P=0.032$, respectively).

Vitamin D supplementation induced a rise in 25(OH)D with a mean treatment effect (95% confidence interval (CI)) of 11.3 (9.2–13.5) ng/mL ($P<0.001$), a rise in $1,25(OH)_2D$ (9.5 (4.9–14.0) pg/mL; $P<0.001$) and a decrease in PTH (−5.9 (−9.4 to −2.2) pg/mL; $P=0.002$) as compared to placebo. The concentrations of sST2 remained unchanged in the whole cohort (0.1 (−0.6 to 0.8) ng/mL; $P=761$) and also in a sub-cohort of study participants with baseline 25(OH)D levels below

Table 1 Baseline characteristics in study A (top) and general characteristics in study B (bottom) according to gender.

Study A	All (*n* = 185)	Men (*n* = 98)	Women (*n* = 87)	*P* value
Age (years)	62.4 (52.9–68.1)	60.9 (52.9–68.1)	63.7 (55.5–68.8)	0.177
BMI (kg/m^2)	29.7 (27.1–32.8)	29.9 (27.0–32.6)	29.3 (27.1–33.2)	0.568
Gender (% female)	47	/	/	/
sST2 (ng/mL)	13.3 (10.0–17.5)	16.2 (11.6–21.2)	11.6 (9.0–15.3)	<0.001
25(OH)D (ng/mL)	21.9 (17.1–25.7)	21.4 (15.2–25.9)	22.7 (17.6–25.6)	0.412
PTH (pg/mL)	49.1 (39.6–62.9)	48.7 (37.9–60.8)	51.4 (41.6–64.0)	0.181
1,25(OH)$_2$D (pg/mL)	48.3 (37.4–63.6)	50.9 (39.2–64.6)	44.6 (33.2–63.0)	0.053
Serum calcium (mmol/L)	2.3 (2.2–2.3)	2.3 (2.2–2.3)	2.3 (2.2–2.4)	0.033
Serum phosphate (mmol/L)	2.9 ± 0.5	2.7 ± 0.5	3.1 ± 0.5	<0.001
eGFR (mL/min/1.73 m^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 B	**All (*n* = 1403)**	**Men (*n* = 994)**	**Women (*n* = 409)**	***P* value**
Age (years)	65.6 (58.6–71.8)	64.9 (57.8–71.0)	67.7 (61.6–73.3)	<0.001
BMI (kg/m^2)	27.3 (25.0–30.1)	27.4 (25.3–30.1)	26.9 (24.4–30.2)	0.008
Gender (% female)	35	/	/	/
sST2 (U/mL)	19.2 (15.4–24.6)	19.8 (16.0–25.4)	17.3 (14.4–22.6)	<0.001
25(OH)D (ng/mL)	15.5 (10.0–22.9)	16.6 (11.3–23.5)	12.6 (8.1–20.6)	<0.001
PTH (pg/mL)	30.0 (22.0–41.0)	29.0 (22.0–39.0)	32.0 (24.0–44.0)	0.001
1,25(OH)$_2$D (ng/L)	33.7 (25.3–42.6)	34.6 (26.3–43.6)	21.1 (22.8–40.3)	<0.001
Serum calcium (mmol/L)	2.3 (2.3–2.4)	2.3 (2.3–2.4)	2.3 (2.3–2.4)	0.683
Serum phosphate (mg/dL)	3.5 (3.1–3.9)	3.4 (3.1–3.7)	3.7 (3.3–4.1)	<0.001
eGFR (mL/min/1.73 m^2)	83.6 (69.2–97.1)	83.6 (69.2–97.1)	83.6 (68.5–98.6)	0.824
IL-6 (ng/L)	3.3 (1.9–6.1)	3.4 (1.9–6.3)	3.3 (2.0–5.7)	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 (1.9–6.1)	3.4 (1.4–9.1)	3.9 (1.6–9.1)	0.160

1,25(OH)D, 1,25-dihydroxyvitamin D; BMI, body mass index; eGFR, estimated glomerular filtration rate; 25(OH)D, 25-hydroxyvitamin D; PRIND, prolonged reversible ischaemic neurologic deficit; PTH, parathyroid hormone; sST2, soluble ST2; TIA, transient ischaemic attack.

