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Short Communication

Vascular calcification in chronic kidney disease associated with pathogenic variants in *ABCC6*

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ABSTRACT

Vascular calcification is prevalent in chronic kidney disease (CKD). Genetic causes of CKD account for 10–20% of adult-onset disease. Vascular calcification is thought to be one of the most important risk factors for increased cardiovascular morbidity and mortality in CKD patients and is detectable in 80% of patients with end stage kidney disease (ESKD). Despite the high prevalence of vascular calcification in CKD, no single gene cause has been described. We hypothesized that variants in vascular calcification genes may contribute to disease pathogenesis in CKD, particularly in families who exhibit a predominant vascular calcification phenotype.

We developed a list of eight genes that are hypothesized to play a role in vascular calcification due to their involvement in the ectopic calcification pathway: *ABCC6, ALPL, ANK1, ENPP1, NT5E, SLC29A1, SLC20A2,* and *S100A12.* With this, we assessed exome data from 77 CKD patients, who remained unsolved following evaluation for all known monogenic causes of CKD. We also analyzed an independent cohort (Ontario Neurodegenerative Disease Research Initiative (ONDRI), n = 520) who were screened for variants in *ABCC6* and compared this to a control cohort of healthy adults (n = 52).

We identified two CKD families with heterozygous pathogenic variants (R1141X and A667fs) in *ABCC6*. We identified 10 participants from the ONDRI cohort with heterozygous pathogenic or likely pathogenic variant in *ABCC6*. Replication in a healthy control cohort did not reveal any variants.

Abbreviations: ABCC6, ATP-binding cassette subtype C, member 6; ACMG, American College of Medical Genetics; ALPL, Alkaline Phosphatase; ANK1, Ankyrin 1; ATP, adenosine triphosphoate; CADD, Combined Annotation Dependent Depletion; CKD, chronic kidney disease; CNV, copy number variant; CT, computed tomography; ENPP1, Ectonucleotide pyrophosphatase 1; ES, exome sequencing; ESKD, end stage kidney disease; ESP, event stream processing; ExAC, Exome Aggregation Consortium; GACI, General Arterial Calcification of Infancy; gnomAD, genome Aggregation Database; HGMD, Human Gene Mutation Database; NGS, Next-generation sequencing; NT5E, Ecto-5-prime nucleotidase; ONDRI, Ontario Neurodegenerative Disease Research Initiative; PPi, inorganic pyrophosphate; Poly2, Polymorphism Phenotypeing version 2; PXE, Pseudoxanthoma Elasticum; SIFT, Sorting Intolerant from Tolerant; SLC19A1, Solute Carrier Family 29 member 1; SLC20A2, Solute Carrier family 20, member 2; S100A12, S100 calcium-binding protein A12.

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Our study provides preliminary data supporting the hypothesis that *ABCC6* may play a role in vascular calcification in CKD. By screening CKD patients for genetic causes early in the diagnostic pathway, patients with genetic causes associated with vascular calcification can potentially be preventatively treated with new therapeutics with aims to decrease mortality.

1. Introduction

Vascular calcification, characterized by calcium mineral deposits in blood vessels, can lead to impaired cardiovascular function and end-organ damage including cardiovascular, kidney and cerebrovascular disease (McCullough et al., 2004). Vascular calcification is thought to be one of the most important risk factors for increased cardiovascular morbidity and mortality in CKD patients (Palit and Kendrick, 2014). It is commonly observed in CKD patients and detectable by computed tomography (CT) in 80 % of patients with end-stage kidney disease (ESKD) (McCullough et al., 2004; Braun et al., 1996; Goodman et al., 2000). This high prevalence is hypothesized to be related to increasing age, dialysis vintage, and the co-existence of diabetes, however the underlying mechanisms of vascular calcification are not completely understood (McCullough et al., 2004; Palit and Kendrick, 2014). Currently there is no definitive treatment for vascular calcification and treatments are predominately palliative with a focus on relieving symptoms.

It is now recognized that genetic causes for CKD account for 10–20 % of adult-onset disease, and detection of single gene (monogenic) causes of disease has the potential to provide insights into disease pathogenesis and potential therapeutic targets (Shimada et al., 2021; Groopman et al., 2019; Dahl et al., 2023). Next-generation sequencing, including exome sequencing (ES), has made it possible to detect genetic causes of CKD, with almost 500 monogenic causes of CKD now described (Groopman et al., 2019). We hypothesize that in families with CKD who have a predominant vascular calcification phenotype **and** who remained unsolved for mutations in genes known to cause CKD, identification of novel candidate genes for CKD in the vascular calcification pathway is possible.

To date, no monogenic causes for CKD with a vascular calcification phenotype have been described. With the high prevalence of vascular calcification in CKD patients, an area of interest is the ectopic calcification pathway, which is a condition that causes calcifications to develop in soft tissues (Fig. 1). Through a literature review, we developed a pre-specified list of 8 potential vascular calcification genes that have been implicated in the ectopic calcification pathway: ATP-binding Cassette Subtype C Member 6 *(ABCC6),* Ectonucleotide Pyrophosphatase 1 *(ENPP1),* Ecto-5-prime Nucleotidase *(NT5E),* Alkaline Phosphatase *(ALPL),* Ankyrin 1 *(ANK1),* Solute Carrier Family 29 Member 1 *(SLC29A1),* Solute Carrier Family 20 Member 2 *(SLC20A2),* and S100 Calcium-binding Protein A12 *(S100A12*) (Supplementary Table 1). Two important genes in this pathway are *ABCC6* and *ENPP1* which are responsible for Pseudoxanthoma Elasticum (PXE) and General Arterial Calcification of Infancy (GACI), respectively (Shimada et al., 2021). Interestingly, animal studies have already highlighted the therapeutic potential through targeting gene dysfunction in both the *ABCC6* and *ENPP1* pathways (Shimada et al., 2021). Herein, we detected variants in both *ABCC6* and *ENPP1* in two families with CKD of unknown etiology, who display a vascular calcification phenotype.

