Association between plasma phosphate/pyrophosphate ratio and CT derived aortic valve calcification score in an unselected cohort of cardiovascular patients

Dénes Juhász[1], Martin Várhegyi[2, 10], Márton Rakovics[3,4], Bálint Szilveszter[1], Ádám Levente Jermendy[1], Edit Dósa[1], Éva Straub[1], Béla Merkely[1], Tamás Arányi[2, 9], Astrid Apor[1], Dávid Szüts[2], Aristomenis Manouras[5,6], Magnus Bäck [5,6,7,8], Flóra Szeri[2*], Anikó Ilona Nagy[1*]

1, Heart and Vascular Centre, Semmelweis University, Budapest, Hungary
2, Institute of Molecular Life Sciences, HUN-REN Research Centre for Natural Sciences, Budapest, Hungary
3, Centre for Translational Medicine, Semmelweis University, Budapest, Hungary
4, ELTE Eötvös Loránd University, Budapest, Hungary
5, Department of Cardiology, Heart and Vascular Center, Karolinska University Hospital, Stockholm, Sweden
6, Department of Medicine Solna, Karolinska Institutet, Stockholm, Sweden
7, Université de Lorraine, Inserm, DCAC, F-54000 Nancy, France
8, Centre Hospitalier Régional Universitaire de Nancy, Vandoeuvre-lès-Nancy, France
9, Department of Molecular Biology, Semmelweis University, Budapest, Hungary
10, Doctoral College, Semmelweis University, H-1085 Budapest, Hungary

*AI Nagy and F Szeri contributed equally to this work

Corresponding author:
Anikó Ilona Nagy
Affiliation: Semmelweis University, Heart and Vascular Centre, Budapest, HUNGARY
Correspondence: 68, Városmajor u. Budapest H-1122
Telephone: +36208259738
e-mail: anychophora@gmail.com

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ABSTRACT

Aims Inorganic pyrophosphate (PPi) is an endogenous inhibitor of soft-tissue calcification. A disturbed equilibrium between pro- and anti-mineralization agents, like extracellular phosphate (Pi) and PPi, have been implicated in the mechanism of aortic valve calcification (AVC). We aimed to investigate the association of the plasma PPi concentration and Pi/PPi ratio with the degree AVC in cardiovascular patients.

Methods and results 154 patients referred for cardiac CT, including 43 individuals with severe aortic stenosis, were prospectively enrolled. The aortic valve calcium score (AVCS) was measured on non-contrast CT images. Plasma PPi level was determined enzymatically.

Of the entire population (age: 67±12 years, 42.5% female), 42% had some degree of AVC (range 9-6641 AU). Plasma PPi showed a significant positive association with plasma Pi and low density lipoprotein cholesterol (LDL-C) concentration and was inversely related to alkaline phosphatase activity. When controlled for age, female patients had higher PPi levels. In univariate analysis, plasma PPi level did not show an association with AVCS, however, the Pi/PPi ratio was significantly positively associated with the degree of AVC (estimate: 1508.1; SE 616.0, p=0.015), along with age, hypertension, plasma lipoprotein(a) concentration and statin treatment, whereas eGFR and LDL-C level showed significant negative associations. In multivariate analysis only age and Pi/PPi ratio remained significant determinant of the AVCS (estimate: 1128.6; SE 562.5, p=0.047).

Conclusion. This is the first study to investigate the association between PPi homeostasis and AVC in humans. The plasma Pi/PPi ratio was significantly positively associated with the AVC load even after adjustment for traditional risk factors.

ABBREVIATIONS

ABCC6	ATP binding cassette subfamily C member 6
ALP	alkaline phosphatase
AS	aortic stenosis
ATP	adenosine triphosphate
AV	aortic valve
AVC	aortic valve calcification
AVCS	aortic valve calcium score
CT	computed tomography
CCTA	cardiac CT angiography
DM	diabetes mellitus
ENPP1	ectonucleotide pyrophosphatase/phosphodiesterase 1
GACI	general arterial calcification of infancy
eGFR	estimated glomerular filtration rate
HT	hypertension
LDL-C	low density lipoprotein cholesterol
Lp(a)	lipoprotein(a)
Pi	phosphate
PPi	inorganic pyrophosphate
PXE	pseudoxanthoma elasticum
SD	standard deviation
SE	standard error
TNAP	tissue non-specific alkaline phosphatase

INTRODUCTION

Aortic stenosis (AS) represents the most common valvular heart disease in developed countries, carries significant morbidity and mortality, and its prevalence increases progressively in the aging populations.(1-3) Despite its abundance, and poor prognosis, the aetiology of AS remains incompletely understood and accordingly, there are no efficient pharmacological therapies capable of modifying disease progression.

The pathogenesis of AS can be divided into two distinct phases. The early initiation stage shares several common characteristics with atherosclerosis. Endothelial damage due to increased mechanical stress is thought to be an important trigger for lipid, in particular, lipoprotein(a) (Lp(a)) and low-density lipoprotein cholesterol (LDL-C) deposition, and consequent inflammation in the aortic cusps.(4-6) Accordingly, observational studies have identified hypertension (HT), LDL-C and Lp(a) as independent risk factors for the development of AS, along with other traditional cardiovascular risk factors, such as male sex, diabetes mellitus (DM) and impaired renal function.(7-11) The second, propagation phase of AS development is characterised by progressive calcium phosphate deposition in the valve that eventually results in reduced cusp mobility.(4-6) This process is thought to be driven by pro-calcific and pro-osteogenic factors.

Phosphate (Pi) has been shown in vitro to activate osteogenic mediators in cultured valvular interstitial cells,(12) and higher serum levels were associated with a greater AVC prevalence(13) and faster AS progression,(14) independent of kidney function.(15)

Inorganic pyrophosphate (PPi) on the other hand is a potent endogenous calcification inhibitor. It is produced through the extracellular hydrolysis of adenosine triphosphate (ATP) by ectonucleotide pyrophosphatase/phosphodiesterase 1 (ENPP1).(16-19) Low systemic PPi levels have been associated with inappropriate connective tissue mineralization in several rare hereditary diseases that affect PPi homeostasis, like pseudoxanthoma elasticum (PXE, predominantly caused by mutations of the ATP binding cassette subfamily C member 6 (ABCC6) transporter that mediates the cellular efflux of ATP) and generalized arterial calcification of infancy (GACI, primarily caused by mutations of the ENPP1 or ABCC6 genes).(20-24) PPi is hydrolysed into Pi by tissue non-specific alkaline phosphatase (TNAP)

in extracellular fluids. An imbalance between extracellular Pi and PPi level emerges as a key regulator for ectopic soft-tissue calcification.(25)

It was recently shown that orally administered PPi sustainably increased plasma PPi levels both in animal models and healthy human volunteers and could efficiently rescue calcification in GACI and PXE mouse models.(26)

Disrupted PPi homeostasis has also been implicated in the pathomechanism of AVC.(21) Two ex-vivo studies demonstrated that AVC was inhibited by PPi in the surrounding media.(27, 28) However, because the methodology of PPi measurement from complex biological samples has only been recently established,(29-31) a potential association between plasma PPi levels and the degree of AVC in vivo has not yet been investigated. We hypothesized that circulating PPi might play a role in the pathomechanism of AVC. The aim of this study was to establish if there is an association between plasma PPi concentration or Pi/PPi ratio and the degree of AVC in a heterogenous cardiovascular patient cohort. Proving a direct link between plasma PPi or Pi/PPi ratio and AVC could provide a potential target for medical treatment of AS by oral PPi supplementation.

