Vitamin D Status in Patients with Atrial Fibrillation and Heart Failure - Is there a Link?

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SUMMARY

Background: The disturbed pleiotropic functions of vitamin D are related to numerous chronic non-skeletal diseases. The role of vitamin D insufficiency/deficiency in cardiovascular diseases (CVD) is controversial. Therefore, the aim was to study the vitamin D status in CVD patients and to reveal possible relationships with CVD risk factors.

Methods: This prospective study includes 93 individuals divided into two groups - patients with CVD (n = 49) and patients at risk for CVD (n = 44) served as controls. The CVD-patients were stratified into AF-group - with paroxysmal or persistent atrial fibrillation and HF-group - with heart failure with preserved ejection fraction, in sinus rhythm. Vitamin D status was assessed by measurement of serum 25-hydroxy-vitamin D (25OHD) using liquid chromatography with mass detection. Gene expression of the regulatory enzyme of vitamin D metabolism, 1-alfa-hydroxylase (CYP27B1), was evaluated by two-step real-time qPCR. Coronary artery calcium scans were performed and coronary artery calcium score (CACS) was calculated. Routine biochemical parameters were extracted from the medical documentation.

Standard statistical methods (descriptive statistics, unpaired Student’s t-test, one-way ANOVA, simple and multiple linear regression analyses) were applied. Statistical significance was considered at p < 0.05.

Results: Serum 25OHD levels of the controls were higher than those of the CVD-patients (37.36 ± 15.10 ng/mL vs. 27.70 ± 11.80 ng/mL, p = 0.008). The vitamin D status worsened with the severity of CVD pathology: significant decrease of 25OHD levels was found in the AF-group (29.56 ± 11.76 ng/mL, p = 0.044) and HF-group (24.47 ± 11.61 ng/mL, p = 0.003) vs. controls (37.36 ± 15.10 ng/mL). Significant reduction in circulating vitamin D levels with the increase of CACS (p = 0.007) was also observed. Linear regression analysis revealed significant negative association for serum 25OHD with CACS for both the entire studied group (p = 0.008) and for CVD patients (p = 0.049). The gene expression of CYP27B1 was down regulated with both the severity of CVD pathology (p = 0.05) and coronary calcium accumulation (p = 0.08). Moreover, we found a significant positive relationship (p = 0.041) between serum 25OHD levels and CYP27B1 gene expression.

Conclusions: Vitamin D deficiency may be an independent cardiovascular risk factor associated with the severity of CVD pathology and increased coronary calcium deposition. The mechanism by which vitamin D itself can affect cardiovascular outcomes remains to be clarified.

KEY WORDS
vitamin D, CYP 27B1, atrial fibrillation, heart failure, coronary artery calcium score

INTRODUCTION
The essential role of vitamin D on calcium homeostasis and bone health is well known [1]. The classical view regarding the regulatory role of vitamin D is linked with its active form, 1,25-dihydroxy vitamin D produced by the renal 1-alfa-hydroxylase. The substrate of the renal enzyme is 25-hydroxy vitamin D (25OHD) synthesized by the liver 25-hydroxylase. The principle circulating storage form of vitamin D, 25OHD, is considered as the best marker of vitamin D status. There is controversy related to defining hypovitaminosis D. Plasma values below 12 ng/mL (30 nmol/L) are considered as severe deficiency related to active bone resorption, while 25OHD concentrations between 20 and 50 ng/mL (50 - 125 nmol/L) are defined as vitamin D sufficiency assuring optimal extra-skeletal health [2].

In the last decades, after the discovery that vitamin D receptor and 1-alfa-hydroxylase are widely distributed in non-renal tissues, most of the research is focused on the pleiotropic effects of vitamin D beyond the bone metabolism and its extra-skeletal effects. Vitamin D plays an important regulatory role in cell proliferation and differentiation, inflammation, apoptosis, fibrosis, and immune response [3]. The disturbances in its novel pleiotropic functions are related to the pathology of numerous chronic non-skeletal diseases such as diabetes, cancer, infectious, autoimmune, kidney, and cardiovascular disease (CVD) [1].

As CVD is one of the most prevalent diseases and accounts for the major cause of death, it is of great importance to clarify the role of vitamin D in their pathogenesis and the relationships with cardiovascular (CV) risk factors such as arterial hypertension, obesity, dyslipidemia, diabetes mellitus, and smoking [4]. Though the relationship between vitamin D insufficiency and CVD is controversial, with positive effects in some observational studies [5] and neutral effects in randomized controlled trials [6,7]. An important feature of atherosclerosis associated with adverse cardiovascular events is vascular calcification (VC). VC is a complex process affecting both major and minor blood vessels characterized by calcium deposits in the vascular walls. The role of vitamin D on VC is not fully clarified and is still controversial. Some epidemiological studies proved that hypovitaminosis D is involved in the pathogenesis of atherosclerosis. On the contrary, others suggest that vitamin D may benefit vascular calcification (VC) and thus may increase CV incidences [8].