2. Methods

2.1. Chronic kidney disease cohort

Patient recruitment. We performed all procedures in compliance with institutional guidelines (Western University Research Ethics Board #115160, approval year 2020). The procedures followed in this study were in accordance with the ethical standards of the responsible committee on human experimentation. Participants were recruited from a Kidney Genetic Clinic if they met one or more of the following inclusion criteria: family history of CKD, extra-renal features of disease, young age of onset of ESKD defined as onset < 50 years or suspected genetic kidney

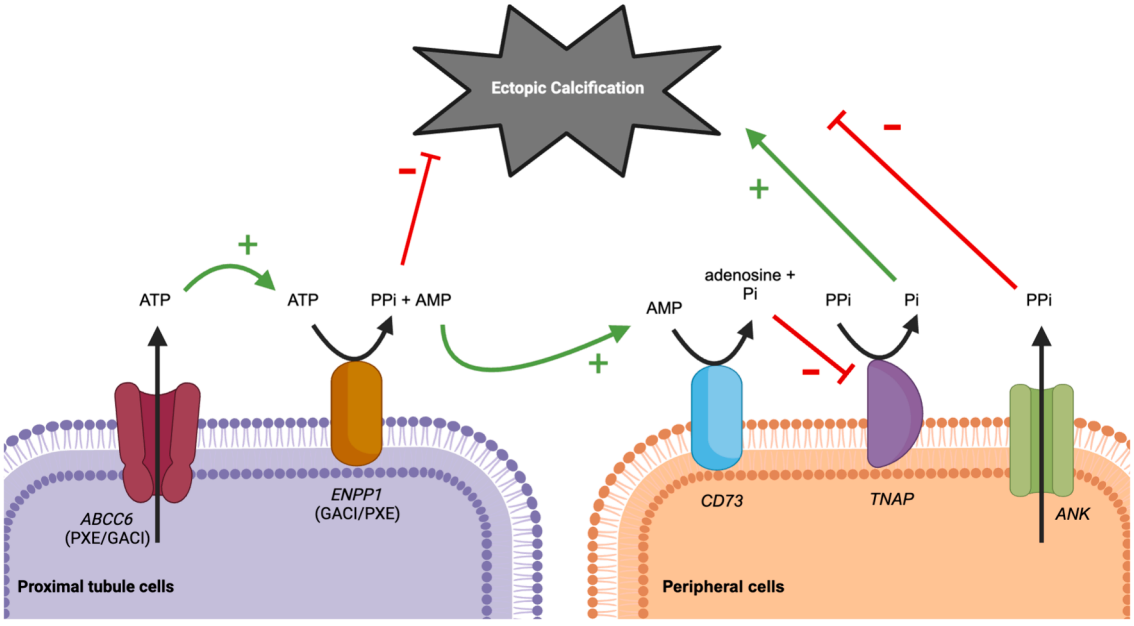

Fig. 1. ATP-binding cassette, subfamily C, member 6 *(ABCC6)* pathway. Positive green arrows represent activation, negative red arrows represent inhibition. Abbreviations: ATP (adenosine triphosphate), AMP (adenosine monophosphate), PPi (inorganic pyrophosphate), Pi (inorganic phosphate), PXE (Pseudoxanthoma elasticum), GACI (generalized arterial calcification in infancy), ENPP1 (Ectonucleotide pyrophosphate/phosphodiesterase 1), CD73 (5′-nucleotidase ecto), TNAP (alkaline phosphatase, biomineralization associated), ANK (Ankyrin).

disease based on phenotype observed. Informed consent was obtained from all participants or the substitute decision maker, as appropriate. Following informed consent from all participants, we recorded detailed clinical and pedigree data using a standardized questionnaire.

DNA extraction. Blood or saliva samples were collected for DNA extraction as previously described (Connaughton et al., 2019). Briefly, DNA was extracted from blood using the QIAGEN Gentra® Puregene® Extraction Kit following the Rapid DNA Isolation from whole blood protocol. Saliva kits from DNAgenoTek were used by participants to collect saliva samples. DNA was extracted from saliva using Oragene®•DNA OG600 series. DNA extraction was completed following the Oragene® DNA Laboratory protocol for manual purification of DNA from 4.0 mL of Oragene®DNA saliva provided by the manufacturer.

Exome Sequencing (ES). ES was completed at the McGill Genome Centre, McGill University (https://www.mcgillgenomecentre.ca/). Using the Lucigen NexSeq® AmpFREE Low DNA Library Kit protocol, next generation sequencing fragment libraries were created. Then Illumina NovaSeq™ Xp protocol (#1000000019358) was used to sequence libraries generating ES data.

Gene List and BED File Generation. First, we overlapped the genomic regions of 478 genes (Connaughton et al., 2019) known to cause CKD with the ES data from 203 participants with CKD recruited to the Southwestern Ontario Renal Genetics Database and Biobank (SWORDGEN #115160). We then performed analysis in any participant in whom **no** pathogenic, likely pathogenic, or variant of uncertain significance (VUS) was identified in any of the 478 kidney disease genes (n = 77). Next, we assessed these 77 participants for variants in a curated list of eight vascular calcification candidate genes namely *ABCC6, ALPL, ANK1, ENPP1, NT5E, SLC29A1, SLC20A2,* and *S100A12* (Supplementary Table 1). This list of candidate genes was generated through extensive literature review. Based on the hypothesis that vascular calcification occurs due to defects in the ectopic calcification pathway, we included candidate genes in this pathway. We also included potential interactors of these genes using functional data confirming a potential interaction with ectopic calcification pathway genes (Bowman and McNally, 2012; Gawdzik et al., 2011; Villa-Bellosta, 2021). To generate BED files for both the known CKD genes (n = 478) and the vascular calcification candidate genes (n = 8), we used the University of California Santa Cruz genome browser with settings for chromosome position hg19, and extra border size +/- 250 bp.

Variant Discovery and Annotation. As previously described, we used a custom automated workflow designed in CLC Bio genomics workbench (CLC bio version 12, QIAGEN Digital Insights) to perform sequence alignment, call variants, and produce targeted region coverage statistics for variant discovery (Johansen et al., 2014). To annotate variant data, we used the ANNOVAR pipeline with customized downloads and filtering strategies as described in the protocol (Wang et al., 2010). Databases RefSeq (hg19), Single Nucleotide Polymorphism database (dbSNP), Genome Aggregation Database (gnomAD), Exome Aggregation Consortium (ExAC), event stream processing (ESP), Human Gene Mutation Database (HGMD), and ClinVar were downloaded, as well as *in silico* predictions including Polymorphism Phenotypeing v2 (Poly2), Combined Annotation Dependent Depletion (CADD), Sorting Intolerant from Tolerant (SIFT), Mutation Taster, and American College of Medical Genetics (ACMG) classification (see web resources below). We also completed copy number variant (CNV) analysis using VarSeq CNV Caller (VarSeq v2.2.1, Golden Helix, Bozeman, MT) (Iacocca et al., 2017).