METHODS

Patient population

Patients referred for clinically indicated cardiac CT angiography (CCTA) were prospectively enrolled between March 2023 and May 2024. The indications for CCTA examinations were coronary CT angiography to exclude coronary artery disease, CCTA as part of TAVI planning and CCTA prior to pulmonary vein isolation (PVI). A flowchart about the enrolment process is provided on **Figure S1**. The participants' demographic and clinical data were collected prior to CCTA, electronic medical records were also used as a complementary source. Patients with conditions that could importantly interfere with the AV calcium load measurement (presence of coronary stent or an AV prosthesis) or the plasma PPi level (glomerular filtration rate (GFR)<30 ml/min/1.73 m^2 according to the referring laboratory result;(32, 33) bisphosphonate, or vitamin K antagonist therapy, which have an established role in the PPi

homeostasis and ectopic mineralization;(34-36) and complicated vein puncture during blood sampling, as tissue injury can affect local PPi concentration) were excluded from the study. None of the included patients had hypo- or hyperparathyroidism, nor were they receiving denosumab or teriparatide treatment for osteoporosis. Based on their aortic valve calcium score (AVCS) patients were allocated into three groups: no AVC (AVCS=0 AU); mild-to-moderate AVC (AVCS 0-1000 AU, representing the initiation phase of AVC); and severe AVC (AVCS>1000 AU, representing the propagation phase of AVC). All patients gave written informed consent. The study was approved by the appropriate Ethics Committee (BMEÜ/3473-1/2022/EKU). The study protocol complied with the declaration of Helsinki.

Blood sampling

Blood samples were drawn from fasting patients immediately prior to CCTA examination. All patients were contacted before enrolment to ensure appropriate preparation (avoidance of consumption anything apart from water at least 8 hours before blood sampling). Blood for PPi measurement was drawn into a K3EDTA vacuum tube using a 21 G or thicker branule. Careful attention was paid to avoid any unnecessary tissue injury that could interfere with PPi measurements. If the vein puncture was not successful at first, the second puncture was performed on the contralateral arm. If the second puncture was not successful, the patient was not included in the study. Complete routine clinical blood tests, including a comprehensive lipid panel with Lp(a) measurement were also conducted.

Inorganic pyrophosphate concentration measurement

The plasma fraction from the blood samples was isolated via two consecutive centrifugations (1000g for 5 minutes at room temperature, repeatedly) and transferred into platelet separation tubes (Centristart® 300.000 MW, Sartorius, Göttingen, Germany). The platelet-free plasma was obtained by further centrifugation at 2200g for 30 minutes at room temperature and stored at -80˚C until PPi measurement (conducted within 3-months).

The PPi content of the plasma was determined enzymatically, using the ATP sulfurylase bioluminescent method. First, PPi was converted into ATP in an assay containing 80µM MgCl2, 50mM HEPES pH 7.4, 32mU/ml ATP Sulfurylase (MO394L, New England Biolabs,

Ipswich, MA, USA) and 8µM adenosine 5´-phosphosulfate (A5508, Sigma-Aldrich, Saint Louis, MO, USA). Samples were incubated at 37°C for 30 minutes, followed by enzyme inactivation at 90°C for 10 minutes. In the next step, ATP levels were evaluated utilizing the BacTiterGlo (Promega, Madison, WI, USA) bioluminescent assay, prepared according to the manufacturer's instructions, and analyzed in a multimode plate reader (PerkinElmer). Plasma PPI concentrations were calculated using calibration standards and correction for initial plasma ATP concentrations.(37) The precision of the PPi assay was determined from replicate measurements with aliquots of platelet free plasma pooled from 3 different patients. The intra-assay precision values were as follows; standard deviation (SD) = 0.037µM, coefficient of deviation (CV) 1.81%; the inter-assay precision was: SD = 0.052µM, CV = 2.54%. The limit of detection (LOD) was 0.17µM, the lower limit of quantification (LLOQ) was 0.52µM. Data were obtained using analytical PPi standards (Biothema, Sweden).

Cardiac computed tomography angiography and calcium load measurement

The CCTA examinations were performed using a dedicated cardiac CT scanner (CardioGraphe, GE Healthcare, Chicago, IL, USA) or a third-generation dual source CT scanner (SOMATOM Force, Siemens Healthineers, Forchheim, Germany) with prospectively ECG triggered axial acquisition mode. A tube voltage of 120 kVp was used in all patients and the tube current was adjusted to patient size.

Before contrast administration native images with slice thickness of 2 mm were acquired in all cases, as part of our clinical routine. AVCS measurements were performed offline on a dedicated workstation equipped with commercially available software (HeartBeat CS, Philips Healthcare, Cleveland, OH, USA) [10], using non-contrast images. A single investigator conducted all measurements, identifying both valve leaflet and annular calcifications on axial slices.

Statistical analysis

Continuous variables were tested for normality using the Shapiro-Wilk test. Normally distributed continuous variables are presented as mean±SD, non-normally distributed variables as median [interquartile ranges]. Categorical variables are presented as

frequencies (percentages). Variables with normal distribution were compared using unpaired Student's T-test. The three AVCS groups were compared by ANOVA test. Variables with non-normal distribution were compared using the Mann-Whitney U test. Frequencies were compared using the Fischer's exact test. Determinants of plasma PPi level and AVCS were assessed using univariate and multivariate linear regression models, with or without interaction effects between explanatory factors. Explanatory variables included in the AVCS predictive models apart from PPi, Pi and Pi/PPi ratio (age, sex, HT, DM, BMI, renal function, statin treatment, LDL-C and Lp(a) level) were chosen based on clinical relevance, established by previous research.(38) The same variables were tested to predict plasma PPi level, in addition to plasma Pi concentration and alkaline phosphatase (ALP) activity, which latter were considered to have a potential impact on plasma PPi.

RESULTS

Patient population

Within the enrolment period, 243 patients were screened. Based on the inclusion and exclusion criteria 171 patients were enrolled, of whom 17 were subsequently excluded due to technical considerations with the CT images (lack of non-contrast images, or intensive artifacts), or the blood sampling and analysis (for details see Figure S1). Accordingly, the final study population consisted of 154 individuals (mean age:67±12 years, female:42.5%).

Sixty-five patients (42%) had some degree of AVC, 39 of them had AVCS>1000 AU. The distribution of the AVCS values in the population are shown on Figure S2. Forty-three patients had severe AS by echocardiographic criteria (defined by an aortic valve area ≤1 cm^2, based on the continuity equation), 39 of them displayed severe AV calcification (AVCS>1000 AU), whereas 4 patients had a predominantly fibrotic phenotype with only moderate degree of AV calcification. Four patients had bicuspid aortic valves, three with massive, one with only trace AVC. Although GFR<30 ml/min/1.73 m^2 according to the referring test was an exclusion criterion, on the day of the examination four patients had GFR values under this cut-off, these patients were included in the analyses. Demographic and clinical data of the population are shown in Table 1.

Group comparisons according to different degrees of AVC

Patients with AVC were older, more frequently hypertensive and had lower GFR, compared to the non-calcified patients. Statin treatment was more common in the heavily calcified group, accordingly their LDL-C level was lower, along with similar Lp(a) values. The plasma Pi and PPi concentration was similar among patients with different degrees of AVC, however, the Pi/PPi ratio was significantly higher among patients with severe AVC (Table1).

Plasma PPi levels and its determinants

The plasma PPi concentration in the cohort was 1.60±0.41 μM, similar in patients with and without AVC (1.61±0.43 vs. 1.59±0.39 μM; p=0.97).