Table 2 Baseline Spearman's correlations of the parameters from study A and study B with sST2 in all subjects included in the study as well as in subjects with 25(OH)D below 20 ng/mL.

	sST2 (in all included subjects)				sST2 (in subjects with 25(OH)D below 20 ng/mL)			
	Men (*N* = 96)		Women (*N* = 87)		Men (*N* = 37)		Women (*N* = 32)	
Study A	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)$_2$D	−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
	Men (*N* = 994)		**Women (*N* = 409)**		**Men (*N* = 633)**		**Women (*N* = 301)**	
Study B	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	−0.143*	<0.001	−0.011	0.851
1,25(OH)$_2$D	−0.031	0.323	−0.026	0.603	−0.092	0.021	−0.027	0.635
PTH	0.084	0.008	−0.008	0.879	0.092	0.021	−0.020	0.735

*Correlation coefficients significant also after Bonferroni correction.
1,25(OH)D, 1,25-dihydroxyvitamin D; 25(OH)D, 25-hydroxyvitamin D; PTH, parathyroid hormone; sST2, soluble ST2.

Table 3 ANCOVA analysis for the effect of vitamin D or placebo treatment on parameters from study A.

Parameter	Group	Baseline	Follow-up	Treatment effect (95% confidence interval)	*P* value
25(OH)D	Vitamin D	23.0 (19.0–26.3)	35.4 (16.3–28.5)	11.3 (9.2–13.5)	<0.001
	Placebo	21.2 (15.2–25.4)	23.0 (30.4–42.1)		
1,25(OH)$_2$D*	Vitamin D	48.7 (38.6–63.6)	58.8 (46.2–69.3)	9.5 (4.9–14.0)	<0.001
	Placebo	46.4 (35.4–63.5)	48.6 (35.4–59.6)		
PTH*	Vitamin D	49.0 (39.6–61.5)	45.5 (37.8–54.3)	−5.9 (−9.4 to −2.2)	0.002
	Placebo	51.4 (39.2–63.9)	50.6 (38.6–66.1)		
sST2*	Vitamin D	13.3 (10.4–17.3)	13.8 (10.6–17.1)	0.1 (−0.6 to 0.8)	0.761
	Placebo	13.5 (9.4–18.3)	12.8 (9.2–17.4)		
sST2 in subjects with baseline 25(OH)D below 20 ng/mL*	Vitamin D	15.5 (9.1–18.3)	14.7 (9.9–19.4)	0.4 (−1.3 to 2.1)	0.639
	Placebo	15.9 (10.1–18.7)	13.7 (10.4–18.0)		
sST2 in men*	Vitamin D	16.5 (13.1–19.2)	15.1 (12.9–20.2)	0.3 (−1.3 to 1.9)	0.753
	Placebo	16.0 (10.7–23.0)	15.4 (10.4–22.4)		
sST2 in women*	Vitamin D	12.3 (9.1–13.7)	11.6 (8.9–13.8)	−0.02 (−1.2 to 1.2)	0.977
	Placebo	11.4 (8.8–16.2)	11.9 (9.0–13.7)		

*Analyses where outliers were present but did not significantly affect the results.
1,25(OH)D, 1,25-dihydroxyvitamin D; 25(OH)D, 25-hydroxyvitamin D; PTH, parathyroid hormone; sST2, soluble ST2.

20 ng/mL (0.4 (−2.1 to 2.1) ng/mL; *P*=0.639) (Table 3) or <15 ng/mL (data not shown).

Since the levels of sST2 differed between genders, we also tested if vitamin D had different effects on sST2 concentrations in men and women. There were no significant treatment effects on sST2 levels neither in men (0.3 (−1.3 to 1.9) ng/mL; *P*=0.753) nor in women (−0.02 (−1.2 to 1.2) ng/mL; *P*=0.977) (Table 3).