The regulatory enzyme 1-alfa-hydroxylase (CYP27B1) expressed in numerous tissues including skin cells, monocytes/macrophages, placenta during pregnancy, prostate, adipocytes cardiac cells, endothelial cells, and vascular smooth muscle cells [9,10] is the most important for the production of the active vitamin D hormone responsible for the effects of vitamin D by binding and signaling through the VDR in target tissues. It is suggested that not only the vitamin D status, but also the local activity of the regulatory enzyme 1-alfa-hydroxylase may be crucial for the extra-skeletal effects of vitamin D hormone. A recent study on obese patients revealed lower expression of CYP27B1 in adipose tissues of obese subjects vs. non-obese despite the lack of differences in plasma 25OHD levels. Down regulated expression of CYP27B1 in adipose tissue of obese patients is considered a reason for the decreased local active vitamin D hormone which results in an increased pro-inflammatory response [10]. The data regarding the gene expression of CYP27B1 enzyme in clinical settings are scarce and usually on biopsy samples, an unpleasant and invasive manipulation. Another approach for testing CYP27B1 gene expression is by using easily accessible peripheral blood mononuclear cells (PBMCs), which are the only site of active gene expression in blood.

A modern treatment of hyperlipidemia and prevention of CV complication is statin therapy. It is well known that myalgia is one of the unwanted effects of statin usage. There are controversial data regarding the association between vitamin D status and myalgia induced by statins. Some recent studies found higher prevalence of myalgia in vitamin D deficient patients. Conversely, others reported no significant relationship between statin-induced myopathy and vitamin D [11].

The aim of the current study was to evaluate the vitamin D status and the expression of 1-alfa-hydroxylase (CYP27B1) in PBMC in CVD patients and to reveal possible relationships with CVD risk factors.

MATERIALS AND METHODS
Study cohort
The present study includes 93 Bulgarian patients of both genders (60 females and 33 males) who were admitted at the Cardiology Clinics of the University Hospital - Varna between October 2018 and January 2020. The patients were devided into two groups - patients with CVD and patients at risk for CVD. The patients in the CVD group (n = 49) were stratified into two subgroups according to their CVD pathology - with paroxismal or persistent atrial fibrillation (AF group) and with heart failure with preserved ejection fraction (ejection fraction > 40%, HFpEF) who were in sinus rhythm at the time of hospitalization (HF group). Patients with estimated moderate to high risk for CVD but without known CVD (n = 44) served as controls. Age and history of CVD, smoking status, presence and treatment of arterial hypertension, and presence and treatment of hyperlipidemia were assessed through a structured interview at admittance in the hospital. Hypertension was defined as blood pressure (BP) > 140/90 mm Hg at the time of examination or with a history of elevated BP.
and on antihypertensive medication. Hyperlipidemia was defined as elevated total cholesterol (TC), low density lipoprotein cholesterol (LDL-C) or triglycerides (TG) according to the ESC Guidelines cutoff values [12] or by lipid lowering medications. All patients had a physical examination including heart rate, blood pressure, weight, height, and waist circumference (WC). WC above 88 cm for women and above 102 cm for men was an indicator for abdominal obesity. Body mass index (BMI) was calculated using the formula weight/height$^2$ and expressed in kg/m$^2$. BMI below 24.9 kg/m$^2$ was considered normal, BMI between 25.0 kg/m$^2$ and 29.9 kg/m$^2$ overweight, and obesity was indicated for BMI above 30 kg/m$^2$ [13]. Patients with proven ischemic heart disease or stroke, cardiomyopathy, type 1 diabetes mellitus, chronic renal disease IV stage or more (with eGFR < 30 mL/min/1.73 m$^2$), known thyroid gland diseases, and active cancer were excluded from the study.