Female patients had higher PPi concentrations compared to men, however the difference did not reach the level of statistical significance (1.68±0.5 vs. 1.55±0.4 μM; p=0.052). While no univariate association between PPi level and age was found, after controlling for age, women had significantly higher estimated PPi level than men (PPi – female association: main effects model estimate 0.149; SE 0.069, p=0.033; female–age interaction estimate -0.005; SE 0.006, p=0.359) (Figure 1A).

In linear regression analysis, plasma PPi levels were significantly positively associated with plasma Pi level and negatively associated with ALP activity (Table 2).

Interestingly, plasma PPi concentration showed a significant positive association with LDL-C in the entire cohort (p=0.003), and among statin naïve patients (p=0.001). Statin use itself did not correlate with plasma PPi concentration. However, when we tested the impact of statin use on the PPi - LDL-C association, we found a significant interaction effect, even when controlling for age. Among statin naïve patients LDL-C was positively associated with PPi, but for patients on statin therapy, the LDL-C - PPi relationship was inverse (statin – LDL-C interaction: estimate -0.241, SE 0.084, p=0.005). (Supplementary file, Figure S3A and and Table S1A). To dissect, whether the observed LDL-C – PPi association was present throughout both phases of AVC, we analysed the mild-to-moderate and severe AVC

subgroups separately. The significant association between the two compounds was limited to patients in the initiation phase of AVC (Table 2, Figure S4).

No significant association was found between plasma PPi and Lp(a) levels, or any of the other tested traditional risk factors of aortic valve calcification, including HT, DM, BMI and GFR (Table 2).

With regard to determinants of the Pi/PPi ratio, BMI and plasma ALP activity showed significant associations, whereas age was a significant determinant only in the highly calcified group. (Table 3)

Patients with bicuspid AVs had similar PPi levels (1.34±0.43 vs. 1.61±0.41, p=0.299), but significantly higher Pi/PPi ratio (0.97±0.36 vs. 0.72±0.18, p=0.012) compared to those with tricuspid AVs, although these comparisons are largely limited by the low number of bicuspid valves.

AVCS and its determinants

The median AVCS among patients with AVC was 1802 AU [128;2917], range: 9-6641 AU. In the overall population, AVCS did not differ between men and women (median: 0 for both; p=0.398). However, when controlling for age, men had higher AVCS values than women (p=0.042) (Figure 1B)

In univariate analysis, AVCS was significantly and positively associated with age, HT, and Lp(a) level, and inversely associated with GFR. In contrast, no association with sex, BMI, DM and plasma Pi or Ca level, or ALP activity was found. (Table 4A, Figure 2)

Looking at the entire cohort, there was a significant negative association between AVCS and plasma LDL-C concentration, whereas AVCS was positively associated with statin use. As AVCS strongly depends on age, we tested whether statin use had a differential impact on the LDL-C-AVCS association in various age groups and found that in general, the association was estimated to be negative, even when controlling for age, except for younger patients on statin therapy, where the interaction model showed that the tendency of the association was weakly positive at high levels of LDL-C. (Supplementary Figure S3B and Table S1B)

Plasma PPi level did not show an association with AVCS. When assessing the impact of patients' age and sex on the relationship between AVCS and PPi, the interaction model

suggested that there was a difference in the AVCS – PPi association by sex for older patients, but only age had a significant effect on AVCS (estimate 120; SE 49.1; p=0.016; all interactions for age-sex and PPi were non-significant) (Supplementary Figure S5 and Table S2). Investigation of a potential effect of plasma LDL-C level and statin use on the association between plasma PPi and AVCS demonstrated no interaction between any of these parameters (Table S3).

Regarding the Pi/PPi ratio, in univariate analysis, it was a significant determinant of the AVCS. When combining all the traditional risk parameters with Pi/PPi ratio in a multiparametric model, only age and the Pi/PPi ratio remained significant predictors of AVCS (Table 4B, Figure 2). The study design inevitably resulted in a highly skewed distribution of AVCS values in our cohort. To control for this circumstance, the regression model was also built using log-transformed AVCS as a sensitivity analysis(39) (Table S4), which provided similar results.

Separate analyses of the determinants of AVCS specifically among patients with severe AS, and among those with GFR $\geq$ 30 mL/min/1.73m^2 according to the index laboratory test are provided in Tables S5 and S6.

DISCUSSION

This is the first study to investigate the association of plasma PPi level and Pi/PPi ratio with AVC in vivo. The main novel findings of the present work conducted on a prospective cohort of 154 cardiovascular patients are the following:1) patients with AVC, particularly those with severe AS, had significantly higher plasma Pi/PPi ratio compared to those without any AVC, despite similar plasma PPi levels; 2) the plasma Pi and PPi levels did not show an association with AVCS, but the Pi/PPi ratio was a significant determinant, even after adjustment for traditional risk factors; 3) circulating PPi concentration showed a significant positive association with LDL-C in the entire cohort as well as among statin naïve patients, but not among those with severe AVC.

Despite the accumulating evidence for the role of disturbed PPi homeostasis and Pi – PPi equilibrium in the pathophysiology of ectopic calcification, data on the relationship of circulating PPi and the Pi/PPi ratio with pathological calcification are scarce. The few studies that measured human plasma PPi level mainly focused on its association with vascular calcification, dominantly in PXE or end stage kidney disease patients. The association between plasma PPi concentration and AV calcium load has not been investigated.

The dependence of plasma PPi concentration on age and sex

In our non-PXE, non-CKD cohort, PPi levels did not significantly vary with age. However, we observed a trend toward decreasing PPi with advancing age in females.

Previous reports have shown a rather counterintuitive positive association between age and plasma PPi concentration in CKD patients, female PXE patients and heterozygous ABCC6 mutation carriers, but not in male PXE patients or in healthy individuals.(32, 40-43)

Regarding the impact of sex, Leftheriotis and colleagues reported that among PXE patients, women had higher PPi levels than men,(40) however, another study including 200 non-PXE children and adolescents found no difference between PPi levels in the two sexes.(42) In the present study, women had higher PPi concentration than men, when controlling for age.

LDL-C and statin associations

In our study, plasma PPi showed a significant positive association with LDL-C. Despite both compounds being attributed central roles in ectopic calcification, the relationship of plasma lipids and PPi was not investigated before.

At the initial stage of AS, characterised by lipid infiltration of the aortic cusps and progressive inflammation in response to lipid oxidation, regions of stippled micro-calcification that colocalize with sites of lipid deposition are observed.(44) The apparent link between lipid accumulation, inflammation and calcification in the process of AVC has led to the hypothesis that statins might be efficient to prevent or decelerate the progression of AS. Statin treatment has been shown to diminish the rate of cardiovascular events in subjects with atherosclerotic disease by lowering LDL-C cholesterol level.(45) At the same time, coronary artery calcification tends to increase with statin treatment,(46, 47) and increased calcification of

valve cells have also been shown ("statin paradox").(48-50) While "plaque stabilization" appears protective against future events in the context of coronary artery disease, this pro-calcific effect might explain why clinical trials failed to show benefit of statins in AS, in terms of disease progression or cardiovascular outcomes.

We found no direct association between statin use and plasma PPi levels, however there was a significant interaction effect of statin use on the PPi - LDL-C association.

On the other hand, with regards to their impact on AVCS, no interaction between PPi, LDL-C and statin use could be demonstrated. Interestingly, the significant association between PPi and LDL-C was limited to patients with mild to moderate AVC and not present in patients with severe AVC.

The complex interplay between phosphate- and lipid homeostasis is also attested by recent evidence demonstrating that plasma Pi influences de novo cholesterol synthesis in vascular smooth muscle cells and macrophages by increasing 3-hydroxy-3-methylglutaryl coenzyme A.(51, 52) This raises the possibility that the well-documented pro-atherogenic effect of hyperphosphatemia might be transmitted through mechanisms other than its direct pro-calcific action.

