

Original Article

Impact of ENPP1 genotype on arterial calcification in patients with end-stage renal failure

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Abstract

Background. Ectonucleotide pyrophosphatase/phosphodiesterase-1 (ENPP1) generates inorganic pyrophosphate, a solute that serves as an essential physiological inhibitor of calcification. Inactivating mutations of ENPP1 are associated with generalized arterial calcification of infancy. We hypothesized the ENPP1 K121Q variant to be associated with increased vascular calcification in patients with end-stage renal failure.

Subjects and methods. We recruited 79 patients with end-stage renal failure undergoing dialysis treatment and genotyped them for the ENPP1 K121Q polymorphism. Next, we matched to each patient with ENPP1 121KQ genotype ($n = 15$) a respective control with ENPP1 121KK genotype by gender, age, diabetes and duration of dialysis treatment. The matching ratio was 1:1. Severity of coronary calcification was quantified by computed tomography, and aortic stiffness was measured by pulse-wave analysis.

Results. Patients with ENPP1 121KQ genotype had a significantly higher coronary calcium score (1385 vs 94; $n = 30$; $P = 0.033$), and also a higher aortic pulse-wave velocity when compared to matched controls with ENPP1 121KK genotype (13.69 m/s vs 9.37 m/s; $P = 0.003$).

Conclusions. Taken together, our study suggests a potential role of the ENPP1 K121Q polymorphism in arterial calcification of patients with end-stage renal failure. Patients heterozygous for the ENPP1 K121Q polymorphism have higher coronary calcification scores and increased aortic stiffness, and may benefit from more intense treatment in order to prevent progression of arterial calcification.

Keywords: aortic stiffness; arterial calcification; insulin resistance; mineral metabolism; pulse-wave velocity; pyrophosphate

Background

Arterial calcification frequently develops in patients with atherosclerosis, diabetes mellitus and end-stage renal failure resulting in an increased risk of cardiovascular events [1–3]. Whereas arterial calcification in atherosclerosis is primarily intimal, calcification in aging, diabetes mellitus and chronic renal failure is predominantly located to the tunica media. Media calcification is due to ectopic ossification and a consequence of transdifferentiation of vascular smooth muscle cells to bone-forming chondrocytic or osteoblastic cells. Similarly to bone remodelling, arterial calcification is subject to regulation by several physiological inhibitors, such as osteopontin, fetuin-A or ectonucleotide pyrophosphatase/phosphodiesterase 1 (ENPP1) [4–7].

ENPP1 is a transmembrane glycoprotein with a short amino-terminal cytoplasmic tail, a single transmembrane domain and a large extracellular carboxyl-terminal domain. It forms disulfide-bond homodimers [8]. This cell surface enzyme generates inorganic pyrophosphate, a solute that regulates cell differentiation and serves as an essential physiological inhibitor of calcification. ENPP1 plays a central role in orthotopic, but also in heterotopic mineral deposition. Inactivating mutations of ENPP1 are associated with generalized arterial calcification of infancy (OMIM no 208000), a spontaneous and frequently lethal form of widely disseminated arterial media calcification [9]. ENPP1-deficient *ttw/ttw* mice develop a skeletal phenotype with spontaneous bone fusion of periarticular and perispinal soft tissues in early life leading to subsequent ossification of the posterior longitudinal

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ligament of the spine [10]. Common variants of ENPP1 such as the K121Q (rs1044498) polymorphism are associated with childhood obesity [11,12], increased risk of type 2 diabetes mellitus [11,13–16], early development of diabetic nephropathy [17] and myocardial infarction [14,18]. These associations were attributed to direct interaction of ENPP1 with the α -subunit of the insulin receptor and the modulation of its subsequent cellular signalling [19–21]. However, this association of common ENPP1 variants with obesity and type 2 diabetes mellitus was not uniformly reproduced in other populations [22–25]. There is only limited information on regulation of ENPP1 expression *in vivo*. Yamada and co-workers have recently shown that the cartilage-specific carminerin promotes chondrocyte calcification during endochondral ossification through transcriptional inhibition of ENPP1 [26].

