Eight novel variants in the *SLC34A2* gene in pulmonary alveolar microlithiasis

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Eight novel variants in the *SLC34A2* gene have been identified in 14 patients with pulmonary alveolar microlithiasis (PAM), which emphasises the importance of the gene in the disease. Furthermore, a genotype–phenotype correlation in PAM may exist. http://bit.ly/3307M1p

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ABSTRACT

Background: Pulmonary alveolar microlithiasis (PAM) is caused by genetic variants in the *SLC34A2* gene, which encodes the sodium-dependent phosphate transport protein 2B (NaPi-2b). PAM is characterised by deposition of calcium phosphate concretions (microliths) in the alveoli leading to pulmonary dysfunction. The variant spectrum of *SLC34A2* has not been well investigated and it is not yet known whether a genotype–phenotype correlation exists.

Methods: We collected DNA from 14 patients with PAM and four relatives, and analysed the coding regions of *SLC34A2* by direct DNA sequencing. To determine the phenotype characteristics, clinical data were collected and a severity score was created for each variant, based on type and localisation within the protein.

Results: We identified eight novel allelic variants of *SLC34A2* in 14 patients with PAM. Four of these were nonsense variants, three were missense and one was a splice site variant. One patient was heterozygous for two different variants and all other patients were homozygous. Four patients were asymptomatic and 10 patients were symptomatic. The severity of the disease was associated with the variant severity.

Conclusions: Our findings support a significant role for *SLC34A2* in PAM and expand the variant spectrum of the disease. Thus, *SLC34A2* variants were detected in all patients and eight novel allelic variants were discovered. An association between disease severity and the severity of the variants was found; however, this needs to be investigated in larger patient populations.

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Introduction

Pulmonary alveolar microlithiasis (PAM) is a rare inherited lung disease with less than 1100 patients reported worldwide since 1933. The disease is characterised by deposition of calcium phosphate concretions (microliths) in the alveoli of the lungs [1]. Symptoms are dyspnoea, nonproductive cough, chest pain and fatigue [2]. The clinical course is variable; in some patients, the disease remains relatively quiescent while in others it progresses to respiratory insufficiency and early death [1]. Lung transplantation is currently the only existing effective treatment [1].

PAM is considered to be an autosomal recessive disorder with high penetrance (OMIM #265 100). The only known causative gene is solute carrier family 34 member 2 (*SLC34A2*) (Entrez Gene ID 10568) [1, 3–5]. It is located on the short arm of chromosome four (4p15.2) and it has 13 exons, of which the first is noncoding. *SLC34A2* encodes a 690 amino acid protein, the sodium-dependent phosphate transport protein 2B (NaPi-2b), which plays a role in inorganic phosphate homeostasis [5–7]. *SLC34A2* is expressed in type II alveolar cells [4, 8], which play an important role in surfactant recycling and catabolism [9], and possibly also in the export from the alveolar space of phosphate liberated from degraded phospholipids. In PAM, dysfunction of NaPi-2b due to genetic variants in *SLC34A2* might therefore cause a defect in cell uptake of phosphate, leading to elevation of phosphate levels in the alveolar lining fluid and deposition of calcium phosphate concretions in the alveoli [4, 10].

Since the identification of the first genetic variants in PAM patients in 2006, a total of 22 different allelic variants have been reported in less than 50 patients (figure 1). The most common DNA alterations in *SLC34A2* involve changes to single or a few nucleotides, but more complex variants have been described including combinations of deletion–insertions and larger deletions [4, 17, 18]. The variants are proposed to lead to protein truncation with a possible decreased protein activity or to gene expression silencing [1, 3, 4, 19, 20]. Furthermore, elimination of the mRNA due to nonsense-mediated decay is another possible outcome [16]. However, no detailed review of clinical data in PAM patients with detected genetic variants has so far been reported. Therefore, no assessments of association between the genotype and the severity of the pulmonary and extrapulmonary symptoms have been made.