Coronary artery calcification measurement
All participants underwent a multislice computed tomography examination in order to assess the presence of coronary artery calcification. Coronary artery calcium scans were performed with a Siemens Somatom Definition (Dual Source 2 x 64) CT scanner using standardized imaging protocols. Thirty to forty consecutive tomographic slices were obtained at 3 mm intervals, 1 cm below the carina and progressing caudally to include the entire coronary tree. Scans were interpreted by a single trained physician blinded to the clinical characteristics of the patients. Coronary artery calcification was defined as a lesion of > 130 Hounsfield units, with an area equal to 3 pixels. Coronary artery calcium score (CACS) was calculated using the Agatston criteria. The presence of coronary artery calcium was defined as an Agatston score > 0 Agatston units (AU). According to the CACS results, the patients were classified into one of the following categories: CACS = 0 AU (absence of coronary calcium), CACS = 1 - 99 AU and CACS ≥ 100 AU. The absence of coronary calcium (CACS = 0 AU) conveys an extraordinarily low cardiovascular risk, irrespective of the number of risk factors. In contrast, the presence of CACS ≥ 100 AU in individuals with moderate risk may reclassify them upward to the high-risk category [14].

Laboratory measurements
Fasting venous blood was drawn at admittance in the clinic. Blood samples were centrifuged at 1,600 g for 15 minutes, blood plasma and serum were separated and stored at -80°C until analysis of specific laboratory parameters. Information for the routine biochemical parameters such as C-reactive protein (CRP), glucose, urea, creatinine, uric acid (UA), total protein (TP), and albumin and lipid profile parameters such as triglycerides (TG), total cholesterol (TC), HDL-cholesterol (HDL-C), and LDL-cholesterol (LDL-C) were extracted from the medical records of the studied patients.

Castelli risk indexes (TC/HDL-C and LDL-C/HDL-C) and estimated glomerular filtration rate (eGFR) were calculated.

Vitamin D measurement
Serum 25OHD levels were measured using a liquid chromatography system coupled with PDA and single quadrupole mass spectrometry detectors (Waters Aquity H class PDA-QDa, Milford, MA, USA) at the Biochemistry Department, Medical University of Varna, Bulgaria.

A working solution of 1 µg/mL hexadeuterated 25-OHDL3 (Sigma-Aldrich Co Ltd, USA) was prepared in methanol as internal standard. The sample preparation involved addition of 50 µL internal standard working solution to 500 µL sample in a 15 mL glass screw top sample tube (VWR International, LLC), and the tubes were vortexed. Ethanol (1,000 µL) was added to precipitate proteins and the vials were vortexed, stored at 4°C for 10 minutes, and centrifuged at 5,000 rpm for 5 minutes. The fat-soluble 25OHD was extracted from the supernatant in triplicate with 7.5 mL n-hexane. The hexane layers were combined and evaporated under gentle stream of nitrogen at room temperature. The dry residue was reconstituted in 100 µL methanol LC-MS grade and filtered through 0.2 µm syringe filter. The filtered samples were transferred to total recovery sample vials and subjected to LC-MS analysis using an automated Waters AQUITY H-class UPLC system equipped with autosampler and QDA-PDA detectors (Waters Corporation, Milford, MA, USA). Filtered sample (5 µL) was injected onto a Waters AQUITY UPLC BEH C18 column (2.1 x 50 mm; 1.7 µm particle size).

The chromatographic mobile phases consisted of methanol:water = 98:2 (v/v), containing 0.1% formic acid and 5 mM ammonium formate (elucent A) and water containing 0.1% formic acid and 5 mM ammonium formate (elucent B). A gradient elution was performed and the column was then re-equilibrated to baseline conditions. The flow rate was 0.30 mL/minute, column temperature was maintained at 45 ± 1°C. A Waters QDa single quadrupole mass spectrometer was used in positive ion mode with electrospray source. The PDA detector was set at 265 nm. Instrument analysis time per sample was 8 minutes. The MS was set at SIR mode, the capillary voltage was 1.3 kV, probe temperature 600°C, cone voltage 15 V. For quantification, pure substances of 25-OHDL and hexadeuterated 25OHD3 as internal standard were used. A working standard solution in methanol with concentration 1 µg/mL was prepared from a commercial internal standard solution with concentration 50 µg/mL (D6-25-Hydroxyvitamin D3 (26,26,27,27,27-D6) solution 50 µg/mL in ethanol, ampule of 1 mL, certified reference material, Cerilliant®, No. H-074, obtained from Merck). Three ions were monitored: for 25-OHDL 383.4Da, 401.35Da and for the internal standard 389.54Da. Hexadeuterated internal standard, 25OHD3, is eluted from the column at 4.87 and 4.95 minutes. System operation, data acquisition, and quantitative data
analysis was performed using Empower v. 3.0 software (Waters Corporation, Milford, MA, USA). The results for the serum concentrations of 25OHD are given in ng/mL.