All previously published clinical studies concerning the genotype–phenotype correlation of the ENPP1 gene were done in patients with a normal calcium phosphate homeostasis [11–16,18,22–25]. Up to date, there are no data available concerning the association of ENPP1 variants with severity of arterial calcification in patients with end-stage renal failure. Cardiovascular mortality in patients on dialysis is about 30 times higher than in general population, and is 10–20 times higher when stratified for age, sex and diabetes [27]. We hypothesized that the ENPP1 K121Q variant might synergistically combine with deranged mineral metabolism and hyperparathyroidism in patients on dialysis leading to extensive arterial calcification. The ENPP1 K121Q variant has lower enzymatic activity than the wildtype variant, and produces less inorganic pyrophosphate, which is a physiological inhibitor of calcification [9]. Genotyping of the ENPP1 gene could thus be of value in the risk stratification for cardiovascular events in patients with end-stage renal failure. Such data would have strong implications for the clinical management of patients with kidney disease.

Subjects and methods

Study population

We enrolled 79 Austrian patients with end-stage renal failure receiving dialysis treatment for at least 6 months at the Department of Internal Medicine of the Innsbruck Medical University. All study participants were Caucasians. The study design was cross-sectional. All cases of acute renal failure were excluded. Other exclusion criteria were underlying malignancy, liver failure, hepatitis B and C. The study was done according to the principles of the Declaration of Helsinki. All participating patients provided written informed consent to the investigation, and the protocol was approved by the Institutional Review Board of the Innsbruck Medical University. DNA was genotyped for the ENPP1 K121Q polymorphism in all 79 study participants. Next, we matched to each patient with ENPP1 121KQ genotype ($n=15$) a respective control with ENPP1 121KK genotype by gender, age (± 2 years), diabetes mellitus, and duration

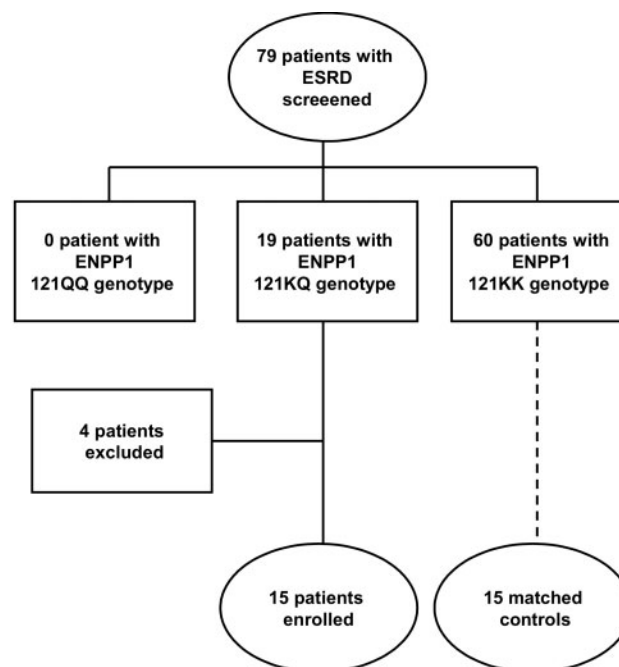


Fig. 1. Flow chart of trial organization. Genotyping of 79 patients on dialysis yielded 60 patients with wildtype genotype, 19 heterozygous patients, and none homozygous for the putative risk variant ENPP1 K121Q. ESRD, end-stage renal disease.

of dialysis treatment ($\pm 1a$) without further information about their co-morbidities (Figure 1). The matching ratio was 1:1. The complete medical records were evaluated only after matching of patients. Four heterozygous patients agreed to genotyping, but refused to give their informed consent for computed tomography analysis in order to avoid radiation dose exposure. The underlying renal diseases in the enrolled patients were diabetic nephropathy ($n=7$ in the group with ENPP1 121KQ genotype; $n=7$ in the wildtype group, respectively), glomerulonephritis ($n=5+4$), adult polycystic kidney disease ($n=2+1$), hypertensive nephrosclerosis ($n=1+2$) and chronic interstitial nephritis ($n=0+1$). Diabetes mellitus was defined by use of any glucose lowering medication, or by fasting glucose levels ≥ 126 mg/dl. Blood pressure and blood samples were taken from all subjects under standardized conditions prior to dialysis treatment. We measured parathyroid hormone, serum calcium and serum phosphate levels, and conventional cardiovascular risk factors, such as lipids, fasting glucose, glycohemoglobine A_{1c} (HbA_{1c}) and C-reactive protein. Time-integrated means of serum calcium, phosphate, cholesterol and triglyceride levels were calculated for the last 6 months. Time-averaged monthly means were used, whenever more than one value per month was documented. The serum fetuin-A concentration was determined using the ELISA provided by Biovendor (Biovendro Laboratory Medicine Inc., Modrice, Czech Republic). The oral therapeutic calcium intake was averaged over the last year. The patients were treated with sevelamer ($n=5$ in the group with ENPP1 121KQ genotype; $n=7$ in the wildtype group, respectively), aluminium hydroxide ($n=4+4$), calcitriol ($n=7+6$), cinacalcet ($n=3+3$) and coumarin anticoagulants ($n=2+2$) for the prior year. The prevalence of pre-existing coronary artery disease was 13% in both study groups.