Variant Analysis. We performed variant calling from ANNOVAR output data as previously described (Wang et al., 2010). Briefly, the data files were filtered to exclude the following: synonymous mutations, variants with frequency > 1 % in gnomAD (v.2.1.1) and ExAC, variants that zygosity called did not match the disease inheritance (i.e., autosomal recessive inheritance but heterozygous variant detection on exome sequencing), and variants classified as benign or likely benign in ClinVar. We used the American College of Medical Genetics (ACMG) guidelines to assess the remaining potential variants (Richards et al.,

Table 1
Genetic and clinical information for families with pathogenic *ABCC6* variants.

Genetic Information										
Gene	**Hg.19 Pos**	**Exon**	**Zygosity**	**c.change, p.change**	**CADD**	**Poly2**	**SIFT**	**Mut Taster**	**gnomAD**	**ACMG Class.; Criteria**
ABCC6	chr16: 16,256,935	24	Het	C3421T, R1141X	42	n/a	n/a	A	0.0012	Path; PVS1, PM2, PP5
ABCC6	chr16: 16,276,732	16	Het	1999delG, A667fs	n/a	n/a	n/a	n/a	0.00002	Path; PVS1, PM2, PP5
ENPP1	chr6: 132,172,368	4	Het	A517C, K173Q	9.2	B	T	P	0.2089	VUS; BP4, BP6

Clinical Information										
Family, Patient	**Variant**	**Sex**	**Age CKD**	**Age ESKD onset/ modality**	**Eye pathology**	**Clinical CKD diagnosis**	**Skin pathology**	**Vascular pathology**	**Other features**	**Protein/ Blood in urine**
F1P09	*ABCC6* A667fs, *ENPP1* K173Q	M	69	n/a	n/a	HTN and diabetes	Eye-lid laxity	HTN	Diabetes, hearing loss	Protein
F1P11	*ABCC6* A667fs, *ENPP1* K173Q	M	40	41, kidney *trans*-plant	Bilateral cataracts, retinal hemorrh-age	HTN and diabetes with kidney stones	Yellowish mucosal nodules in mouth	HTN, VC, bilateral BKA	Diabetes	n/a
F2P18	*ABCC6* R1141X	M	28	29, kidney *trans*-plant	Bilateral cataracts	GN, Alport	Elastosis perforans serpignosa, yellowish mucosal nodules in mouth	n/a	Hearing loss, osteo-arthritis	n/a
F2P23	*ABCC6* R1141X	F	24	n/a	n/a	GN, Alport	n/a	n/a	n/a	Both

Adenine (A), American College of Medical Genetics (ACMG), Arginine (Arg), BKA (below-knee amputation), Chromosome change (c.Change), Combined Annotation Dependent Depletion (CADD), deleterious (D), Exome Aggregation Consortium (ExAC), F (female), Genome reference consortium human build 37 position (Hg19Pos), GN (glomerulopathy), HTN (hypertension), LB (likely benign), M (male), n/a (not available), path (pathogenic), polymorphism phenotyping v2 (Poly2), protein change (p.Change), reference SNP (rs), single nucleotide polymorphism identification (SNP ID), Sorting Intolerant from Tolerant (SIFT), The Genome Aggregation Database (gnomAD), VC (vascular calcification).

2015). We included only variants classified as pathogenic or likely pathogenic as per ACMG guidelines in the final analysis. All variants included in this paper were validated using Ensembl 111. Extended methods for variant annotation and analysis can be found in the Supplementary Methods section.

Sanger sequencing. To confirm the *ENPP1* variant, we designed Sigma Millipore primers (Supplementary Table 2). We performed PCR and sanger sequencing using a 3730 Applied Biosystems Sequencer. We confirmed all pathogenic and likely pathogenic variants in the *ABCC6* genes through targeted variant testing available through Prevention Genetics (preventiongenetics.com).

2.2. Ontario Neurodegenerative Disease Research Initiative (ONDRI) Replication cohort

ONDRI participants. Due to the rarity of monogenic diseases, we sought to identify other disease cohorts who had undergone assessment for variants in any of the 8 vascular calcification candidate genes. The Ontario Neurodegenerative Disease Research Initiative (ONDRI) includes data from 520 participants who have undergone targeted next-generation sequencing (NGS) for 80 candidate genes which includes the gene *ABCC6*. Although not ascertained for a kidney disease phenotype, the ONDRI cohort included individuals with neurodegenerative and cerebrovascular diseases. As previously described, blood samples were collected from 520 participants after informed consent, in accordance with the Research Ethics Boards at Hamilton General Hospital (Hamilton, Ontario, Canada); McMaster (Hamilton, Ontario, Canada); Parkwood Hospital (London, Ontario, Canada); London Health Sciences Centre (London, Ontario, Canada); The Ottawa Hospital (Ottawa, Ontario, Canada); University Health Network- Elizabeth Bruyère Hospital (Ottawa, Ontario, Canada); Baycrest Centre for Geriatric Care (Toronto, Ontario, Canada); Centre for Addiction and Mental Health (Toronto, Ontario, Canada); St Michael's Hospital (Toronto, Ontario, Canada); Sunnybrook Health Sciences Centre (Toronto, Ontario, Canada); and Toronto Western Hospital (Toronto, Ontario, Canada). Formal diagnoses and demographic data were recorded by participants' clinicians upon enrollment in the study, in accordance with ONDRI standard operating protocols (Farhan et al., 2017).

ONDRI sequencing. Genomic DNA was isolated from blood samples collected from each participant. All samples underwent targeted next generation sequencing using the ONDRISeq neurodegenerative disease gene panel, which covered 80 genes previously associated with various neurodegenerative and cerebrovascular diseases, including *ABCC6*. Full methodology of DNA isolation, sequencing with the ONDRISeq panel, and raw sequencing data processing were previously described (Farhan et al., 2016; Dilliott et al., 2018). We filtered data to include variants in the *ABCC6* gene from ONDRISeq. Only variant classified as likely pathogenic and pathogenic as per ACMG guidelines were included in the final analysis.

ONDRI clinical analysis. The demographic and medical history of all ONDRI participants was obtained upon enrollment and underwent thorough quality control processing (Sunderland et al., 2023). Through retrospective database review we extracted data relevant to participants' clinical history in all participants carrying variants in the *ABCC6* gene. As kidney disease was not a main outcomes measure in the ONDRI cohort, data related to kidney phenotype was extracted from descriptive terms completed in the physician comments form. We extracted data on relevant kidney pathologies from the descriptive data including at least one of the following terms: kidney stones, renal impairment, renal failure, chronic kidney disease, acute renal injury, hydronephrosis, renal cysts, renal colic, hematuria, or urinary obstruction.