To minimize the matrix effects such as the influence of triglyceride levels on the extraction process and LC-MS analysis, we monitored the overall sample preparation procedure including the pre-analytical part (protein precipitation, extraction, concentration) and LC-MS analysis by using commercial lyophilized quality controls and calibrators (6 different concentration levels plus 1 blank), based on human serum (MassCheck® 25-OH-Vitamin D3/D2 Serum Control, Bi-Level, No. 0221 and 6PLUS1® Multilevel Serum Calibrator Set 25-OH-Vitamin D3/D2, No. 62039 obtained from Chromsystems, Germany). The controls and the calibrators were handled and measured in the same manner as the patient samples. It is well known that 25OHD levels vary by season. As only 16 (16.1%) of our patients were tested during the summer, we assume that the average 25OHD levels for the subgroups would be not biased by the seasonal variations. Serum concentrations of 25OHD were classified as deficient (< 20 ng/mL), intermediate (20 - 29 ng/mL), or optimal (> 30 ng/mL), based on prior studies and classifications from The Endocrine Society and according to the decisions of the First International Conference on Controversies in Vitamin D held in 2017 [2].

Peripheral blood mononuclear cell collection
For the purpose of gene expression analysis, peripheral blood mononuclear cells (PBMC) were isolated from freshly drawn whole blood samples collected in lithium heparin vacutainer tubes. PBMC separation was carried out by density gradient centrifugation using Ficoll separation medium (density 1.077 g/L) and LeucoSep™ centrifuge separation tubes (GreinerBioOne, Austria) following the manufacturer’s instructions.

Gene expression analysis
RNA was extracted from the collected PBMCs with Trizol reagent (Invitrogen®, Thermo Fisher Scientific, USA). First strand cDNA synthesis was performed with 0.1 µg of total RNA using Thermo Scientific M-MuLV reverse transcriptase (USA) following the steps of the manufacturer’s instructions.

Quantitative gene expression analysis was performed using two-step real-time qPCR. Each reaction was performed in triplicate and amplified in a reaction mix containing SYBR Green qPCR 1 x Master Mix with ROX (KAPA SYBR FAST qPCR Kit, KAPA BIOSYSTEMS, USA) and 0.3 µM of each primer. Primer sequences used for the Real-Time qPCR were mRNA specific with the following sequencing:

<table>
<thead>
<tr>
<th>mRNA</th>
<th>Forward</th>
<th>Reverse</th>
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<tbody>
<tr>
<td>U6</td>
<td>GAGCTTGGCAGACATCCAGGC;</td>
<td>GGCTCAGATTTGCG TTGTCAT;</td>
</tr>
<tr>
<td>1-alfa-hydroxylase</td>
<td>CYP27B1</td>
<td>CYP27B1</td>
</tr>
<tr>
<td>CYP27B1</td>
<td>GAGCTTGGCAGACATCCAGGC;</td>
<td>GGCTCAGATTTGCG TTGTCAT;</td>
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Analysis was performed on Applied Biosystems® 7500 Real-Time qPCR instrument (USA). The amount of mRNA of each gene of interest was normalized according to the amount of mRNA encoding U6 as an internal control. Gene expression levels were calculated using 2^(-ΔΔCt) method [15]. Data is presented as relative units mRNA (RU mRNA).

Statistical analysis
Data were presented as mean ± standard deviation (SD), or percentage (%), as appropriate.

Data analysis was performed on GraphPad Prism v. 8.3 and SPSS v. 23. Standard statistical methods such as descriptive statistics, unpaired Student’s t-test for normally distributed parameters, and one-way ANOVA with Bonferroni correction were used. Chi-squared test or Fisher’s exact test were applied for categorical data. The relationships between continuous variables were evaluated by Spearman’s correlation analysis. Simple and multiple linear regression analyses were applied to test the relationships between 25OHD concentration or 1-alfa-hydroxylase gene expression as dependent variables and other tested parameters. Statistical significance was considered at p < 0.05.

The study was approved by the local Ethical Committee at the Medical University of Varna (Protocol No. 75/07.06.2018). Written informed consent was obtained from all participants in the study.

RESULTS

Characteristics of the studied patients
The mean ± SD age of all studied patients was 62.0 ± 12.0 years; the controls comprising non-CVD participants were 53.7 ± 10.1 years, the mean age of the CVD group was 70.1 ± 7.3 years (70.1 ± 7.8 years for AF patients and 70.1 ± 6.54 years for HF group). The females dominated in our study. They were 65.7% of the total studied patients, 61.7% in the control non-CVD group, and 69.2% in the CVD group. Most of the participants (88.8%) had arterial hypertension (83.7% of the controls and 94.6% of the CVD patients); 80.0% of all studied subjects had hyperlipidemia (88.4% of the controls and 70.3% of the CVD patients). One-third (33.8%) of the subjects were obese and almost half of them were abdominally obese (55.8%). Of all studied patients, 32.3% were on statin medication and, of those, 27.7% were controls and 36.5% were CVD patients. Among all studied patients, 17.2% were vitamin D deficient, 36.6% had vitamin D insufficiency, and 46.2% had optimal 25OHD levels.
Vitamin D Status in CVD Patients

Figure 1a, b. Serum 25OHD levels stratified by CVD pathology.