Although the explanation of the strong association between plasma PPi and LDL-C significantly impacted by statin use remains to be understood, it highlights the complexity of the promoting and modifying factors of the active process of AVC.

Plasma versus local PPi

In univariate analysis, plasma PPi level did not show an association with the degree of AVC. Although decreased extracellular PPi concentration has been proposed as a unifying pathomechanism of ectopic calcification, whether circulating PPi levels can predict pathological calcification remains elusive. Several genetic disorders that feature ectopic calcification in association with dysregulated extracellular PPi homeostasis, like PXE and GACI, are characterised by reduced plasma PPi levels. Plasma PPi concentration in these patients often correlate with the severity of ectopic calcification.(53, 54) However, it has become clear that circulating PPi level does not necessarily reflect local tissue production.(37, 55) It was demonstrated in experimental models, that normal circulating PPi

levels can overcome the absence of local PPi production in various mutant backgrounds,(56) and vice versa, local PPi production in the vessel wall can partially prevent vascular calcification in the presence of low plasma PPi concentration.(54, 56) While it is yet unclear which components of PPi homeostasis act locally and which influence plasma levels, the lack of direct association between plasma PPi and AVCS might be explained by the complex regulation and effects of local and systemic PPi concentrations.

Additionally to its anti-mineralization function, PPi has also been attributed a signalling role to influence gene expression and cellular behaviour in mineralizing cells.(57) This dual role—both as a direct inhibitor of crystal formation and as a regulator of calcification-related gene expression—offers a new perspective on how PPi homeostasis could be essential in preventing pathological calcification and potentially provides a pathway amenable for medical intervention.

Clinical perspective

Orally administered PPi substantially raised plasma PPi and could significantly rescue the calcification phenotype in PXE and GACI mouse models.(26) PPi is a non-toxic, physiologic metabolite, recognized as safe by the US Food and Drug Administration (FDA) and widely used in the food industry.

The PROPHECI phase II randomized trial is now ongoing,(58) to provide safety and efficacy data of oral PPi administration to halt calcification in PXE. In the light of our results, PPi also holds major promise as a potential therapeutic strategy to prevent or decelerate aortic valve calcification (Central illustration).

An important aspect of therapeutic PPi supplementation that needs consideration is its potential impact on osteogenic processes. PPi plays a complex role in bone metabolism, with both pro-osteogenic and anti-mineralization effects. Low doses of PPi can stimulate osteogenic differentiation, however, high doses, through inhibition of hydroxyapatite formation, may lead to hypomineralized bone and osteomalacia.(59) Therefore, therapeutic modulation of PPi homeostasis must ponder the balance between inhibition and promotion of mineralization in various tissues.

Limitations

Our cohort size is limited, although among the few publications looking at interaction between plasma PPi level and tissue calcification, the present work represents the largest. Our aim was to study a cohort with a wide range of AVC load and numerous cardiovascular risk factors, however only a handful of patients had moderate AVC (AVCS 0-1000). Therefore, the distribution of AVCS in our study population is rather uneven which might importantly influence the results. A single CT examination provides cross-sectional information about AVC in the context of risk factors, but in the absence of repeated scans, the rate of progression could not be studied. We reconstructed the CCTA images with 2 mm slice thickness, which could slightly affect the measured AVCS values; however, this is unlikely to alter the overall conclusions.

Plasma PPi homeostasis is delicately regulated. There is a known circadian fluctuation of plasma PPi concentration, additionally individual PPi concentration variability is increased after food consumption and intense exercise.(60) In our study meticulous attention was paid to standardise the conditions of PPi sampling, by careful preparation of the patients before blood draw and avoidance of any unnecessary tissue injury. Nonetheless, repeated sampling and PPi measurement could have further refined the individual patients' average PPi level. However, exactly due to the labour-intensive preparation, sampling and biochemical analysis, this was not feasible. Vitamin D and parathyroid hormone levels are also known to influence Pi homeostasis and might affect AV calcification. Although no patient had clinically manifest hypo- or hyperparathyroidism, unfortunately, these parameters were not directly measured in our study, which must be acknowledged as a limitation.

As discussed above, plasma PPi level might not reliably reflect local PPi concentration. Direct PPi measurement in the valvular milieu could have provided valuable information, however, unfortunately no method for determining extracellular PPi concentration in tissues is currently available.

Conclusion

There is a major unmet clinical need to identify pharmacological treatments capable of modifying AVC. This is the first study to relate AVC to the Pi/PPi imbalance. Our results provide

further insight into the complexity of PPi homeostasis and its role in pathological mineralization and pave the way for further research to fill the gaps in our knowledge regarding the role of PPi in aortic valve calcification. Anti-calcific therapies e.g. by PPi supplementation hold major promise as a method of treatment to prevent or decelerate AS progression.

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Conflict of interest: FSz is co-inventor on patent applications related to the use of pyrophosphate for therapeutic applications. Application NLA2017471, entitled 'Oral Pyrophosphate For Use In Reducing Tissue Calcification', is continued as WO 2018/052290, EP 3 512 530 B1, and US 11,504,395 B2.

Data availability statement

The data underlying this article will be shared on reasonable request to the corresponding author.

REFERENCES

1. Nkomo VT; Gardin JM; Skelton TN; Gottdiener JS; Scott CG; Enriquez-Sarano M. Burden of valvular heart diseases: a population-based study. Lancet. 2006;368(9540):1005-11.
2. Osnabrugge RL; Mylotte D; Head SJ; Van Mieghem NM; Nkomo VT; LeReun CM, et al. Aortic stenosis in the elderly: disease prevalence and number of candidates for transcatheter aortic valve replacement: a meta-analysis and modeling study. J Am Coll Cardiol. 2013;62(11):1002-12.
3. Vavilis G; Back M; Barany P; Szummer K. Epidemiology of Aortic Stenosis/Aortic Valve Replacement (from the Nationwide Swedish Renal Registry). Am J Cardiol. 2022;163:58-64.
4. Dweck MR; Boon NA; Newby DE. Calcific aortic stenosis: a disease of the valve and the myocardium. J Am Coll Cardiol. 2012;60(19):1854-63.
5. Goody PR; Hosen MR; Christmann D; Niepmann ST; Zietzer A; Adam M, et al. Aortic Valve Stenosis: From Basic Mechanisms to Novel Therapeutic Targets. Arterioscler Thromb Vasc Biol. 2020;40(4):885-900.
6. Akahori H; Tsujino T; Masuyama T; Ishihara M. Mechanisms of aortic stenosis. J Cardiol. 2018;71(3):215-20.
7. Katz R; Wong ND; Kronmal R; Takasu J; Shavelle DM; Probstfield JL, et al. Features of the metabolic syndrome and diabetes mellitus as predictors of aortic valve calcification in the Multi-Ethnic Study of Atherosclerosis. Circulation. 2006;113(17):2113-9.
8. Kaiser Y; van der Toorn JE; Singh SS; Zheng KH; Kavousi M; Sijbrands EJG, et al. Lipoprotein(a) is associated with the onset but not the progression of aortic valve calcification. Eur Heart J. 2022;43(39):3960-7.
9. Khurrami L; Moller JE; Lindholt JS; Urbonaviciene G; Steffensen FH; Lambrechtsen J, et al. Cross-sectional study of aortic valve calcification and cardiovascular risk factors in older Danish men. Heart. 2021;107(19):1536-43.
10. Yan AT; Koh M; Chan KK; Guo H; Alter DA; Austin PC, et al. Association Between Cardiovascular Risk Factors and Aortic Stenosis: The CANHEART Aortic Stenosis Study. J Am Coll Cardiol. 2017;69(12):1523-32.
11. Vavilis G; Back M; Occhino G; Trevisan M; Bellocco R; Evans M, et al. Kidney Dysfunction and the Risk of Developing Aortic Stenosis. J Am Coll Cardiol. 2019;73(3):305-14.
12. Lu C; Dong X; Yu WP; Ding JL; Yang W; Gong Y, et al. Inorganic phosphate-osteogenic induction medium promotes osteogenic differentiation of valvular interstitial cells via the BMP-2/Smad1/5/9 and RhoA/ROCK-1 signaling pathways. Am J Transl Res. 2020;12(7):3329-45.
13. Linefsky JP; O'Brien KD; Sachs M; Katz R; Eng J; Michos ED, et al. Serum phosphate is associated with aortic valve calcification in the Multi-ethnic Study of Atherosclerosis (MESA). Atherosclerosis. 2014;233(2):331-7.