2.3. Healthy control replication cohort

To compare the frequency of pathogenic and likely pathogenic vascular calcification candidate genes, we repeated analysis in a healthy, non-CKD population. This cohort is an in-house cohort of 52 unaffected family members. All participants were recruited to the Southwestern Ontario Renal Disease Genetics Registry Biobank (SWORD-GEN), as unaffected family members of participants with suspected genetic kidney disease (WREM #115160). All individuals completed a medical questionnaire and at the time of recruitment had no reported evidence of chronic kidney disease. All individuals underwent ES, variant annotation and analysis as described in the above methods.

Statistical analysis. We summarized the demographic and clinical characteristics using mean and standard deviation for continuous variables, and frequencies and percentages for categorical variables. We compared the difference in demographic and clinical characteristics by *ABCC6* genotype using Wilcoxon rank-sum test for continuous variables and Fisher's exact test for categorical variables. All statistical analysis was performed using statistical software R studio (version 2023.06.2 Build 561). All tests were two-sided with a significance level of $p < 0.05$.

2.4. Website resources

University of California Santa Cruz (UCSC) Genome Browser https://genome.ucsc.edu/.

Databases RefSeq (hg19) https://www.ncbi.nlm.nih.gov/refseq/.

Single Nucleotide Polymorphism database (dbSNP) https://www.ncbi.nlm.nih.gov/snp.

Genome Aggregation Database (gnomAD) https://gnomad.broadinstitute.org.

Exome Aggregation Consortium (ExAC) https://exac.broadinstitute.org.

Exome Sequencing Project (ESP) https://esp.gs.washington.edu/drupal.

Human Gene Mutation Database (HGMD) https://apps.ingenuity.com/ingsso.

ClinVar https://www.ncbi.nlm.nih.gov/clinvar.

PolyPhen-2 http://genetics.bwh.harvard.edu/pph2/.

Combined Annotation Dependent Depletion (CADD) https://cadd.gs.washington.edu/.

Sorting Intolerant from Tolerant (SIFT) https://sift.bii.a-star.edu.sg/.

Mutation Taster https://www.mutationtaster.org/.

American College of Medical Genetics (ACMG) classification (PMID: 25741868).

Prevention Genetics https://www.preventiongenetics.com/.

3. Results

We detected pathogenic variants in two vascular calcification candidate genes in two unrelated families with CKD of unknown cause. Both families were unsolved for variants in any of the 478 genes known to cause CKD. In Family 1, a heterozygous pathogenic variant (c.1999delG:p.A667fs) in the gene *ABCC6* (GenBank: NM_001171) was detected. An additional heterozygous variant of uncertain significance (VUS) (c.A517C:p.K173Q) in the gene *ENPP1* (GenBank: NM_006208) was detected in Family 1 (**Supplementary** Figure 1). In Family 2, a heterozygous pathogenic variant in the *ABCC6* gene (c.C3421T:p.R1141X) was detected (Table 1). All variants were validated using Ensembl 111 (Supplementary Figs. 2 & 3).

3.1. Family 1

A 55-year-old male (Family 1, Patient 11) of European descent was diagnosed with CKD at the age of 40 years. He progressed rapidly to ESKD within one year of diagnosis. His assumed etiology of CKD was diabetes mellitus type 2 which he developed at the age of 33 years, and hypertension which was first noted at 39 years. A kidney biopsy was never performed. Kidney imaging revealed significant vascular