Genotyping

The single nucleotide polymorphism rs1044498 causing the amino acid exchange K121Q was detected by polymerase chain reaction (PCR) restriction fragment length polymorphism (RFLP) analysis, as previously described [28]. This polymorphism is also referred to as K173Q variant due to two upstream in-frame translation initiation sites. Briefly, the PCR was performed using the primers 5'-CTG TGT TCA CTT TGG ACA TGT TG -3' and 5'-GAC GTT GGA AGA TAC CAG GTTG-3'. The PCR product aliquot was digested with *Ava*II (New England Biolabs, Ipswich, MA, USA) recognizing the K121Q variant. DNA fragments were analysed by electrophoresis on a 2% agarose gel. As an internal quality control, we genotyped all samples also by primer extension of PCR products with detection of the allele-specific products by 5' nuclease allelic discrimination (Taqman) assays [29]. The genotyping results by RFLP and Taqman were identical.

Computed tomography (CT)

A CT examination was performed during a single inspiratory breathhold using a 16-row detector CT scanner (Sensation 16, Siemens Medical Systems, Forchheim, Germany) and the following scan parameters: detector collimation 16 mm × 1.5 mm, gantry rotation time 420 ms, tube output 125 mAs/120 kV. Transaxial images with an effective slice width of 3 mm were reconstructed at increments of 1.5 mm with a medium-smooth convolution kernel (B 35 f) by using retrospective electrocardiogram gating at the time point of least cardiac motion (30–40% or 60–80% of RR-interval) dependent on heart rate. The calcium score for coronary arteries, the ascending and descending aorta, aortic valve and mitral valve was calculated by multiplying the calcification area in mm² by a density score determined from the peak CT scan number (Agatston score) [30] with a dedicated software (Calcium Score, Syngo Heart View) on a computed workstation (Siemens Medical Systems, Germany) by one experienced reviewer (G.F.) blinded to genotype profiling. Scores were determined for each main epicardial coronary artery, and the total calcium score was defined as the sum of the values of all lesions identified.

Pulse-wave analysis

After dialysis and 10 min of supine rest, carotid-femoral pulse-wave velocity (PWV) was determined using the Sphygmocor system (AtCor Medical, Sydney, Australia) by sequentially recording ECG-gated carotid and femoral artery waveforms by high-fidelity applanation tonometry. Surface distances from the carotid sampling site to the suprasternal notch and from the suprasternal notch to the femoral artery were measured as straight lines between these points on the body surface using a tape measure. The pressure wave transit time was calculated using a foot-of-the-wave to foot-of-the-wave method. PWV was then calculated by dividing the distance to the distal site (*D*) by the pressure wave transit time (*t*) as follows: $PWV = D/t$ (m/s) [31]. PWV was measured by one experienced reviewer (E.Z.) blinded to genotype profiling and coronary calcium score. All measurements were made in triplicates and mean values used in subsequent analysis. Pulse wave analysis was only performed

in patients with rhythmic cardiac action (*n* = 20), but not in patients with atrial fibrillation.