14. Candellier A; Diouf M; Bohbot Y; Bennis Y; Pasquet A; Goffin E, et al. Association Between Serum Phosphate Levels and Progression of Aortic Stenosis. JACC Adv. 2024;3(8):101096.
15. Rajamannan NM. Serum phosphate concentrations: a novel pre-clinical biomarker for cardiovascular calcification. J Am Coll Cardiol. 2011;58(3):298-9.
16. Côté N; El Husseini D; Pépin A; Guauque-Olarte S; Ducharme V; Bouchard-Cannon P, et al. ATP acts as a survival signal and prevents the mineralization of aortic valve. J Mol Cell Cardiol. 2012;52(5):1191-202.
17. Côté N; El Husseini D; Pépin A; Bouvet C; Gilbert LA; Audet A, et al. Inhibition of ectonucleotidase with ARL67156 prevents the development of calcific aortic valve disease in warfarin-treated rats. Eur J Pharmacol. 2012;689(1-3):139-46.
18. Shimada BK; Pomozi V; Zoll J; Kuo S; Martin L; Le Saux O. ABCC6, Pyrophosphate and Ectopic Calcification: Therapeutic Solutions. Int J Mol Sci. 2021;22(9).
19. Rebbe NF; Tong BD; Finley EM; Hickman S. Identification of nucleotide pyrophosphatase/alkaline phosphodiesterase I activity associated with the mouse plasma cell differentiation antigen PC-1. Proc Natl Acad Sci U S A. 1991;88(12):5192-6.
20. Favre G; Laurain A; Aranyi T; Szeri F; Fulop K; Le Saux O, et al. The ABCC6 Transporter: A New Player in Biomineralization. Int J Mol Sci. 2017;18(9).
21. Bäck M; Michel JB. From organic and inorganic phosphates to valvular and vascular calcifications. Cardiovasc Res. 2021;117(9):2016-29.
22. Ringpfeil F; Lebwohl MG; Christiano AM; Uitto J. Pseudoxanthoma elasticum: mutations in the MRP6 gene encoding a transmembrane ATP-binding cassette (ABC) transporter. Proc Natl Acad Sci U S A. 2000;97(11):6001-6.
23. Bergen AA; Plomp AS; Schuurman EJ; Terry S; Breuning M; Dauwerse H, et al. Mutations in ABCC6 cause pseudoxanthoma elasticum. Nat Genet. 2000;25(2):228-31.
24. Rutsch F; Ruf N; Vaingankar S; Toliat MR; Suk A; Höhne W, et al. Mutations in ENPP1 are associated with 'idiopathic' infantile arterial calcification. Nat Genet. 2003;34(4):379-81.
25. Villa-Bellosta R; Egido J. Phosphate, pyrophosphate, and vascular calcification: a question of balance. Eur Heart J. 2017;38(23):1801-4.
26. Dedinszki D; Szeri F; Kozák E; Pomozi V; Tőkési N; Mezei TR, et al. Oral administration of pyrophosphate inhibits connective tissue calcification. EMBO Mol Med. 2017;9(11):1463-70.
27. Rathan S; Yoganathan AP; O'Neill CW. The role of inorganic pyrophosphate in aortic valve calcification. J Heart Valve Dis. 2014;23(4):387-94.
28. Rattazzi M; Bertacco E; Iop L; D'Andrea S; Puato M; Buso G, et al. Extracellular pyrophosphate is reduced in aortic interstitial valve cells acquiring a calcifying profile: implications for aortic valve calcification. Atherosclerosis. 2014;237(2):568-76.
29. Rubera I; Clotaire L; Laurain A; Destere A; Martin L; Duranton C, et al. A Plasma Pyrophosphate Cutoff Value for Diagnosing Pseudoxanthoma Elasticum. Int J Mol Sci. 2024;25(12).
30. Lundkvist S; Niaziorimi F; Szeri F; Caffet M; Terry SF; Johansson G, et al. A new enzymatic assay to quantify inorganic pyrophosphate in plasma. Anal Bioanal Chem. 2023;415(3):481-92.

31. Veiga-Lopez A; Sethuraman V; Navasiolava N; Makela B; Olomu I; Long R, et al. Plasma Inorganic Pyrophosphate Deficiency Links Multiparity to Cardiovascular Disease Risk. Front Cell Dev Biol. 2020;8:573727.
32. O'Neill WC; Sigrist MK; McIntyre CW. Plasma pyrophosphate and vascular calcification in chronic kidney disease. Nephrol Dial Transplant. 2010;25(1):187-91.
33. Villa-Bellosta R; O'Neill WC. Pyrophosphate deficiency in vascular calcification. Kidney Int. 2018;93(6):1293-7.
34. Nollet L; Van Gils M; Verschuere S; Vanakker O. The Role of Vitamin K and Its Related Compounds in Mendelian and Acquired Ectopic Mineralization Disorders. Int J Mol Sci. 2019;20(9).
35. Drake MT; Clarke BL; Khosla S. Bisphosphonates: mechanism of action and role in clinical practice. Mayo Clin Proc. 2008;83(9):1032-45.
36. Fülöp K; Jiang Q; Wetering KV; Pomozi V; Szabó PT; Arányi T, et al. ABCC6 does not transport vitamin K3-glutathione conjugate from the liver: relevance to pathomechanisms of pseudoxanthoma elasticum. Biochem Biophys Res Commun. 2011;415(3):468-71.
37. Jansen RS; Duijst S; Mahakena S; Sommer D; Szeri F; Váradi A, et al. ABCC6-mediated ATP secretion by the liver is the main source of the mineralization inhibitor inorganic pyrophosphate in the systemic circulation-brief report. Arterioscler Thromb Vasc Biol. 2014;34(9):1985-9.
38. Bäck M; Larsson SC. Risk factors for aortic stenosis. e-Journal of Cardiology Practice: European Society of Cardiology; 2020.
39. Owens DS; Katz R; Takasu J; Kronmal R; Budoff MJ; O'Brien KD. Incidence and progression of aortic valve calcium in the Multi-ethnic Study of Atherosclerosis (MESA). Am J Cardiol. 2010;105(5):701-8.
40. Leftheriotis G; Navasiolava N; Clotaire L; Duranton C; Le Saux O; Bendahhou S, et al. Relationships between Plasma Pyrophosphate, Vascular Calcification and Clinical Severity in Patients Affected by Pseudoxanthoma Elasticum. J Clin Med. 2022;11(9).
41. Van Gils M; Depauw J; Coucke PJ; Aerts S; Verschuere S; Nollet L, et al. Inorganic Pyrophosphate Plasma Levels Are Decreased in Pseudoxanthoma Elasticum Patients and Heterozygous Carriers but Do Not Correlate with the Genotype or Phenotype. J Clin Med. 2023;12(5).
42. Bernhard E; Nitschke Y; Khursigara G; Sabbagh Y; Wang Y; Rutsch F. A Reference Range for Plasma Levels of Inorganic Pyrophosphate in Children Using the ATP Sulfurylase Method. J Clin Endocrinol Metab. 2022;107(1):109-18.
43. Kozák E; Bartstra JW; de Jong PA; Mali WPTM; Fülöp K; Tőkési N, et al. Plasma Level of Pyrophosphate Is Low in Pseudoxanthoma Elasticum Owing to Mutations in the ABCC6 Gene, but It Does Not Correlate with ABCC6 Genotype. J Clin Med. 2023;12(3).
44. Otto CM; Kuusisto J; Reichenbach DD; Gown AM; O'Brien KD. Characterization of the early lesion of 'degenerative' valvular aortic stenosis. Histological and immunohistochemical studies. Circulation. 1994;90(2):844-53.
45. Collins R; Reith C; Emberson J; Armitage J; Baigent C; Blackwell L, et al. Interpretation of the evidence for the efficacy and safety of statin therapy. Lancet. 2016;388(10059):2532-61.