Data are presented as mean ± SD (statistical significance was indicated at p < 0.05). CVD patients comprise both AF and HF patients. CVD - cardiovascular disease, AF - atrial fibrillation, HF - heart failure, 25OHD - 25-hydroxy vitamin D.

Figure 2a, b. Serum 25OHD levels stratified by CACS.

Data are presented as mean ± SD (statistical significance was indicated at p < 0.05). CACS > 0 represents presence of coronary calcium deposits. CACS = 0 represents no coronary calcium. CACS - coronary arterial calcium score, 25OHD - 25-hydroxy vitamin D.
Figure 3a, b. Associations between 25OHD levels and CACS.

A. - for the entire studied group, p = 0.008, B. - for the CVD patients, p = 0.049 (statistical significance was indicated at p < 0.05).
CACS - coronary arterial calcium score, 25OHD - 25-hydroxy vitamin D.

Figure 4a, b. CYP27B1 gene expression in PBMC of the studied patients stratified by CVD pathology.

Data are presented as mean ± SD (statistical significance was indicated at p < 0.05). CVD patients comprise both AF and HF patients.
CVD - cardiovascular disease, AF - atrial fibrillation, HF - heart failure, 25OHD - 25-hydroxy vitamin D.

Serum 25OHD levels and CVD pathology

Serum 25OHD levels are presented in Figures 1a and b. Serum 25OHD levels of the entire cohort were 32.38 ± 14.34 ng/mL, close to the upper limit (30 ng/mL) for vitamin D insufficiency. The controls revealed 35.0% higher 25OHD levels than those of the CVD-patients (37.4 ± 15.1 ng/mL vs. 27.7 ± 11.8 ng/mL, respectively; Figure 1a). In the control group, 9.1% of the studied
Vitamin D Status in CVD Patients

Figure 5. CYP27B1 gene expression in PBMC of the studied patients stratified by CACS.

Data are presented as mean ± SD (statistical significance was indicated at p < 0.05). CACS > 0 represents presence of coronary calcium deposits. CACS = 0 represents no coronary calcium. CACS - coronary arterial calcium score.

Figure 6. Associations between 25OHD levels and CYP 27B gene expression in PBMC.

Statistical significance was indicated at p < 0.05. 25OHD - 25-hydroxy vitamin D.
Figure 7a, b. Changes in 25OHD levels and in CYP27B1 gene expression in PBMC in the studied patients in accordance with statin medication.

A. Changes in 25OHD serum levels in controls and CVD patients with and without statin medication.
B. Changes in CYP27B1 gene expression in PBMC of controls and CVD patients with and without statin medication. Data are presented as mean ± SD (statistical significance was indicated at p < 0.05).

CVD - cardiovascular disease, 25OHD - 25-hydroxy vitamin D.

subjects were vitamin D deficient, 29.5% were insufficient, and 61.4% had optimal vitamin D serum concentrations. The CVD patients revealed almost three times higher percentages of vitamin D deficiency (24.5%); 42.9% were insufficient and those with optimal 25OHD levels were two-fold lower (32.7%). A significant decrease of 25OHD levels was found in the AF-group (29.56 ± 11.76 ng/mL, p = 0.044) and HF-group (24.47 ± 11.61 ng/mL, p = 0.003) vs. controls (37.36 ± 15.10 ng/mL). The vitamin D status was most severely aggravated in the HF group as 72.2% had vitamin D deficiency (44.4%) and insufficiency (27.8%). When the participants were stratified according to CVD pathology, a gradual and significant decrease of serum 25OHD with CVD severity was observed (Figure 1b).