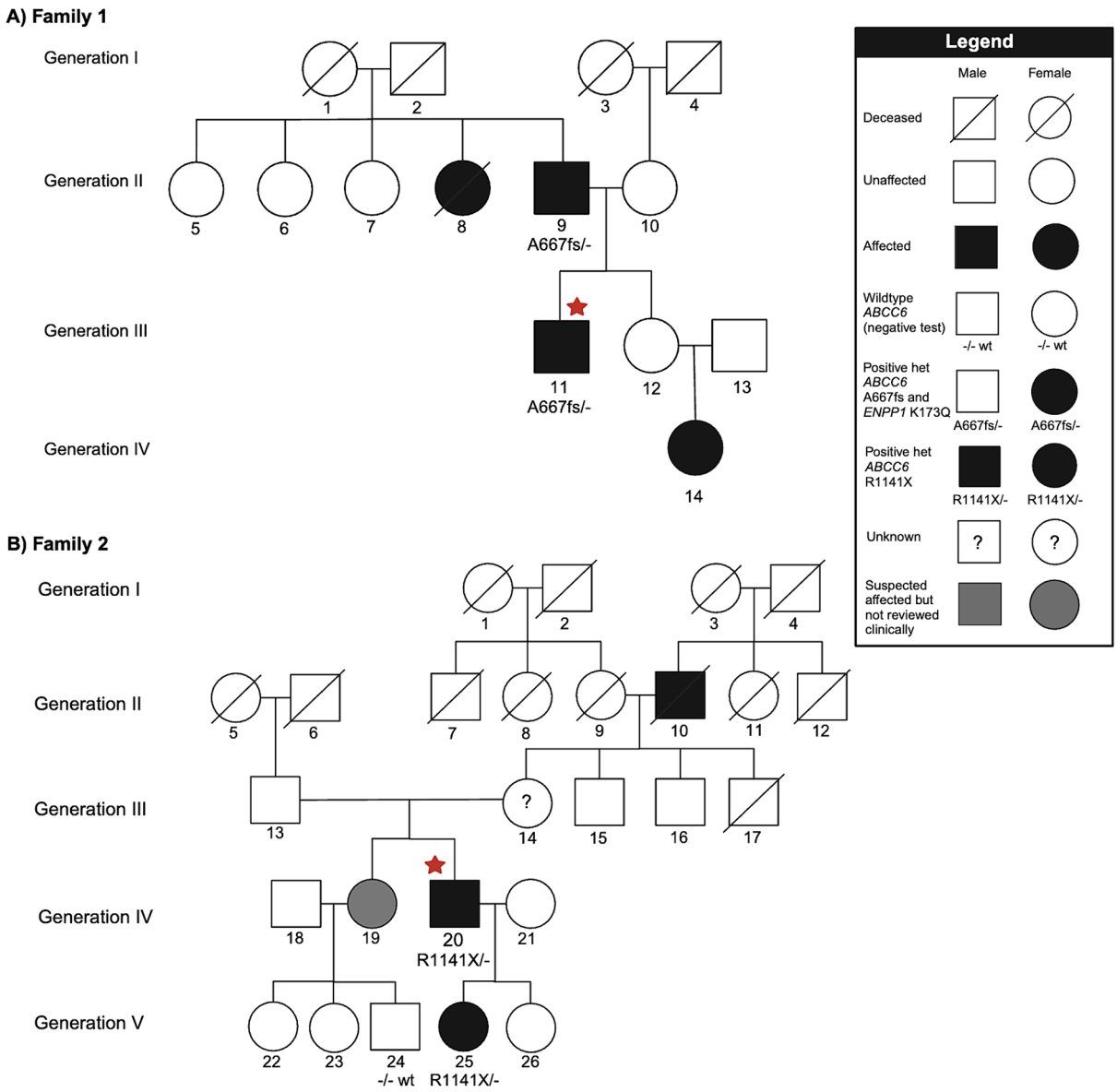

Fig. 2. *ABCC6* family pedigrees. A) Family 1 with heterozygous *ABCC6* variant p.A667fs, and heterozygous *ENPP1* variant K173Q. B) Family 2 with heterozygous *ABCC6* variant p.R1141X. Additionally, one individual is represented in grey, as it was reported by the family that they have hematuria and proteinuria, though no clinical data was available and they were unable to be reviewed for this study. The red star represents the probands for each family. The legend provided in the figure dictates both pedigrees. Abbreviations: *ABCC6* (ATP binding cassette subfamily C member 6), A667fs (Alanine frameshift mutation at amino acid position 667) *ENPP1* (Ectonucleotide pyrophosphatase/ phosphodiesterase 1), Het (heterozygous), K173Q (Lysine to glutamate amino acid substitution at position 173), R1141X (arginine termination mutation at amino acid position 1141), wt (Wildtype). Figure created using BioRender.

calcification including calcification of the renal and pelvic vessels (**Supplementary Figure 4A-B**). He also had a history of recurrent kidney stones. Noteworthy was the co-occurrence of severe peripheral vascular disease ultimately resulting in bilateral below the knee amputations in his 50th decade. His ophthalmological history was significant for reduced visual acuity, with early onset cataracts at the age of 52 and retinopathy resulting in retinal hemorrhage requiring vitrectomy. On skin examination, multiple yellow mucosal nodules were noted suggestive of the pseudoxanthoma elasticum (PXE) phenotype.

On review of his family pedigree, he had a positive family history of CKD on the paternal side with his father, paternal aunt, and paternal niece all having CKD (Fig. 2**A**). The index participants' father (Family 1, Patient 9) developed CKD at the age of 69 years, which was presumed to be secondary to diabetes and hypertension. His kidney disease was progressive, and at the age of 85 years he had CKD stage 5. Other history was significant for hearing loss in his 70s. He too had reduced visual acuity with bilateral cataracts. On examination he had laxity of the skin around the eyelids of uncertain etiology.

A pathogenic heterozygous frameshift variant in *ABCC6* (c.1999delG:p.A667fs) was detected in the proband (Family 1, Patient 11) as well as a heterozygous VUS in *ENPP1* (c.A517C:p.K173Q). Upon familial variant testing, the proband's father was also found to carry both variants (Table 1).

3.2. Family 2

A 56-year-old Caucasian male (Family 2, Patient 20) was diagnosed with CKD at the age of 28 years, presumed to be caused by Alport Syndrome due to the presence of hearing impairment in his 20s, and a urine dipstick positive for blood and protein. No kidney biopsy was performed. He progressed to ESKD at 29 years. His past medical history was significant for reduced visual acuity with bilateral cataracts,