46. Raggi P; Davidson M; Callister TQ; Welty FK; Bachmann GA; Hecht H, et al. Aggressive versus moderate lipid-lowering therapy in hypercholesterolemic postmenopausal women: Beyond Endorsed Lipid Lowering with EBT Scanning (BELLES). Circulation. 2005;112(4):563-71.
47. Schmermund A; Achenbach S; Budde T; Buziashvili Y; Förster A; Friedrich G, et al. Effect of intensive versus standard lipid-lowering treatment with atorvastatin on the progression of calcified coronary atherosclerosis over 12 months: a multicenter, randomized, double-blind trial. Circulation. 2006;113(3):427-37.
48. Wu B; Elmariah S; Kaplan FS; Cheng G; Mohler ER. Paradoxical effects of statins on aortic valve myofibroblasts and osteoblasts: implications for end-stage valvular heart disease. Arterioscler Thromb Vasc Biol. 2005;25(3):592-7.
49. Rutkovskiy A; Malashicheva A; Sullivan G; Bogdanova M; Kostareva A; Stensløkken KO, et al. Valve Interstitial Cells: The Key to Understanding the Pathophysiology of Heart Valve Calcification. J Am Heart Assoc. 2017;6(9).
50. Monzack EL; Masters KS. A time course investigation of the statin paradox among valvular interstitial cell phenotypes. Am J Physiol Heart Circ Physiol. 2012;303(7):H903-9.
51. Zhou C; He Q; Gan H; Zeng T; Liu Q; Moorhead JF, et al. Hyperphosphatemia in chronic kidney disease exacerbates atherosclerosis via a mannosidases-mediated complex-type conversion of SCAP N-glycans. Kidney Int. 2021;99(6):1342-53.
52. Hénaut L; Massy ZA. Phosphate meeting cholesterol-consequences for cardiovascular disease in chronic kidney disease? Kidney Int. 2021;99(6):1264-7.
53. Ralph D; van de Wetering K; Uitto J; Li Q. Inorganic Pyrophosphate Deficiency Syndromes and Potential Treatments for Pathologic Tissue Calcification. Am J Pathol. 2022;192(5):762-70.
54. Ralph D; Levine M; Millán JL; Uitto J; Li Q. Weighing the Evidence for the Roles of Plasma Versus Local Pyrophosphate in Ectopic Calcification Disorders. J Bone Miner Res. 2023;38(4):457-63.
55. Li Q; Kingman J; van de Wetering K; Tannouri S; Sundberg JP; Uitto J. Abcc6 Knockout Rat Model Highlights the Role of Liver in PPi Homeostasis in Pseudoxanthoma Elasticum. J Invest Dermatol. 2017;137(5):1025-32.
56. Lomashvili KA; Narisawa S; Millán JL; O'Neill WC. Vascular calcification is dependent on plasma levels of pyrophosphate. Kidney Int. 2014;85(6):1351-6.
57. Bourne LE; Davies BK; Millan JL; Arnett TR; Wheeler-Jones CPD; Keen JAC, et al. Evidence that pyrophosphate acts as an extracellular signalling molecule to exert direct functional effects in primary cultures of osteoblasts and osteoclasts. Bone. 2023;176:116868.
58. Clotaire L; Rubera I; Duranton C; Gal J; Chamorey E; Humeau H, et al. The PROPHECI trial: a phase II, double-blind, placebo-controlled, randomized clinical trial for the treatment of pseudoxanthoma elasticum with oral pyrophosphate. Trials. 2025;26(1):30.
59. Orriss IR; Arnett TR; Russell RG. Pyrophosphate: a key inhibitor of mineralisation. Curr Opin Pharmacol. 2016;28:57-68.

60. Khursigara G; Huertas P; Wenkert D; O'Brien K; Sabbagh Y. Effects of food, fasting, and exercise on plasma pyrophosphate levels and ENPP1 activity in healthy adults. Bone. 2023;171:116750.

TABLES

Table 1. Baseline demographic, clinical, and imaging data of the patient cohort stratified by aortic valve calcium score.

	Entire population (n=154)	No AVC (AVCS=0) (n=89)	Mild-to moderate AVC (0<AVCS<1000) (n=26)	Severe AVC (AVCS>1000) (n=39)	p-value
Demographic data and comorbidities					
Age (years)	67.0±12.2	61.1±12.0*	71.3±7.6ǂ	77.4±4.8#	**<0.001**
Female (n, %)	65 (42.5)	33 (37.5)	15 (57.7)	17 (43.6)	0.187
BMI (kg/m^2)	28.4±4.7	28.4±4.3	29.2±5.1	27.9±5.2	0.598
Hypertension (n, %)	100 (64.9)	48 (53.9)*	21 (80.8)	31 (79.5)#	**0.004**
Diabetes (n, %)	31 (20.1)	12 (13.5)	8 (30.8)	11 (28.2)	0.054
Statin treatment (n, %)	63 (40.9)	26 (29.2)	12 (46.2)	25 (64.1)#	**0.001**
eGFR [mL/min/1.73m^2]	73.4±16.6	78.4±12.8*	72.3±15.4ǂ	62.8±20.0#	**<0.001**
Laboratory data					
LDL-C (mmol/l)	3.14±0.94	3.34±0.93	3.13±0.95ǂ	2.67±0.80#	**0.001**
Lp(a) (g/l)	0.42±0.54	0.37±0.40	0.34±0.42	0.61±0.78	0.603
ALP (U/l)	79.8±40.6	76.1±24.4	78.9±30.7	88.7±67.1	0.921
Ca	2.50 ± 0.10	2.49 ± 0.09	2.51 ± 0.11	2.50 ± 0.11	0.616
Pi (mmol/l)	1.11±0.19	1.08±0.19	1.16±0.19	1.14±0.16	0.085
PPi (µmol/l)	1.60±0.41	1.61±0.43	1.67±0.40	1.53±0.39	0.435
Pi/PPi	0.73±0.19	0.70±0.16	0.73±0.20	0.80±0.24#	**0.040**
Imaging data					
AVCS (AU)	0 [0-1102]	0 [0-0]	87 [22-275]	2626 [2126-3811]	

Data presentation: frequencies (percentages), or mean±standard deviation or, median [1st and 3rd quartiles]. ALP, alkaline phosphatase; AVC, aortic valve calcification; AVCS, aortic valve calcium score; BMI, body mass index; eGFR, estimated glomerular filtration rate; LDL-C, low density lipoprotein; Lp(a), lipoprotein(a); ALP, alkaline phosphatase; Ca, serum calcium; Pi, inorganic phosphate; PPi, inorganic pyrophosphate.