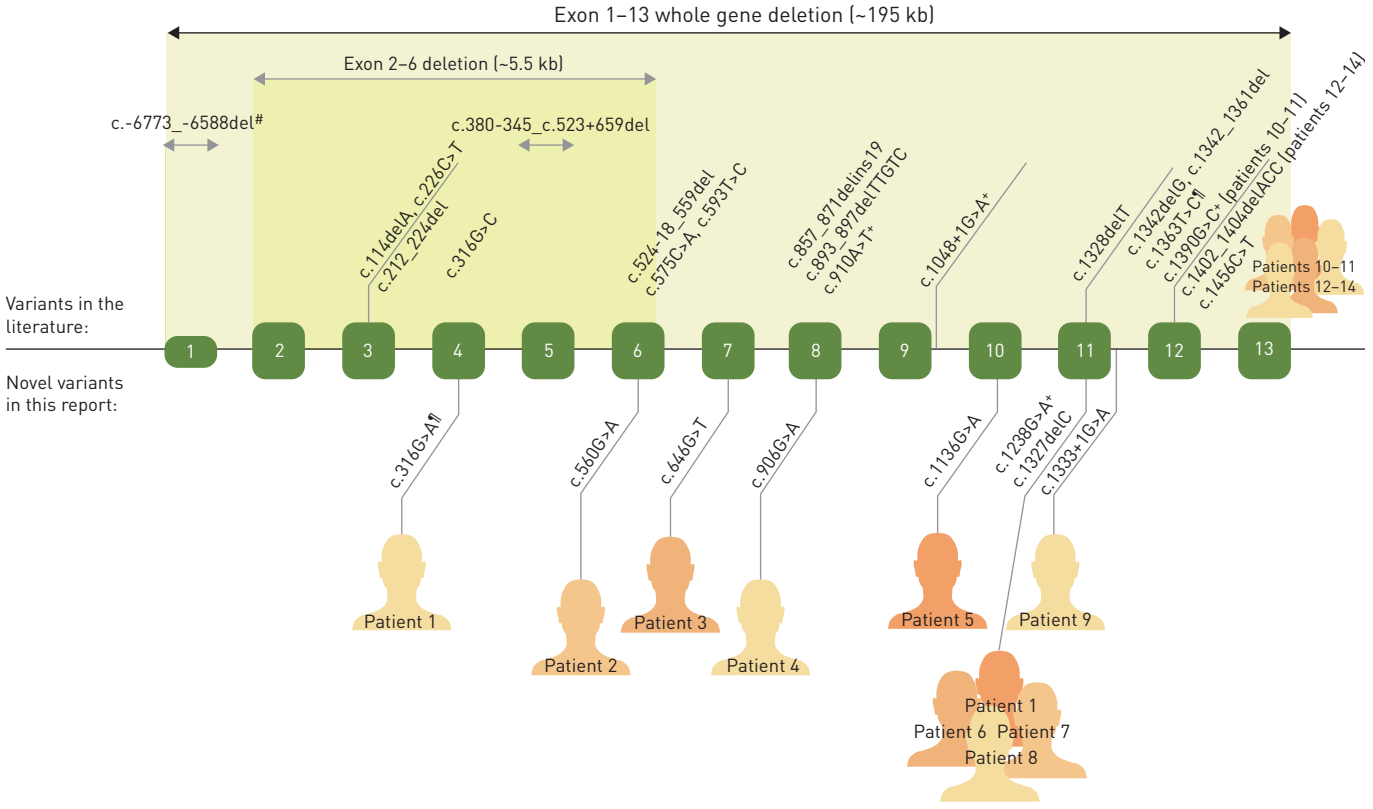

FIGURE 1 Allelic variants in *SLC34A2* in patients with pulmonary alveolar microlithiasis (PAM) in the literature [2–4, 11–23] and novel variants presented in this study. Patients 13 and 14 are previously described with the variant c.1402_1404delACC [2]. Variants are present in homozygous form unless otherwise stated. Narrow boxes are for noncoding exons and wider boxes are for coding exons. Exons, introns and deletions are not drawn to scale. #: involving the *SLC34A2* promoter region; ¶: compound heterozygous; +: homozygous and compound heterozygous.