Secondly, we wanted to determine if seasonal variation is present in sST2 levels. General characteristics of the study B cohort can be found in Table 1 (bottom; study B) and parameter values of subjects subgrouped according to gender as well as the meteorological definition of the seasons can be found in the supplement (Supplementary Table 4, see section on supplementary data given at the end of this article). We found significant differences in age, BMI, sST2, 25(OH)D, PTH, 1,25(OH)$_2$D and serum phosphate.

We found significant correlations of sST2 levels with 25(OH)D (r_s=−0.106, *P*<0.001; significant after Bonferroni correction) in men, but not with 1,25(OH)$_2$D and PTH; r_s=−0.031, *P*=0.323; r_s=0.084, *P*=0.008; respectively, both not significant after Bonferroni correction), while no significant associations were found in women (r_s=−0.055, *P*=0.271; r_s=−0.026, *P*=0.603; r_s=−0.008, *P*=0.879; for 25(OH)D, 1,25(OH)$_2$D and PTH, respectively) within the whole cohort (Table 2; study B). We made a similar observation in the sub-cohort of study participants with baseline 25(OH)D levels below 20 ng/mL (and <15 ng/mL; data not shown). We also detected significant correlations of sST2 with eGFR in both men and women (r_s=−0.194, *P*<0.001 and r_s=−0.194, *P*<0.001, respectively).

Monthly variations were present in 25(OH)D levels in men and in women, with and without HF (*P*<0.001 for all subgroups). There was a significant effect on sST2 in men with HF (*P*=0.44), while sST2 levels remained unaffected by the seasons in men without HF and women with and without HF (*P*=0.895, *P*=0.512 and *P*=0.948, respectively). When adjusted for age, eGFR, BMI, IL-6 levels, T2DM status, smoking status, any previous myocardial infarctions, any previous strokes, transient ischaemic attacks and/or prolonged reversible ischaemic neurologic deficits, the monthly seasonal variances remained significant in all subgroups for 25(OH)D (*P*<0.001 for all groups), while they were not significant for sST2 in any of the subgroups (for men with HF, *P*=0.073, and without HF, *P*=0.803; for women with HF, *P*=0.948, and without HF, *P*=0.512.

Median 25(OH)D and sST2 concentrations with 95% CI in men and women, with and without HF are shown in Fig. 1. The levels of 25(OH)D peaked in August in both men and women with HF (median 23.2 µg/L and 20.8 µg/L respectively), in July in men without HF (median 24.4 µg/L) and in August in women without HF (median 27.0 µg/L). The lowest 25(OH)D values were observed in January in men with HF (median 8.1 µg/L), in February in women with HF (median 6.6 µg/L) and in March in men and women without HF (median 10.9 µg/L and 7.1 µg/L, respectively).