*Indicates significant difference between groups 1 and 2, # Indicates significant difference between groups 1 and 3, ǂ Indicates significant difference between groups 2 and 3.

Table 2. Univariate regression coefficients for determinants of plasma PPi levels

Variable	All patients		Mild-to-moderate AVC		Severe AVCS	
	Estimate	p-value	Estimate	p-value	Estimate	p-value
Age	-0.0013 (0.0027)	0.644	-0.0057	0.603	0.0240	0.067
Female sex	0.1307(0.0668)	0.052	0.2029	0.210	0.0708	0.577
BMI	0.0102 (0.0071)	0.152	0.0049	0.762	0.0148	0.221
Hypertension	-0.0275 (0.0698)	0.695	-0.1067	0.604	0.1347	0.386
Diabetes	-0.0929 (0.0839)	0.270	-0.1190	0.497	0.0817	0.559
eGFR	-0.0020 (0.0020)	0.314	0.0003	0.950	-0.0054	0.083
Statin treatment	-0.0389 (0.0677)	0.567	-0.0683	0.674	-0.0961	0.463
LDL-C	0.0787 (0.0350)	**0.026**	0.1656	**0.047**	-0.0041	0.958
Lp(a)	-0.0260 (0.0624)	0.677	0.2586	0.182	-0.0386	0.635
Pi	0.7098 (0.1698)	**<0.001**	0.5610	0.189	0.0142	0.972
Ca	0.2875 (0.3374)	0.395	0.0076	0.992	0.2188	0.716
ALP	-0.0020 (0.0008)	**0.013**	0.0018	0.513	-0.0015	0.106

AVC, aortic valve calcification; AVCS, aortic valve calcium score; BMI, body mass index; eGFR, estimated glomerular filtration rate; LDL-C, low density lipoprotein; Lp(a), lipoprotein(a); Pi, inorganic phosphate; PPi, inorganic pyrophosphate; ALP, alkaline phosphatase; Ca, calcium

Table 3. Univariate regression coefficients for determinants of plasma Pi/PPi ratio

	All patients		Mild-to-moderate AVC		Severe AVC	
Variable	Estimate (SE)	p-value	Estimate (SE)	p-value	Estimate (SE)	p-value
Age	0.0014 (0.0013)	0.270	0.0037 (0.0054)	0.502	-0.0197 (0.0076)	**0.013**
Female sex	-0.0001 (0.0315)	0.998	0.0496 (0.0814)	0.549	-0.0423 (0.0780)	0.591
BMI	-0.0066 (0.0033)	**0.046**	-0.0072 (0.0080)	0.379	-0.0105 (0.0073)	0.161
Hypertension	-0.0107 (0.0326)	0.744	-0.1055 (0.1006)	0.305	-0.1017 (0.0947)	0.290
Diabetes	0.0548 (0.0386)	0.158	0.0501 (0.0873)	0.571	-0.0227 (0.0862)	0.793
eGFR	-0.0002 (0.0009)	0.817	0.0001 (0.0027)	0.958	0.0007 (0.0020)	0.722
Statin treatment	0.0376 (0.0315)	0.234	0.0568 (0.0805)	0.488	-0.0068 (0.0809)	0.933
LDL-C	-0.0225 (0.0165)	0.173	-0.0281 (0.0429)	0.519	0.0315 (0.0486)	0.521
Lp(a)	0.0271 (0.0291)	0.353	-0.1169 (0.0957)	0.233	0.0189 (0.0501)	0.709
Pi	0.3440 (0.0789)	**<0.001**	0.4698 (0.1950)	**0.024**	0.6431 (0.2274)	**0.008**
PPi	-0.3417 (0.0258)	**<0.001**	-0.3656 (0.0710)	**<0.001**	-0.5269 (0.0538)	**<0.001**

Ca	-0.0605 (0.1581)	0.702	0.4525 (0.3571)	0.217	-0.2523 (0.3681)	0.497
ALP	0.0016 (0.0004)	**<0.001**	0.0007 (0.0013)	0.583	0.0015 (0.0005)	**0.006**

BMI, body mass index, eGFR, estimated glomerular filtration rate; LDL-C, low density lipoprotein; Lp(a), lipoprotein(a); Pi, inorganic phosphate; PPi, inorganic pyrophosphate; ALP, alkaline phosphatase; Ca, calcium

Table 4. Univariate (A) and multivariate (B) regression models on AVCS.

A,

Univariate		
Variable	Estimate (Std. Error)	p-value
Age	56.5 (8.8)	**<0.001**
Sex (female)	1.8 (245.1)	0.994
BMI	-30.1 (25.7)	0.243
Diabetes	88.3 (300.4)	0.769
Hypertension	624.4 (247.4)	**0.013**
eGFR	-26.4 (6.9)	**<0.001**
Statin treatment	833.9 (235.5)	**0.001**
LDL-C	-386.3 (124.5)	**0.002**
Lp(a)	452.1 (222.8)	**0.044**
ALP	4.4 (3.0)	0.140
Ca	169.6 (1224.1)	0.890
Pi	551.2 (646.3)	0.395
PPi	-443.8 (290.9)	0.129
Pi/PPi	1508.1 (616.0)	**0.015**

B,

Multivariate		
Variable	Estimate (Std. Error)	p-value
Age	49.0 (11.2)	**<0.001**
Sex (female)	-335.9 (240.4)	0.165
BMI	-13.4 (24.3)	0.581
Diabetes	-448.7 (281.6)	0.113
Hypertension	218.5 (246.7)	0.377
eGFR	-4.3 (7.5)	0.567
Statin treatment	248.5 (261.3)	0.343
LDL-C	-111.1 (144.8)	0.444
Lp(a)	345.0 (201.7)	0.089
Pi/PPi	1128.6 (562.5)	**0.047**

1AVCS, aortic valve calcium score; BMI, body mass index; eGFR, estimated glomerular filtration rate; LDL-C, low
2density lipoprotein; Lp(a), lipoprotein(a); PPi, inorganic pyrophosphate, Pi, inorganic phosphate.
3

FIGURE LEGENDS

Figure 1. Dependence of plasma PPi and AVCS on age and sex.

Panel A: Estimated association of PPi and age in men and women, based on a regression model allowing for interaction between age and sex. Panel B: Estimated association of AVCS and age in men and women, based on a regression model allowing for interaction between age and sex. Dashed lines indicate 95% confidence intervals. AVCS, aortic valve calcification; PPi, Plasma inorganic pyrophosphate concentration.

Figure 2. Determinants of AVCS is univariate (A) and multivariate (B) analysis.

AVCS, aortic valve calcium score; BMI, body mass index; DM, diabetes mellitus; HT, hypertension; eGFR, estimated glomerular filtration rate; LDL-C, low density lipoprotein; Lp(a), Lipoprotein(a); Pi, inorganic phosphate; PPi, inorganic pyrophosphate.

Central illustration. Mechanisms of aortic valve calcification and a proposed role for PPi substitution.

ANKH, human protein product of the progressive ankylosis gene; PPi, inorganic pyrophosphate; Pi, inorganic phosphate; ATP, adenosine triphosphate; ABCC6, ATP binding cassette subfamily C member 6; ENPP-1, ectonucleotide pyrophosphatase/phosphodiesterase 1; TNAP, tissue non-specific alkaline phosphatase; HMGCR, 3-hydroxy-3-methyl-glutaryl coenzyme A reductase.