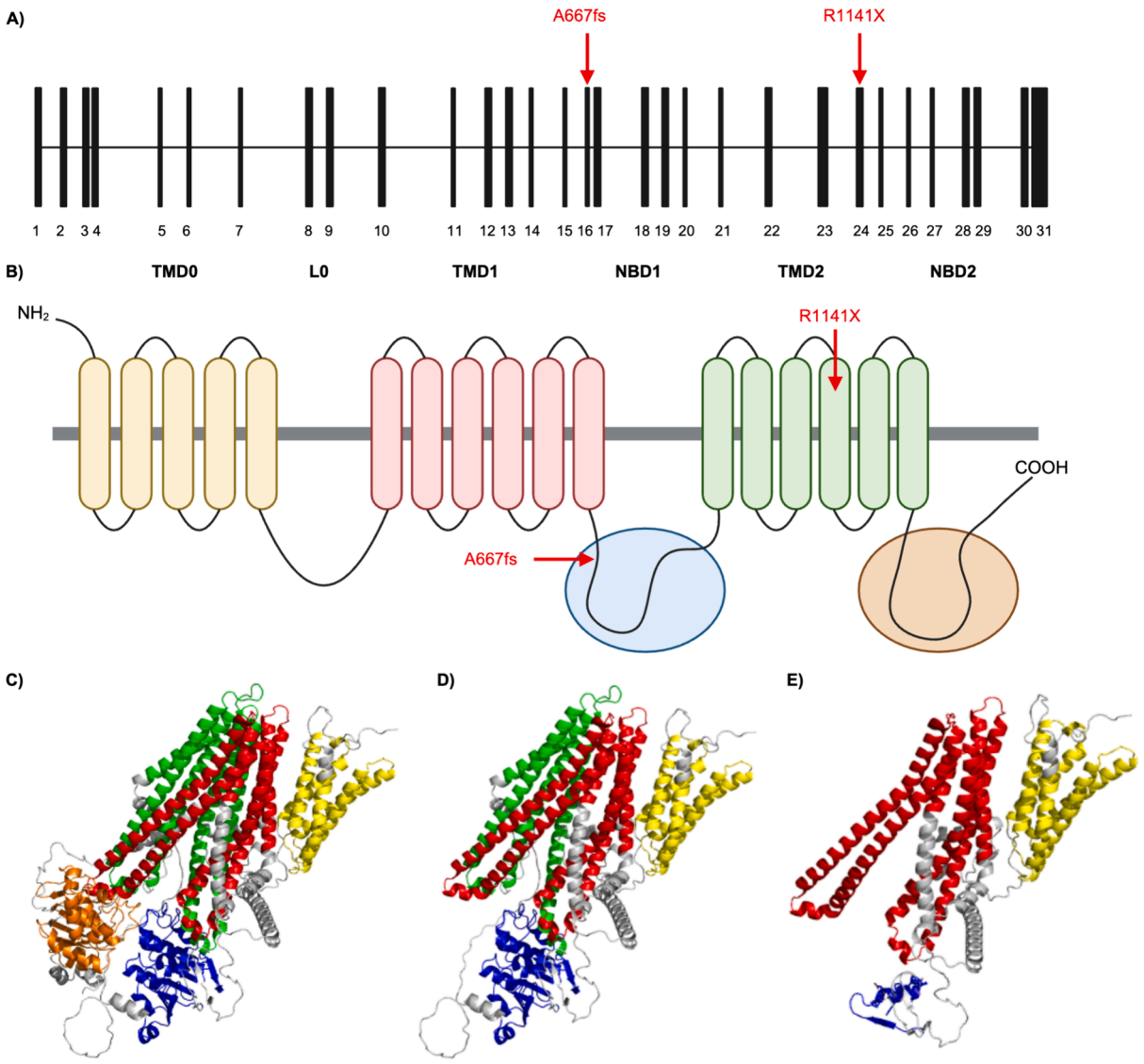

Fig. 3. *ABCC6* Structure. (A) 31 exons of *ABCC6*. (B) Domains of *ABCC6*. (C) 3D model of wild-type protein structure. (D) 3D model of *ABCC6* with arginine 1141 termination mutation. (E) 3D model of *ABCC6* with alanine 667 frameshift mutation. Red arrows represent locations of the mutations detected in this cohort. Domains are represented as the following: transmembrane binding domain 0, transmembrane binding domain 1, transmembrane binding domain 2, nucleotide binding domain 1, and nucleotide binding domain 2, coloured in yellow, red, green, blue, and orange respectively. Undefined areas are coloured in grey. Images A and B were made using BioRender. Images C-E were made using PYMOL, UniProt code is 095255, PDB file downloaded from AlphaFold Protein Structure Database.

sensorineural hearing loss, and osteoarthritis. Skin examination revealed lesions consistent with elastosis perforans serpiginosa along with yellowish lip mucosal nodules, again consistent with the skin manifestations of PXE (**Supplementary Figure 4C-E**).

There was also a positive family history of CKD with his mother (Family 2, Patient 14) having CKD of unknown etiology and his sister (Family 2, Patient 19) having proteinuria and hematuria, although they were not available for clinical review (Fig. 2**B**). His 28-year-old daughter (Family 2, Patient 25) was diagnosed with CKD at the age of 24, with proteinuria and hematuria first noted on urine dipstick in her teens. Her CKD was presumed to be secondary to Alport Syndrome, although no prior genetic testing had been performed. Her estimated glomerular filtration rate remained at 99 mL/min/1.73 m^2 in her 20 s.

No mutations in any of the genes known to cause CKD including the Alport Syndrome genes (*COL4A3*, *COL4A4*, and *COL4A5*), were detected. Upon evaluation for vascular calcification genes, a pathogenic heterozygous nonsense variant in *ABCC6* (c.C3421T:p.R1141X) was detected in the index patient and his affected daughter.

3.3. Structural analyses

ABCC6 is a 165kD protein composed of 1503 amino acids and 31 exons (Shimada et al., 2021). This protein has three transmembrane domains (TMD), two linker domains, and two nucleotide binding domains (NBD) (Verschuere et al., 2021). NBDs are typical of ABC transporters, binding and hydrolyzing ATP, which allows for conformational changes of the protein during transport activity (Shimada et al., 2021). There is also a PDZ-like sequence located at the C-terminus, which is critical for regulated trafficking and membrane localization (Xue et al., 2014). The mutation identified in family 1 (c.1999delG:p.A667fs) occurs in the 16th exon in the first NBD, whereas the mutation identified in family 2 (c.C3421T:p.R1141X) occurs in the 24th exon in the second TMD (Fig. 3**A-B**). In both identified variants, there is a predicted loss of the C-terminus, including the PDZ domain. Through 3D modelling, we demonstrated that both variants result in a termination codon; R1141X is a substitution for a termination codon, and A667fs causes a frameshift that introduces a termination codon 15 amino acids after the frameshift occurs (Fig. 3**C-E**). Both variants, A667fs and R1141X, are predicted to

result in loss of functional domains, including deletion of most of the NBD1 domain in the A667fs variant, and deletion of NBD2 and part of TMD2 domain in the R1141X variant.

3.4. Ontario Neurodegenerative Disease Research Initiative (ONDRI) cohort analysis

We identified 10 participants with pathogenic or likely pathogenic heterozygous variant in *ABCC6* in the ONDRI cohort (Supplementary Tables 3–5). *ABCC6* positive participants had a higher prevalence of vascular cognitive impairment (60 % *versus* 30 %, $p = 0.08$) and a higher prevalence of retinoschisis (retinal detachment) (10 % *versus* 0 %; $p = 0.02$). There was a higher mean calcium level observed in participants with *ABCC6* mutations (2.4 mmol/L versus 2.3 mmol/L, $p = 0.005$), and a higher mean serum creatinine value (115.9 μmol/L versus 77.9 μmol/L; $p = 0.54$) though medians were similar (73.5 interquartile range (IQR) 19, versus 76 IQR 22). Kidney pathologies were reported more frequently in *ABCC6* positive participants (20 % vs. 5 %; 0.08); kidney stones (10 % versus 4 %; $p = 0.30$) and physician reported "partial renal failure" (10 % versus 0 %; $p = 0.02$). As this was a retrospective, cross-sectional study, no further data was available to determine the trend in kidney function over time.

3.5. Healthy replication cohort

To determine the frequency of variants in vascular calcification candidate genes in a healthy control, we replicated the analysis in an in-house cohort of healthy individuals ($n = 52$). We detected no pathogenic or likely pathogenic variants in any of the eight candidate vascular calcification genes in this cohort (Supplementary Table 6).