The specific objectives of our study were to expand the allelic spectrum of *SCL34A2* in patients with PAM and to evaluate a possible genotype–phenotype correlation. We present the genotype and phenotype characteristics in a series of PAM patients and contribute important new knowledge of the variant spectrum of *SLC34A2*. In addition, we investigate the correlation between the clinical phenotype and the localisation of the genetic variant and variant type.

Patients and methods

Patients

Fourteen patients with PAM and four relatives were included in this case series, from seven medical centres between 2014 and 2017. The clinical diagnosis of PAM was based on either radiographic appearance, a typical histological picture in lung biopsy, or the presence of microliths in bronchoalveolar lavage fluid (BALF). The project protocol was approved by the Central Denmark Region Committees on Biomedical Research Ethics (1-10-72-10-14). Sampling was performed according to clinical practice and the regulations in each country, complying with the principles of the Declaration of Helsinki. Written informed consent was obtained from the subjects.

Sampling and data analyses

Total DNA was extracted from venous blood (5–10 mL in EDTA) or saliva using standard protocols. Genetic analysis included direct DNA sequencing of PCR-amplified coding regions and flanking splice sites of the *SLC34A2* gene (see supplementary material and supplementary table E1). Sequencing data was analysed using Mutation Surveyor Loc48 version 3.20 (SoftGenetics LLC, State College, PA, USA) with the *SLC34A2* DNA sequence as a reference (Ensembl Transcript ID ENST00000382051.7 (GRCh38.p12 assembly)). We screened for the prior inclusion of all variants in public databases of known genetic variation, including the Genome Aggregation Database (GnomAD) [24], the Exome Variant Server, the NHLBI GO Exome Sequencing Project (ESP) [25], the 1000 Genomes Project [26] and the Database of Single Nucleotide Polymorphisms (dbSNP) [27], as well as through searches of the scientific literature [28] and the Human Gene Mutation Database (HGMD Professional 2019.1) [29]. Variants with a frequency in the general population above 0.1% were considered to be common and accordingly were not considered causative for PAM.

The severity of the disease was stated as a "clinical disease severity score" based on a composite measure of nine clinical parameters. Each parameter was scored between zero and one points (pulmonary hypertension (PH), chest pain, fatigue, clinical progression, limitations of daily activities and incapability to work) or between zero and two points (forced vital capacity (FVC), diffusing capacity of the lung for carbon monoxide (D_{LCO}) and dyspnoea) (see supplementary table E2). The maximum obtainable score was 12 and it is presented as a ratio with a maximum average score of 1.33. The severity of the disease was graded into three groups: mild (0–0.44), moderate (0.45–0.88) and severe (0.89–1.33).

The variants were interpreted *in silico* with MutationTaster2 (www.mutationtaster.org) [30], PANTHER version 13.1 (http://pantherdb.org) [31], Polyphen-2 version 2.2.2r398 (http://genetics.bwh.harvard.edu/pph2/) [32], PROVEAN version 1.1.3. (http://provean.jcvi.org) [33] and Human Splicing Finder version 3.1 (www.umd.be/HSF3/) [34]. The variants were classified according to American College of Medical Genetics and Genomics (ACMG) and Association for Molecular Pathology (AMP) criteria [35]. In addition, the variants were classified into three severity groups: mild (0 points), moderate (1 point) and severe (2 points), on the basis of their predicted effect on NaPi-2b function based on the variant type and localisation within the protein. Additional details regarding this classification are provided in the supplementary material.

Statistical analysis

The calculations were performed in Stata 11.2 (StataCorp 2009, College Station, TX, USA). Normality was inspected by QQ-plots of data. Pairwise correlation was tested with Spearman's rank correlation. A p-value of less than 0.05 was considered statistically significant.