Serum 25OHD levels and coronary arterial calcium deposition
Significant reduction in circulating vitamin D levels with the increase of coronary arterial calcium score (CACS), was indicated (Figure 2a, b).
The participants with arterial calcium deposits (CACS > 0) revealed significantly lower 25OHD (29.25 ± 13.14 ng/mL vs. subjects with CACS = 0 AU (37.40 ± 15.42 ng/mL, p = 0.007; Figure 2a). In patients with highest CACS, a substantial decrease in serum 25OHD (25.56 ± 12.57 ng/mL) by 41.04% compared to the patients with CACS = 1 - 99 AU (36.05 ± 11.58 ng/mL) and by 46.3% vs. those with CACS = 0 AU (37.40 ± 15.42 ng/mL; Figure 2b) was found. The patients with highest CACS exhibited vitamin D insufficiency with values close to the lower reference limit of 20 ng/mL. This group had the highest percentage of vitamin D deficient patients (40.0%), 25.7% were vitamin D insufficient and only 34.3% were within the optimal range.
Linear regression analysis revealed significant negative association for serum 25OHD with CACS for both the entire studied group (Figure 3a) and for CVD patients (Figure 3b).

Serum 25OHD levels and risk factors for CVD
Linear regression analysis showed a tendency for a decrease in 25OHD levels with BMI, abdominal obesity, and duration of hypertension for both controls and CVD patients. More than half (66.7%) of the patients with obesity (BMI ≥ 30 kg/cm²) were vitamin D deficient (25.0%) or insufficient (41.7%). The results for the patients with abdominal obesity are the same - 64.7% of them represent vitamin D deficiency (21.6%) or insufficiency (43.1%). The presence of arterial hypertension is also related with poor vitamin D status - 53.8% subjects were vitamin D deficient (20.0%) or insufficient (33.8%) vs. 0.0% vitamin D deficiency in normotensive patients. Multiple linear regression analysis using 25-
Vitamin D Status in CVD Patients

OHD serum levels as dependent variable revealed significant associations with TG (β = -0.45, p = 0.04) and WC (β = -0.33, p = 0.045) in the moderate to high-risk patients without CVD. For the CVD patients, we found statistically significant associations between serum 25-OHD and TC/HDL ratio (β = -0.60, p = 0.05) and a tendency of associations between serum 25OHD and LDL (β = -0.43, p = 0.09). Significant associations between serum 25OHD concentrations and metabolic risk factors such as TC, LDL-C, HDL-C, Castelli indexes, and UA were not established.

Expression of CYP27B1
Analysis of 1-alfa-hydroxylase (CYP27B1) gene expression in PBMC revealed a tendency for downregulation (p = 0.07) in CVD patients (Figure 4a). The same tendency was observed when the CVD patients were stratified according to CVD pathology into AF and HF groups. The gene expression of 1-alfa-hydroxylase decreased significantly with CVD severity. It reached lowest values in HF patients (Figure 4b).
Analyzing CYP27B1 gene expression according to coronary arterial calcium deposits, we found a significant downregulation of CYP27B1 in the patients with high CACS (Figure 5). These results are not surprising as serum 25OHD was inversely associated with CACS for all studied groups (Figure 3a, b). Moreover, we found a significant positive relationship (p = 0.041) between serum 25OHD levels and CYP27B1 gene expression (Figure 6).

The effect of statin treatment
Testing the effect of statin treatment on 25OHD serum levels and on gene expression of CYP27B1, we found a tendency for a decrease of the tested parameters between patients on statin medication and those without such therapy. More pronounced and significant decrease of serum 25OHD and downregulation of CYP27B1 was established in the group of CVD patients (Figure 7a, b).

DISCUSSION
Vitamin D is a prohormone formed in skin by UV irradiation of 7-dehydrocholesterol. Its active form vitamin D hormone (calcitriol, 1,25-dihydroxycholecalciferol) depends on the concentration of the circulating form 25-OHD, formed in the liver and on the activity of the kidney regulatory enzyme 1-alfa-hydroxylase. Serum 25-OHD levels are the only reliable indicator for the assessment of vitamin D status. It has been established that the active form of vitamin D can be formed not only in the kidney, but also in non-renal tissues, which explains its non-calcemic extra-renal effects on muscle strength and on cardiovascular, nervous, and immune systems [1]. Therefore, vitamin D deficiency has been shown to raise the incidence of multiple chronic diseases including CVD such as hypertension, coronary artery disease, and heart failure.

We aimed to study whether there is a relationship of vitamin D status with CVD pathology, the degree of coronary arterial calcium deposition, assessed by CACS, as well as with CVD risk factors in adult patients. We found a significant decrease of serum 25OHD in CVD patients vs. controls. Moreover, the vitamin D status was worsened with the severity of CVD pathology as lowest 25OHD levels were detected in the HF-group. Of course, partly the observed differences between CVD patients and controls in vitamin D levels may be explained by the different age. As the controls in our study are younger, it is more likely that they will have higher levels of 25OHD. However, more than 38% of them are either vitamin D deficient or insufficient. Therefore, we believe that age is not the only factor that may explain our results.