4. Discussion

4.1. ABCC6 phenotype

We report four individuals from two families with CKD who carry heterozygous pathogenic variants in the gene *ABCC6*. *ABCC6* is a vascular calcification candidate gene through its involvement in the ectopic vascular calcification pathway (Fig. 1). Mutations in *ABCC6* are associated with the heritable multi-system disease pseudoxanthoma elasticum (PXE) (OMIM #603234). PXE can occur in both the autosomal recessive and autosomal dominant forms. The pathogenesis is assumed to be a result of accumulation of abnormal calcified elastic fibers which can affect the skin, eyes, and cardiovascular system (Ringpfeil et al., 2001). Despite the multiple system involvement associated with PXE, to date, kidney disease has not been considered part of the disease spectrum (OMIM #177850). Interestingly, both heterozygous, *ABCC6* variants, A667fs in Family 1 and R1141X in Family 2, have been reported in patients with PXE (ClinVar ID 433256 and 6559, respectively). Both variants are rare, though R1141X is the most common *ABCC6* variant, predominantly seen in European populations, with a gnomAD frequency of 0.0014; A667fs has a frequency of 0.00002. In addition, heterozygous R1141X carriers display many of the clinical features of the PXE spectrum including angioid streaks, laxity of the skin, vascular disease, and arterial hypertension, with a 4.2x higher risk of coronary artery disease (Garcia-Fernandez et al., 2008; Schulz et al., 2006; Trip et al., 2002). A study of heterozygous *ABCC6* carriers found that, similar to the participants in our cohort, 24 % had retinal alterations; 26 % had lower limb atherosclerosis and subsequent peripheral vascular disease; 26 % had mineral deposits in the kidneys, liver, prostate, or spleen; and 46 % of males had calcifications of the kidney (Nollet et al., 2022).

4.2. ABCC6 and kidney disease

In this report, the diagnosis of PXE was not suspected prior to genetic testing. Following reverse phenotyping, after the detection of the *ABCC6* variants, clinical features were noted which were consistent with the PXE disease spectrum including skin and eye manifestations, and radiographic evidence of vascular calcification. Increasingly, data suggests that kidney disease phenotypes may be underestimated in PXE, though the association is still sparse (Letavernier et al., 2018). For example, a recent study demonstrated a high prevalence of kidney stones (40 %) in an established PXE cohort (Letavernier et al., 2018). Recurrent kidney stones and papillary calcification have been linked to the development of CKD, as well as cardiovascular disease, suggesting that PXE and vascular calcification may have a common underlying mechanism leading to both cardiovascular and kidney disease (Ringpfeil et al., 2001). Others suggest that *ABCC6* heterozygosity should be considered a risk factor for kidney stone formation, as was seen in Family 1 (Nollet et al., 2022). In addition, there have been case reports of PXE patients with ESKD, glomerulonephritis, nephrocalcinosis, and renovascular hypertension, presumably related to small elastic fiber fragmentation and mineralization in renal arteries (D'Marco et al., 2020). In this study, compared to healthy controls, we found a higher prevalence of pathogenic and likely pathogenic variants in *ABCC6* in the ONDRI cohort, which was ascertained for individuals with cerebrovascular and cardiovascular disease. Interestingly, the predominant neurogenerative phenotype in *ABCC6* positive participants was vascular cognitive impairment. Although limited by small numbers and low statistical power, we observed a higher prevalence of kidney pathologies including kidney stones in participants with *ABCC6* mutations as well as higher serum calcium and serum creatinine levels. Taken together, these findings raise the possibility that phenotypic expansion of PXE disease spectrum to include a vascular calcification kidney disease phenotype is warranted.

4.3. ABCC6 pathogenesis

In terms of pathogenesis, we propose that *ABCC6*, a member of the ABC transporter proteins, which is expressed almost exclusively in the kidney and liver (ABCC6 Protein Expression Summary - The Human Protein Atlas Available online: https://www.proteinatlas.org/ENSG0000009, 2024), plays a role in vascular calcification by mediating the cellular efflux of ATP (Fig. 1) (Shimada et al., 2021). At the cell surface, ATP is hydrolyzed by the ectoucleotidases *ENPP1* and *CD73*, to PPi and adenosine. PPi is an inhibitor of mineralization, therefore it is hypothesized that the large efflux of ATP from *ABCC6*, which is rapidly converted to PPi by *ENPP1*, prevents calcification (Shimada et al., 2021). Interestingly, *ABCC6* knockout mice have evidence of renal calcification, suggesting that systemic PPi deficiency can cause calcium phosphate supersaturation leading to papillary calcification (Letavernier et al., 2018). The mice also experience increased creatinine at 8 months, leading to kidney failure, suggesting that kidney dysfunction is not only a consequence of vascular disease in PXE, but also a contributor (Gorgels et al., 2005). In addition, *ABCC6* knockout rats have been found to express 70 % less PPi than wild type controls, while human subjects with *ABCC6* knockout mutations have been found to have low plasma PPi concentration (Terry, 2020). Therefore, it is hypothesized that pathogenic variants in *ABCC6* cause decreased transportation of ATP to the extracellular space in the proximal tubule cells of the kidney, which subsequently reduces the amount of PPi, leading to an increase in calcification in the kidney.

4.4. The role of ENPP1

In Family 1, an additional variant was detected in the gene *ENPP1*. Recessive mutations in *ENPP1* are associated with GACI (OMIM# 208000), which is characterized by calcification of arterial elastic fibers and associated fibrotic myointimal proliferation of muscular arteries (Shimada et al., 2021). Considering the heterozygous state of the *ENPP1* variant in Family 1, we do not assume causation due to this variant alone. However, since *ABCC6* and *ENPP1* are functionally related in

ectopic calcification pathway (Fig. 1) (Shimada et al., 2021; Terry, 2020), the finding raises the possibility of a digenic effect which may worsen the vascular calcification phenotype observed. Certainly, in Family 1, a severe vascular calcification phenotype was evident with early onset peripheral vascular disease. This hypothesis is further supported by data that demonstrates heterozygous carriers of the K121Q variant in *ENPP1* have very high coronary calcium scores (Eller et al., 2008). In addition, mouse models with both *ABCC6* and *ENPP1* haploinsufficiency have been found to develop ectopic calcification comparable to that observed in homozygous mice carrying either recessive mutations in *ABCC6* or *ENPP1* (Li et al., 2014).