Pyrophosphate assay

Inorganic pyrophosphate was measured by enzymatic assay using uridine-diphosphoglucose (UDPG) pyrophosphorylase as described previously [32]. A sample (20 µl) was added to 100 µl of reaction buffer that contained 90 mM KCl, 5 mM MgCl₂, 70 mM Tris-HCl (pH 7.60), 10 µM NADPH, 3.7 µM UDPG, 0.25 U/ml UDPG pyrophosphorylase, 2.5 U/ml (Type X from baker's yeast), phosphoglucomutase (from rabbit muscle), 0.5 U/ml glucose-6-phosphate dehydrogenase (Type XV from baker's yeast) and 0.15 µCi/ml [¹⁴C]UDPG. After 30 min at 37°C, 200 ml of 2% activated charcoal was added on ice with occasional stirring to bind residual UDPG. After centrifugation, the radioactivity in 200 µl of supernatant was counted. [¹⁴C]UDPG was obtained from Perkin-Elmer (Boston, MA, USA). All other reagents were from Sigma Aldrich Chemicals (St Louis, MO, USA).

Statistical analyses

Normally distributed quantitative values are expressed as mean ± SD, variables missing normal distribution as median (25th–75th percentile). Differences in mean values between the two groups with different ENPP1 genotype were determined by unpaired Student's *t*-test, those of medians by Mann-Whitney U test. χ^2 -test was used to compare categorical variables. *P*-values < 0.05 (two-tailed) were considered to indicate statistical significance. Statistical analyses were performed using SPSS version 13.5 (SPSS, Inc., Illinois, USA).

Results

The minor allele frequency of the ENPP1 K121Q polymorphism was 0.137 in the presented population of end-stage renal failure. Genotyping of 79 patients with chronic renal failure yielded 60 patients with wildtype genotype, 19 heterozygous patients, and none homozygous for the putative ENPP1 risk variant K121Q (Figure 1). The allele distribution in our population was in Hardy-Weinberg equilibrium. Next, we matched to each patient with ENPP1 121KQ genotype a control with ENPP1 121KK genotype by gender, age, diabetes mellitus and duration of dialysis treatment in a 1:1 study design (Figure 1). The clinical and biochemical characteristics of the study participants are shown in Table 1. There were no significant differences in blood pressure, glycohaemoglobine levels, smoking habits, parathyroid hormone, serum calcium, serum phosphate, fetuin-A levels and dose of oral therapeutic calcium between the two groups with different ENPP1 genotypes. Table 2 shows the calcium score of coronary arteries, ascending and descending aorta and the heart valves in these patients with end-stage renal failure. Patients with ENPP1 121KQ genotype had significantly higher coronary calcium scores than matched controls

Table 1. Clinical and biochemical characteristics of patients

| Characteristics | ENPP1 121KK (<i>n</i> = 15) | ENPP1 121KQ (<i>n</i> = 15) | <i>P</i> -value |
|---|------------------------------------|------------------------------------|--------------------|
| Gender | 6/9 | 6/9 | 1.000 ^a |
| Age (years) | 67 (46–73) | 64 (47–71) | 0.775 ^a |
| Duration of ESRD (months) | 28 (8–62) | 31 (18–53) | 0.683 ^a |
| Diabetes mellitus (<i>n</i> , %) | 7 (47%) | 7 (47%) | 1.000 ^a |
| Body mass index (kg/m ²) | 27.1 ± 4.4 | 26.6 ± 5.5 | 0.781 |
| Current smoking (<i>n</i> , %) | 3 (20%) | 2 (13%) | 0.775 |
| Systolic blood pressure ^b (mmHg) | 131 ± 28 | 139 ± 20 | 0.386 |
| Diastolic blood pressure ^b (mmHg) | 71 ± 15 | 73 ± 13 | 0.650 |
| Creatinine (mg/dl) | 8.6 ± 2.7 | 7.7 ± 1.9 | 0.301 |
| Albumin (g/l) | 39 ± 4 | 40 ± 6 | 0.411 |
| Glycohaemoglobine (%) | 6.3 ± 1.4 | 6.2 ± 1.3 | 0.786 |
| Total-cholesterol ^b (mg/dl) | 179 ± 32 | 155 ± 35 | 0.059 |
| LDL-cholesterol ^b (mg/dl) | 103 ± 33 | 89 ± 34 | 0.274 |
| HDL-cholesterol ^b (mg/dl) | 55 (41–69) | 47 (42–49) | 0.285 |
| Triglycerides ^b (mg/dl) | 195 (110–295) | 130 (111–203) | 0.436 |
| Homocysteine (μmol/l) | 32 (24–60) | 26 (14–36) | 0.108 |
| CRP (mg/l) | 0.30 (0.24–0.73) | 0.62 (0.22–0.93) | 0.461 |
| Interleukin-6 (ng/l) | 7.0 (2.7–17.5) | 7.4 (6.6–10.5) | 0.586 |
| Calcium ^b (mmol/l) | 2.11 ± 0.12 | 2.18 ± 0.16 | 0.160 |
| Phosphate ^b (mmol/l) | 1.92 ± 0.53 | 2.03 ± 0.34 | 0.514 |
| Parathyroid hormone (ng/l) | 328 ± 193 | 410 ± 184 | 0.244 |
| Fetuin-A (g/l) | 0.23 ± 0.06 | 0.26 ± 0.04 | 0.159 |
| Dose of oral calcium (g/a) | 9 (0–219) | 0 (0–255) | 0.902 |
| Dialysate Calcium (mmol/l) | 1.31 ± 0.18 | 1.39 ± 0.22 | 0.447 |
| Calcitriol (<i>n</i> , %) | 6 (40%) | 7 (47%) | 0.775 |
| Sevelamer (<i>n</i> , %) | 7 (47%) | 5 (33%) | 0.539 |
| Aluminium hydroxide (<i>n</i> , %) | 4 (27%) | 4 (27%) | 1.000 |
| Cinacalcet (<i>n</i> , %) | 3 (20%) | 3 (20%) | 1.000 |
| Statin (<i>n</i> , %) | 4 (27%) | 7 (47%) | 0.367 |