sST2 concentrations were highest in May in men with HF (median 24.7 U/mL), in February in women with HF (median 22.6 U/mL) in January in men without HF (median 20.2 U/mL) and in March in women without HF (median 19.2 U/mL). The lowest levels of sST2 were found in June in men with HF (median 19.4 U/mL), in August
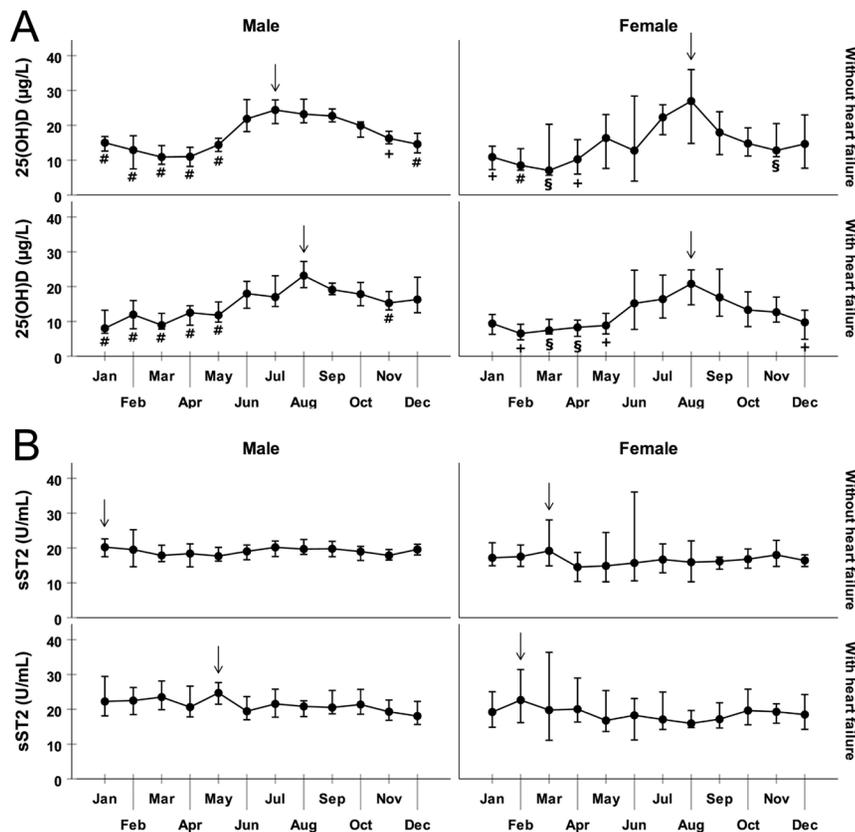
Figure 1

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 (↓); $^{\S}P < 0.05$; $^{+}P < 0.01$; $^{\#}P < 0.001$. The data were log transformed before being used in ANOVA analyses. Outliers were present in all ANOVA analyses but did not significantly affect the results.

in women with HF (median 20.0 U/mL), in May in men without HF (median 17.7 U/mL) and in April in women without HF (median 14.5 U/mL).

Discussion

While several studies (16, 17, 18, 19, 20, 21, 22) were pointing towards the possibility of sST2 levels being regulated by vitamin D, our findings based on two non-related studies do not support an interrelation of sST2 levels with vitamin D levels or an influence of seasons on sST2 levels in subjects at cardiovascular risk. We found no effects of vitamin D intervention on the serum levels of sST2 in study A, even though both 25(OH)D and 1,25(OH)$_2$D levels increased and PTH levels decreased in the treatment group. It is possible that we did not observe any vitamin D effects because the participants of study A had relatively low sST2 levels and in general did not suffer from severe CVD. For example, in patients with HF or myocardial infarction, in which sST2 level elevations have been shown to be higher (6, 7), the effects of vitamin D treatment might have been more pronounced. Also, vitamin D treatment might affect the

sST2-producing cells and change sST2 secretion locally in the target cell surroundings, as shown in experiments with primary human cells in culture (19), but the changes in sST2 secretion were not large enough to be detectable as differences in systemic levels. Additionally, also the fact that the subjects in study A were vitamin D insufficient and not severely vitamin D deficient might have contributed to our observed results. In general, the most plausible explanation could be that vitamin D treatment had no effect on sST2 secretion, which would be in tune with several recent meta-analyses of vitamin D RCT studies showing limited or no benefits on CVD events, mortality and various surrogate cardiovascular parameters (26).

The second aim of our study was to determine if sST2 levels follow a seasonal pattern relatable to that of 25(OH)D. We predicted a distinct pattern of sST2 throughout the year, following that of vitamin D (25(OH)D), if sST2 levels were affected by vitamin D levels. Another possibility was the occurrence of its own distinct yearly pattern as observed with the commonly used cardiovascular biomarker NT-proBNP. It has been shown to be periodically elevated in summer months in primary care patients, although its seasonal pattern did not seem to match that of vitamin D, hypertension, total cholesterol or BMI (27).