Graphical abstract

AVC, aortic valve calcification; AVCS, aortic valve calcium score; PPi, inorganic pyrophosphate; Pi, inorganic phosphate; BMI; body mass index; DM; diabetes mellitus; HT; arterial hypertension; eGFR; estimated glomerular filtration rate; LDL-C; low-density lipoprotein cholesterol; Lp(a), lipoprotein (a).

Figure 1. Dependence of plasma PPi and AVCS on age and sex

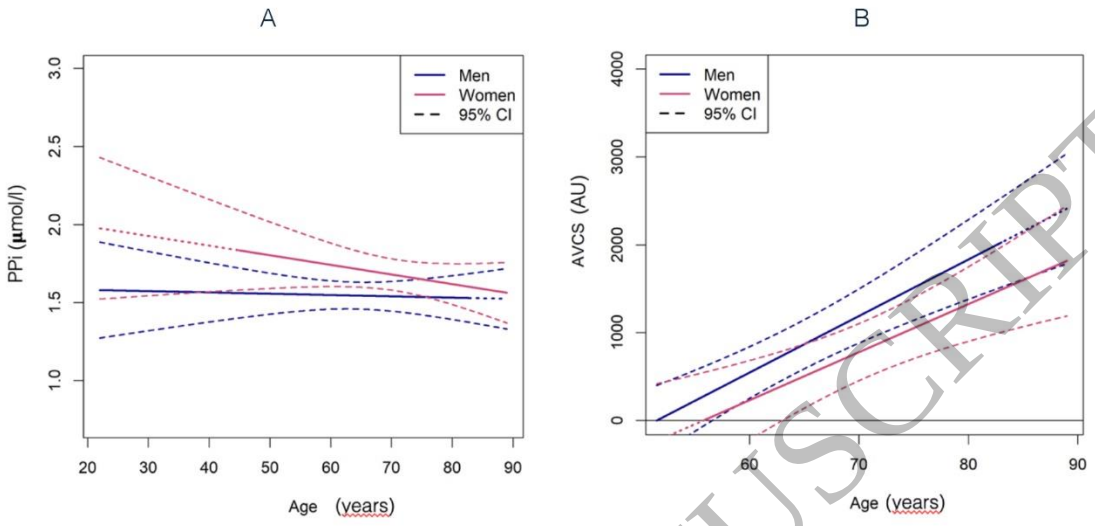
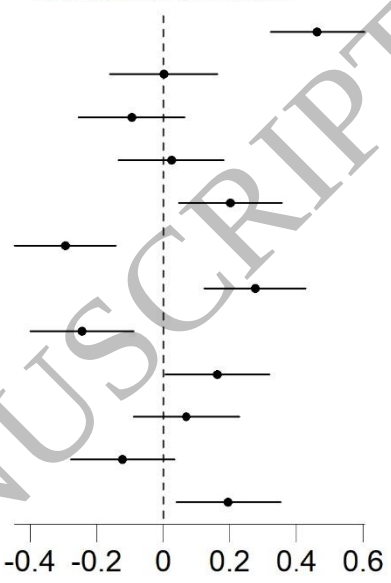


Figure 1
160x95 mm (x DPI)

Figure 2. Determinants of AVCS is univariate (A) and multivariate (B) analysis

A

Variable	Std. Coefficient (SE)	p-value
Age	0.463 (0.072)	<0.001
Female	0.001 (0.082)	0.994
BMI	-0.095 (0.081)	0.243
DM	0.024 (0.081)	0.769
HT	0.201 (0.079)	0.013
eGFR	-0.295 (0.078)	<0.001
Statin treatment	0.276 (0.078)	0.001
LDL	-0.244 (0.079)	0.002
Lp(a)	0.162 (0.080)	0.044
Pi	0.069 (0.081)	0.395
PPi	-0.123 (0.080)	0.129
Pi/PPi	0.195 (0.080)	0.015



B

Variable	Std. Coefficient (SE)	p-value
Age	0.402 (0.092)	<0.001
Female	-0.112 (0.080)	0.165
BMI	-0.042 (0.076)	0.581
DM	-0.121 (0.076)	0.113
HT	0.070 (0.079)	0.377
eGFR	-0.048 (0.084)	0.567
Statin treatment	0.082 (0.086)	0.343
LDL	-0.070 (0.091)	0.444
Lp(a)	0.124 (0.072)	0.089
Pi/PPi	0.146 (0.073)	0.047

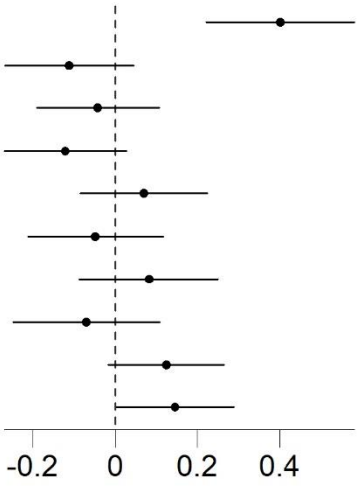


Figure 2
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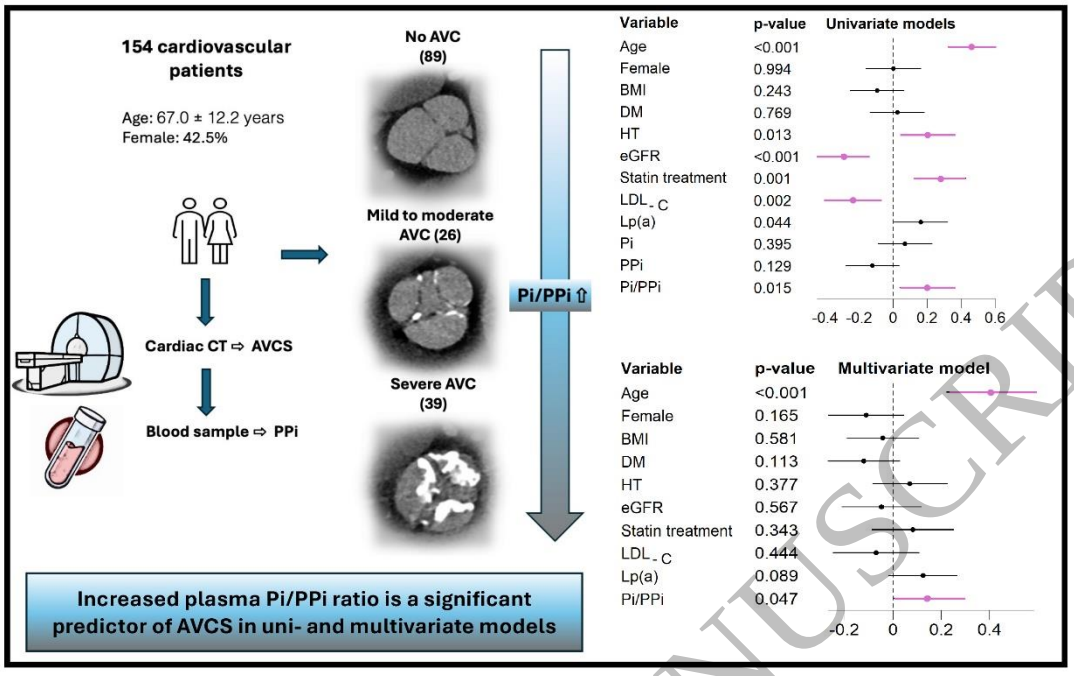

Graphical Abstract