^aMatching variable.
^btime integrated measurements over the last 6 months.

with ENPP1 121KK genotype (1385 vs 94; *n* = 30; *P* = 0.033; Figure 2A and Table 2). There were three patients with a calcium score less than 10 in the group of patients with ENPP1 121KQ genotype, and four patients in the group with ENPP1 wildtype genotype, respectively. The coronary calcium score increased with age and duration of dialysis treatment, and it increased disproportionately in patients with ENPP1 risk genotype (Figure 2B and C). Aortic valve calcification was present in 17 of 30 patients (57%) and mitral valve calcification in 16/30 (53%). We did not find any significant differences in the calcium scores of the aortic or mitral valve between the two ENPP1 genotype groups.

Aortic pulse-wave velocity is often associated with presence and quantity of coronary artery calcium [33], and it is increased in patients with end-stage renal disease [34]. Therefore, we also performed a pulse-wave analysis in our population of dialysis patients in order to investigate the impact of ENPP1 polymorphism K121Q on aortic stiffness. Aortic pulse-wave velocity

Table 2. Calcium score in patients on dialysis with different ENPP1 genotypes

| Calcium score | ENPP1 121KK (<i>n</i> = 15) | ENPP1 121KQ (<i>n</i> = 15) | <i>P</i> -value |
|-------------------|---------------------------------|---------------------------------|--------------------|
| Coronary arteries | 97 (0–1130) | 1385 (203–2854) | 0.033 ^a |
| Ascending aorta | 3 (0–146) | 86 (0–1842) | 0.367 |
| Descending aorta | 1263 (0–2357) | 2228 (0–9695) | 0.345 |
| Aortic valve | 172 (0–740) | 123 (0–397) | 0.624 |
| Mitral valve | 33 (0–5507) | 198 (0–2712) | 0.683 |

Data presented as median (25th to 75th percentile).
^aindicating statistical significance of *P* = 0.033.