Further, since study B, as opposed to study A, also included patients with HF, more pronounced effects might have been observed. Contrary to our predictions, the results showed that sST2 levels remained unchanged throughout the year in study B although the levels of 25(OH)D followed the expected seasonal patterns (28, 29, 30) in both genders. Although one of the unadjusted statistical tests found significant differences in men with HF, after adjusting for several parameters including age and eGFR, we believe these differences are not biologically relevant (for example, the biggest difference in adjusted log transformed sST2 level means was 0.132 log(U/mL) between the months March and July). In general, we also observed similar results when the subjects in study B were subgrouped according to the meteorological definition of the seasons (Supplementary Fig. 2) and when comparing the shapes of the graphs (Supplementary Fig. 3). This observation, in addition to the fact that the results obtained by analysing study B that stem from a separate cohort than the vitamin D treatment experiments, reiterates that vitamin D seems to have minimal or no effect on systemic levels of sST2. In addition, our results support the notion that sST2 levels might not fluctuate as a result of external environmental influences, such as changes in ambient temperature that have been proposed to play a role in the observed seasonality of CVD (1).

We also observed differences between genders in the month in which the levels of 25(OH)D started to rise when also taking into account whether these patients suffered from HF. This was observed earlier in women (May) than in men (June) without HF and could have been a consequence of different outdoor activity levels throughout the year between men and women without HF in our study population. In subjects of both genders with HF 25(OH)D levels started to rise in June, indicating that HF itself probably impacts the outdoor activity of the subjects to a similar degree. Nonetheless, we found higher median 25(OH)D levels in men when not stratifying for HF. A similar observation was also made in a cohort of patients undergoing coronary angiography and concluded that gender differences in 25(OH)D levels might have a relevant role in severity of CAD (31).

Interestingly, we found significant associations of sST2 concentrations with 25(OH)D in study B in men but not in women, while we found no correlations in study A. The lack of associations of vitamin D-related parameters in study A may be due to the fact that the subjects did not have as severe cardiovascular disease complications as in study B and the study published by Gruson *et al.* This study in HF patients, where associations with sST2

were analysed in the whole cohort without stratification into genders (16), made similar observations to ours in study B and found correlations not only with 25(OH)D and PTH, but also with 1,25(OH)$_2$D. Substantially lower 1,25(OH)$_2$D levels have been observed in HF (32), which was also found for the HF patients in the aforementioned study (24.0 pg/mL) (16), compared to our study (48.3 pg/mL in study A and 33.7 pg/mL in study B). This difference might have contributed at least in part to the lack of observed associations in studies A and B. The contrasting associations found in studies A and B might also be explained by the difference in cohort size and this difference in size could also be the reason we found contrasting differences in baseline parameter levels between men and women in our two studies.

Our study has some limitations, namely, that the results stem from *post hoc* analyses of both studies A and B. In addition, the inclusion of vitamin D insufficient hypertensives only in study A and patients referred for coronary angiography in study B could mean that the conclusions cannot be readily extrapolated to the general population. While a growing body of research is available on the role of sST2 in hypertension and several other CVD, it is not as established to be a potential prognostic or diagnostic marker as in HF. Despite that, the results on the effects of seasonality on sST2 we obtained from hypertensives with and without HF were very similar. Of note, measurements of sST2 in study A and B were performed with different methods which are not standardised and at present cannot be directly compared to each other. Nonetheless, both showed the expected differences in sST2 levels between the genders (33, 34), with higher sST2 levels measured in men than in women, confirming the reliability of both measurements. Other strengths of our study include the RCT design of study A with a successful vitamin D intervention, the cohort size of study B with data collected over several years, as well as broad and reliable parameter measurements in both studies A and B. Lastly, the results from two non-related cohorts pointing towards the same conclusion further underline the validity of our findings.

In summary, we show that systemic sST2 levels are not influenced by vitamin D treatment in vitamin D-insufficient hypertensive subjects. We also show that sST2 levels do not change in parallel to 25(OH)D levels and exhibit no significant fluctuations throughout the year in subjects referred to coronary angiography irrespective of whether they did or didn't have HF. Our study provides the first evidence that vitamin D levels are not interrelated with systemic sST2 levels in subjects with cardiovascular

disease. While it still remains to be elucidated, if the same holds true for the general population, our study gives further confidence that sST2 could be used as a biomarker also in the clinical setting throughout the year.

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