4.5. Therapeutics and clinical implications

Numerous studies in both CKD and ESKD populations have investigated therapeutic targets to attenuate vascular calcification through non-targeted treatments including non-calcium-based phosphate binders, cinacalcet (Raggi et al., 2011) and more recently, the myo-inositol hexaphosphate, SNF472 (Raggi et al., 2020). Identification of monogenic causes of vascular calcification within the CKD population now offers the potential to directly target key players in the ectopic calcification pathway. For example, considering a reduced function hypothesis in the setting of heterozygous loss-of-function mutations in *ABCC6*, drugs such as PTC-124 (Welch et al., 2007), which target premature termination codons due to nonsense or frameshift mutations as seen in this study, may offer a direct therapeutic target by restoring full-protein synthesis. The benefits of PTC-124 on certain *ABCC6* variants, including one detected in our study (c.C3421T:p.R1141X), have already been shown in *ABCC6* zebrafish morpholino models (Zhou et al., 2013). Zhou et al., 2013 demonstrated that PTC-124 facilitated read-through of the loss of function variants with subsequent rescue after restoration of full-length protein synthesis (Zhou et al., 2013). Another potential therapeutic target is enzyme replacement of key components in the vascular calcification pathway. In a preclinical trial in patients with mutations in *ENPP1,* as seen in Family 2, soluble recombinant human NPP1 (rhNPP1-Fc) replacement led to reduced ectopic calcification, improved plasma PPi levels, and reduced death in rat models of disease (Albright et al., 2015). Another therapeutic option to consider is either supplementation or augmentation of downstream effectors, including PPi level, to prevent subsequent ectopic calcification. Studies in both mice and humans have shown that oral supplementation of PPi can significantly decrease calcification (Dedinszki et al., 2017), although clinical trials have yet to demonstrate efficacy in disease population. In addition, bisphosphonate, which mimics the structure of PPi, may hold promise on the assumption that they inhibit enzymes that utilize pyrophosphates, ultimately leading to an increase in PPi level (Drake et al., 2008). Overall, with the development of new therapeutics, it is essential to assess families with CKD for genetic causes early in the diagnostic pathway to allow for personalized disease management and the prevention of vascular calcification.

4.6. Limitations

Although this study demonstrates the novel detection of vascular calcification genes in patients with CKD, it is not without limitations. Our study was limited by the lack of follow-up data from the ONDRI cohort, limiting our ability to determine whether participants developed CKD over time. In addition, the lack of renal imaging and use of descriptive terms such as “partial renal failure” to identify participants with CKD, limits our ability to assess for the coexistence of vascular calcification and CKD. Our study also lacks generalizability to the CKD cohort, as the ONDRI cohort includes patients with a predominant cerebrovascular phenotype. We did however observe an excess of pathogenic variants in *ABCC6* in the ONDRI cohort compared to healthy controls. Considering the potential for multisystem involvement in a vascular calcification phenotype, this finding warrants further evaluation to determine if the end-organ damage observed in the ONDRI cohort, namely cerebrovascular and cardiovascular disease, is mediated through a common vascular calcification pathway. It is possible, given the dominant mode of inheritance, that variable expressivity or reduced penetrance is contributing to the varying phenotypic expression of disease. The present study is also limited by the small sample size of participants with *ABCC6* variants, as well as the lack of functional analysis to confirm the pathogenesis of the detected variants.

4.7. Future directions

Future studies should focus on functional studies to decipher the exact role of *ABCC6* and *ENPP1* in vascular calcification and for the potential selectively in end-organ damage observed in these cohorts. In addition, although heterozygous mouse models for PXE support our hypothesis that kidney disease occurs in association with *ABCC6* mutations (Li et al., 2017), further studies to demonstrate the function of the exact variants, R1141X/- and A667fs/-, are required. Additionally, future studies should aim to investigate the role of *ABCC6* as a potential therapeutic target for vascular calcification, as well as the efficacy of new therapeutics for treatment of vascular calcification in patients with *ABCC6* variants.

5. Conclusion

We identified mutations in *ABCC6* and *ENPP1* in two families with CKD of unknown etiology with a vascular calcification phenotype. In both families, features of the PXE phenotype were detected following reverse phenotyping including eye pathologies, severe peripheral vascular disease, and skin manifestations. In an additional disease cohort (ONDRI cohort), ascertained for cerebrovascular and cardiovascular disease, we found an excess of pathogenic and likely pathogenic variants in the gene *ABCC6* in individuals with vascular cognitive impairment, compared to healthy controls. We propose that in the heterozygous state, loss-of-function variants in *ABCC6* may contribute to a kidney disease phenotype mediated through a common vascular calcification pathway ultimately leading to end organ damage of the kidneys, heart, or brain. Overall, with the development of new therapeutics for vascular calcification, it is essential to assess families with CKD for genetic causes early in the diagnostic pathway to allow for personalized disease management and the prevention of vascular calcification.

CRediT authorship contribution statement

Clara Schott: Writing – review & editing, Writing – original draft, Visualization, Investigation, Formal analysis, Data curation, Conceptualization. **Allison A. Dilliott:** Writing – review & editing, Investigation, Formal analysis, Data curation. **Jian Wang:** Writing – review & editing, Software, Formal analysis, Data curation. **Adam D. McIntyre:** Writing – review & editing, Investigation, Formal analysis, Data curation. **Surim Son:** Formal analysis. **Samantha Colaiacovo:** Writing – review & editing, Resources, Data curation. **Cadence Baker:** Writing – review & editing, Project administration, Data curation. **Lakshman Gunaratnam:** Writing – review & editing, Resources. **Andrew A. House:** Writing – review & editing, Resources. **Shih-Han Susan Huang:** Writing – review & editing, Resources. **Hariharan Iyer:** Resources. **John Johnson:** Resources. **Khaled Lotfy:** Writing – review & editing, Resources. **Mario Masellis:** Resources. **Douglas P. Munoz:** Resources. **Faisal Rehman:** Writing – review & editing, Resources. **Pavel Roshanov:** Resources. **Richard H. Swartz:** Resources. **Matthew A. Weir:** Writing – review & editing, Resources. **Robert A. Hegele:** Writing – review & editing, Validation, Supervision, Software, Resources, Methodology. **Dervla M. Connaughton:** Writing – review & editing, Writing – original draft, Validation, Supervision, Resources, Methodology, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Author Contributions

This project was conceived by CS and DMC. Data curation was completed by CS, AAD, JW, SC, CB, and DMC. Formal analysis was completed by CS, AAD, JW, ADM and SS. DMC is responsible for funding acquisition. CS and AAD completed investigations. DMC and RAH were responsible for methodology development. CB was responsible for project administration. Resources were provided by AH, DMC, DPM, FR, HI, JJ, KL, LG, MM, MW, PR, RAH, RHS, SC, and SH. Software was provided by JW and RAH. Supervision and validation were provided by RAH and DMC. Visualizations were created by CS. Writing of the original draft was completed by CS and DMC, review and editing were completed by CS, AAD, JW, ADM, SC, CB, RAH, and DMC. All authors provided final approval of the version to be submitted.

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Appendix A. Supplementary material

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