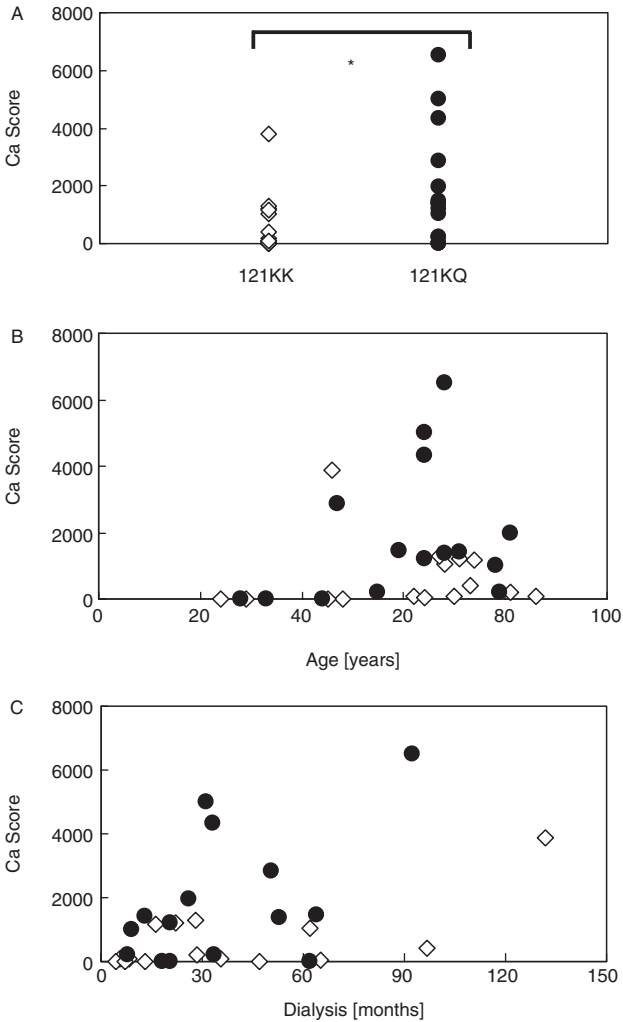


Fig. 2. Coronary calcium score in patients with different ENPP1 genotypes. Coronary calcium score (Ca Score) values are shown as a function of ENPP1 genotype (A), age (B), and duration of dialysis treatment (C). Closed circles represent patients with ENPP1 121KQ genotype, open diamonds stand for patients with ENPP1 121KK genotype. *indicates statistical significance of *P* = 0.033.

was significantly higher in patients heterozygous for the ENPP1 K121Q variant when compared to matched controls with wildtype ENPP1 genotype (13.69 ± 3.24 m/s vs 9.37 ± 1.90 m/s; *n* = 20; *P* = 0.003; Figure 3). Moreover, aortic pulse-wave velocity

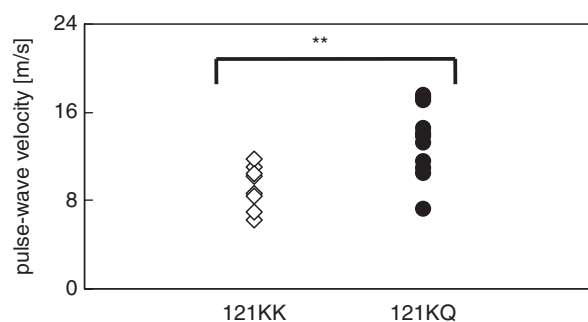


Fig. 3. Aortic pulse-wave velocities in patients with different ENPP1 genotypes. Aortic pulse-wave velocities are shown as a function of ENPP1 genotype. Closed circles represent patients with ENPP1 121KQ genotype, open diamonds stand for patients with ENPP1 121KK genotype. ** indicates statistical significance of $P=0.003$.

correlated with the calcium score of the ascending ($r=0.46$, $P=0.048$) and the descending aorta ($r=0.49$, $P=0.033$).

We also measured inorganic pyrophosphate, which is the enzymatic product of ENPP1. The mean plasma concentration of inorganic pyrophosphate was slightly lower in carriers of the ENPP1 K121Q variant ($2.43 \pm 0.63 \mu\text{mol/l}$) when compared to patients with wildtype genotype ($2.72 \pm 1.11 \mu\text{mol/l}$; $P=0.387$). However, this difference was not statistically significant.