Although numerous studies have confirmed an association between vitamin D and CVD [16], a cause-effect relationship between both remains unclear. Lack of consensus exists about the relationship of 25OHD and HF and its effect on HF and the consequences of 25OHD deficiency [17]. Recent studies reported high prevalence of 25OHD deficiency in HF patients [18], an inverse association between low 25OHD and new biomarkers of chronic heart failure such as BNP, N-terminal (NT)-pro-BNP, and a higher rate of hospitalization due to HF [19]. Controversial results are reported regarding vitamin D deficiency and E/E’ ratio [20] and no significant association with left ventricular diastolic performance [21] were also observed.

The associations between 25OHD status and AF are also contradictory. In agreement with our results are the data of a recent comparative study on vitamin D status in patients with different types AF and controls. This study revealed lower 25OHD levels for AF patients and a significant relationship between vitamin D deficiency and non-valvular AF [22]. Recent studies on patients with persistent or permanent AF and non-AF controls revealed significantly lower serum 25OHD levels in AF patients [23]. These data are also consistent with our results for the AF patients. On the contrary, others reveal no relationship between vitamin D and AF [24].

The exact mechanism by which 25OHD deficiency results in poor clinical outcomes in HF patients is still not clear. Down regulation of the renin-angiotensin-aldosterone system (RAAS) by 25OHD found in animal models leads to the hypothesis that there could be an inverse relationship between 25OHD and RAAS activation in humans. It is hypothesized that 25OHD deficiency leads to RAAS hyper activation and as a result to left ventricular remodeling and worsening of HF [16,17]. Moreover, 25OHD deficiency results in increased production and release of inflammatory cytokines which may directly affect the myocardium and thus favor cell apoptosis, hypertrophy, fibrosis, ventricular remodeling, and may lead to negative inotropic effects [25]. Studies on the effect of vitamin D supplementation on the activ-
ity of RAAS reveal a decrease of plasma renin and aldosterone in HF patients and are in agreement with these assumptions [26]. The mechanisms explaining the effect of vitamin D on the atrium are also not fully understood. One hypothesis is related to the regulatory role of vitamin D on RAAS which is involved in the development of the fibrotic substrate facilitating AF onset [16]. On the other hand, vitamin D by its immune modulatory and anti-inflammatory effects can affect the inflammatory pathway in AF pathogenesis [27]. Beside the endocrine effects of vitamin D, there are studies reporting a direct electrophysiological effect of vitamin D on the atrium [28]. Vitamin D deficiency negatively correlated with atrial electromechanical delay, and it can be reduced by supplementation with vitamin D [29]. We found an inverse association between 25OHD serum levels and Agatston CACS. Patients with CACS > 100 AU revealed significantly lower 25OHD levels as compared to those with CACS = 1 - 99 AU and CACS = 0 AU. Their 25OHD serum levels were close to the lower reference limit for vitamin D sufficiency of 30 ng/mL [2]. The role of vitamin D on vascular calcification is still not fully understood and remains controversial. The common insight for the protective role of vitamin D on vasculature is based on its anti-inflammatory effects and prevention of endothelium dysfunction [8]. This protective role vitamin D exerts by several mechanisms: it maintains endothelium integrity, inhibits the release of pro-inflammatory cytokines, which mediate endothelium stress, dysfunction, and, as a result, vascular calcification; reduces the expression of vascular endothelium growth factor and of matrix metalloproteinase 2 and 9, calcification promoters; and directly inhibits the osteogenic differentiation of vascular smooth muscle cells [8]. A recent insight on the role vitamin D in the mechanisms of vascular calcification is focused on its modulatory effect on osteoblastic differentiation of mesenchymal stem cells and stimulation of the adhesion and migration of endothelial progenitor cells. Moreover, vitamin D mitigates the adipocyte transformation in mesenchymal stem cells and thus reduces inflammation related to vascular calcification [30]. In clinical settings it was shown that vitamin D deficiency correlates with monocyte infiltration in vascular adventitia in patients with coronary arterial disease and thus promotes inflammation [31]. The inverse association of vitamin D deficiency with obesity and arterial hypertension as risk factors for CVD is well established. Our findings of a tendency for a decrease of 25OHD levels with the duration of arterial hypertension are in agreement with the results of several large studies showing that vitamin D deficiency leads to hyperactivity of RAAS and thus contributes to development and progression of arterial hypertension [17]. The inverse association between 25OHD and obesity indexes such as BMI and WC in our patients is in agreement with the findings of other studies [32]. It had been hypothesized that the reverse link between 25OHD levels and obesity may be due to vitamin D deposition in adipose tissue, resulting in lower circulating 25OHD levels in the blood. A one-directional causal relationship, proven by Vimaleswaran et al., indicates that obesity leads to lower vitamin D levels. They also concluded that obesity could be considered a causal risk factor for 25OHD deficiency, accounting for approximately one third of vitamin D deficiency [33]. In our study, we demonstrated that 1-alfa-hydroxylation of PBM C gradually diminished with both the severity of CVD pathology and coronary calcium accumulation. Moreover, we found a positive and significant relationship between serum 25OHD levels and 1-alfa-hydroxylase expression in PBMC. Unlike the renal 1-alfa-hydroxylase, the non-renal enzyme including that expressed in PBMC, not regulated by parathyroid hormone and calcium, is dependent on serum levels of 25OHD, and is not suppressed by calcitriol [34]. Lower expression of CYP27B1 in PBMC with CVD severity and CACS may result in lower enzyme activity and subsequently, in lower concentrations of the active vitamin D metabolite in the blood. Both the downregulation of CYP27B1 and vitamin D insufficiency may lead to deficient production of the active form of vitamin D and compromised anti-inflammatory and immune modulatory function. Altogether, these molecular events may contribute to vascular calcification and CVD deepening. To the best of our knowledge, studies regarding the expression of CYP27B1 in PBMC in CVD patients and its associations with serum 25OHD and vascular calcification are scarce. Our study is one of the few attempting to clarify this problem. It is a matter of debate how statin medication affects vitamin D status. Some studies report elevated 25OHD levels after statin therapy [35], others show that statins do not affect vitamin D levels [36]. However, there are studies indicating a significant decrease in 25OHD concentration subsequent to statins therapy [11]. Our results also reveal significantly lower 25OHD levels in both controls and CVD patients after statin treatment. Moreover, a significant downregulation of CYB27B, the regulatory enzyme in vitamin D activation, in PBMC was indicated for the same groups of patients. The most frequently observed side effects of statins are on the muscular skeletal system [17]. Numerous studies indicated that vitamin D deficiency worsens the side effects of statin treatment on muscles, and they established that a cutoff level of 15 ng/mL are predictive for high frequency of myalgia development induced by statins [37]. One of the launched hypotheses explaining the elevated 25OHD levels following statin medication is based on the statin-suppressed activity of 3-hydroxy-3 methyl glutaryl coenzyme A reductase (HMGCoAR), the main regulatory enzyme for de novo cholesterol synthesis in the liver. The proponents of this idea claim that the inhibition of HMGCoAR by statins results in increased 7-hydrocholesterol levels, a common precursor of 25OHD [38,39]. This sounds unlikely as 7-hydrocholesterol is
formed at the end of cholesterol synthesis and if the HMGCoAR is suppressed its levels would be diminished as well as the levels of 25OHD. It would be reasonable for patients starting statin therapy to be screened for vitamin D deficiency/insufficiency and to start vitamin D supplementation with the aim to reduce the toxic effects of statins on muscles. The current study concerns a hot topic related to the associations between vitamin D deficiency and widespread CVD. It includes a diverse cohort of both moderate and high-risk patients without confirmed CVD and CVD patients with different pathology. To our knowledge this is the first study analyzing the possible relationship between CVD pathology and coronary calcium deposition, assessed by CT scan and CYP27B1 expression in PBMC, easily accessible from blood samples. In addition, we examined the relationships between vitamin D status and CYP27B1 gene expression with numerous demographic and lifestyle variables along with established cardiovascular risk factors. One of the limitations of our study is the relatively small number of patients. Due to the observational nature of this study, we were unable to distinguish whether the observed associations were causal.

CONCLUSION

It can be concluded that vitamin D deficiency may be an independent cardiovascular risk factor that is associated with the severity of CVD pathology and increased coronary calcium deposition. In the studied Bulgarian population, the vitamin D status was worsened with the severity of CVD pathology and the degree of coronary calcium deposition. 25OHD serum levels were positively related to 1-alfa-hydroxylase gene expression in PBMC. The gene expression of this regulatory enzyme of the vitamin D activation pathway was down regulated with both the severity of CVD pathology and coronary calcium accumulation. While it seems plausible that vitamin D deficiency can be considered a surrogate marker for poorer health status observed in patients with moderate to high risk for CVD and present CVD, The mechanism by which vitamin D itself can affect cardiovascular outcomes remains to be clarified. Further studies are also needed to assess whether the gene expression of 1-alfa-hydroxylase in PBMC may be used as a reliable biomarker for CVD.

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Declaration of Interest:
The authors report no potential conflict of interest.

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