Conclusions

Vascular calcification is not just a passive physico-chemical deposition of calcium phosphate, but a tightly regulated process under the control of several inducers and inhibitors [35]. The presented data suggest a potential role of ENPP1 in the arterial calcification of patients with end-stage renal failure. Patients heterozygous for the ENPP1 K121Q variant had significantly higher coronary calcium scores ($P=0.033$) and significantly higher aortic pulse-wave velocities ($P=0.003$) than matched controls with wildtype ENPP1 genotype. Both parameters are predictors of cardiovascular mortality in dialysis patients, and are associated to each other [33,36–38]. ENPP1 inhibits soft tissue calcification by generation of inorganic pyrophosphate. Pyrophosphate binds tightly to hydroxyapatite, thus preventing further crystallization [7,39]. Mutations of ENPP1 that reduce or eliminate enzymatic production of inorganic pyrophosphate lead to extensive ectopic calcifications in both mice and humans [9,10]. Also, the 121Q variant has been shown to be associated with insulin resistance, most likely through interference with the insulin receptor [21]. From the present data it is not clear, whether insulin resistance or altered pyrophosphate metabolism caused the aortic stiffness and coronary calcification in our study population. Although matching for diabetes mellitus, we cannot exclude that the association of arterial calcification and ENPP1 K121Q

polymorphism is due to interference with insulin signalling. Arterial calcification in turn is responsible for stiffening of the arteries leading to increased left ventricular cardiac afterload and diminished coronary perfusion [40]. The ENPP1 K121Q polymorphism is also associated with ischaemic heart disease in the general population [14,18].

Whereas complete loss-of-function mutations of the ENPP1 gene cause widely disseminated media calcification in childhood [9], vascular calcification in our population with the ENPP1 K121Q polymorphism and end-stage renal failure increased progressively with age and duration of dialysis treatment. Coronary-artery calcification is common and progressive in young adults with end-stage renal disease, who are undergoing dialysis over years [2]. Therefore, genotyping for risk factors of vascular calcification in young patients on dialysis could theoretically offer the possibility of pharmacological intervention to prevent further media calcification. Stringent controls for hypercalcaemia, hyperphosphataemia and hyperparathyroidism would be warranted in these genetically determined risk groups. Furthermore, these patients might benefit from treatment with sevelamer, which was associated with a significant survival benefit as compared to calcium-containing phosphate binders in the recent clinical study by Block *et al.* [41]

This study also demonstrates a strong association between aortic pulse-wave velocity and ENPP1 genotypes and a borderline correlation of pulse-wave velocity with aortic calcification. However, aortic calcium score was not significantly different between the two study arms. This discrepancy is probably due to the small population size. Furthermore, aortic sclerosis and stiffness may precede overt aortic calcification *in vivo*.

Coronary artery and valvular calcification are often associated to each other [42,43]. Therefore, we also quantified aortic and mitral valve calcification in our population. The prevalence of valvular calcification in our population (57% and 53% for aortic and mitral valve, respectively) was higher when compared to reported data (32%) [43]. However, no difference between the two groups with different ENPP1 genotypes was found, suggesting that ENPP1 does not play a role in the pathogenesis of valvular calcification in patients with end-stage renal failure.

A major limitation of this study is the small sample size, probably increasing the risk of a type 2 statistical error. Nevertheless, ENPP1 genotypes were significantly associated with severity of calcification in coronary arteries and increased aortic pulse-wave velocity. Additionally, the association between genotype and phenotype may also occur by linkage disequilibrium with another gene locus nearby. However, the concept of arterial calcification due to complete loss-of-function mutations of the ENPP1 gene is already validated [9], and the genotype-phenotype association of ENPP1 polymorphisms in adults with deranged mineral metabolism seems a consequent pathophysiological extrapolation. The lack

of homozygous carriers of the ENPP1 K121Q variant is another limitation of this study, as it does not permit to perform a gene-dosis analysis. We looked for allelic distribution and homozygous patients also in a healthy control population of similar age and ethnicity (Linz Peripheral Disease LIPAD study) [44], and found only seven homozygous carriers of the ENPP1 K121Q variant in 433 patients and an allelic frequency of 0.141 in the LIPAD population. Thus, the low allelic frequency of the K121Q variant in our population with end-stage renal failure was reproduced in another population of Austrian origin.

Taken together, the presented data suggest a potential role of genetic markers in the identification of persons at risk for vascular calcification and might serve as rationale to design large prospective clinical studies in patients with incident haemodialysis to determine the effect of ENPP1 variants on cardiovascular morbidity and mortality in patients with end-stage renal failure. Genotyping of ENPP1 may thus contribute to personalize pharmacological treatment in patients with end-stage renal failure according to their genetic predisposition for vascular calcification.

Conflict of Interest statement. None declared.

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