Results

Clinical findings

Demographics and clinical characteristics

Table 1 and supplementary table E3 summarise the clinical characteristics and demographics of the patients. The mean±SEM age was 39.6±5.9 years and age at diagnosis varied from 0 to 66 years. The median follow-up time was 12 years (range: 4–39 years). Patients 4, 8 and 10 were diagnosed with PAM in the setting of familial testing. In six patients, PAM was an incidental diagnosis based on chest radiographs taken for another purpose. The patients were from Denmark (n=2), France (n=1), Italy (n=2), Norway (n=2), Spain (n=3) and the USA (n=4). Patients were Caucasian (n=13) or Arabian (n=1). Seven patients had at least one known relative with PAM including two pairs of siblings (patients 7 and 8 as well as patients 10 and 11). Consanguinity was reported in three patients.

TABLE 1 Clinical characteristics of the patients

Patient ID	Sex	Age years		Symptoms	CT involvement[#] %	FVC % predicted	D_{LCO} % predicted	Microliths		Clinical course[¶]	PAM specific therapy
		Current	At diagnosis					In biopsy	In BALF		
1	F	39	22	Dyspnoea, chest pain	No data	93	63	No data	Not performed	Stable	None
2	F	9	5	None	>50	89	63	Not performed	Not performed	Stable	None
3	F	–[+]	66	Dyspnoea, cough, asthenia	No data[§]	57	23	No data	No data	Progress	ALD (70 mg·week^{-1}), PSE (10 mg·day^{-1}) Unknown effect[+]
4	F	40	34	Dyspnoea	>50[f]	80	51	Yes	Yes	Progress	STS (12.5 g·month^{-1}; 9 months) No effect[##]
5	M	54	46	None	>50[¶¶]	92	60	Yes	Yes	Progress	None
6	F	37	No data	Dyspnoea, cough	No data	No data	No data	No data	No data	No data	No data
7	F	–[++]	20	Dyspnoea, chest pain, asthenia	>50	52	38	No data	Not performed	Progress	None
8	F	52	23	Dyspnoea, chest pain, asthenia, cough	>50	52	25	No data	Not performed	Progress	None
9	M	58	19	Dyspnoea, cough	>50	No data	No data	Yes	Not performed	Progress	None
10	M	9	9 months	None after age four[§§]	20–50	102	87	Not performed	Not performed	Stable	None
11	F	14	5	None after age four[§§]	>50	94	74	Yes	No data	Stable	None
12	F	69	51[ff]	Dyspnoea, asthenia, cough	No data	45	29	Not performed	Not performed	Progress	LPD (2 years) Serum phosphate decreased
13	M	32	16	Dyspnoea, chest pain, asthenia, cough	>50	No data	No data[###]	Yes	No	Progress	EHDP (200 mg·day^{-1}; 1.5 years) No effect
14	M	62	50	Dyspnoea, asthenia	>50	87	33	Yes	No	Progress	PDN (2 months), EHDP (200 mg·day^{-1}; 6 months) No effect

CT: computed tomography; FVC: forced vital capacity; D_{LCO}: diffusing capacity of the lung for carbon monoxide; BALF: bronchoalveolar lavage fluid; PAM: pulmonary alveolar microlithiasis; ALD: alendronate; PSE: prednisone; STS: sodium thiosulfate; LPD: low phosphate diet; EHDP: disodium etidronate; PDN: prednisolone. [#]: CT involvement was based on a clinical estimation from each medical centre and was not standardised; [¶]: clinical course was evaluated on symptoms, pulmonary function tests (PFTs) and radiographical appearance; [+]: patient was lost to follow-up at age 66; [§]: patient was known with parenchymal calcifications from age 10; [f]: extensive radiographic progression, slow clinical progression; [##]: treatment with STS in this patient was previously reported by Taille *et al.* [36]; [¶¶]: radiographic abnormalities detected at the age six; [++]: patient died at age 47; [§§]: patient experienced respiratory symptoms including broncho-obstructive crises and recurrent pneumonias until the age of four, but has been asymptomatic since; [ff]: patient was misclassified with sarcoidosis for 20 years prior to the diagnosis of PAM; [###]: patient could not cooperate with pulmonary function tests (PFTs) or spirometry due to chest pain. Pulmonary function tests at the time of diagnosis revealed signs of mild restrictive pattern and normal D_{LCO}.

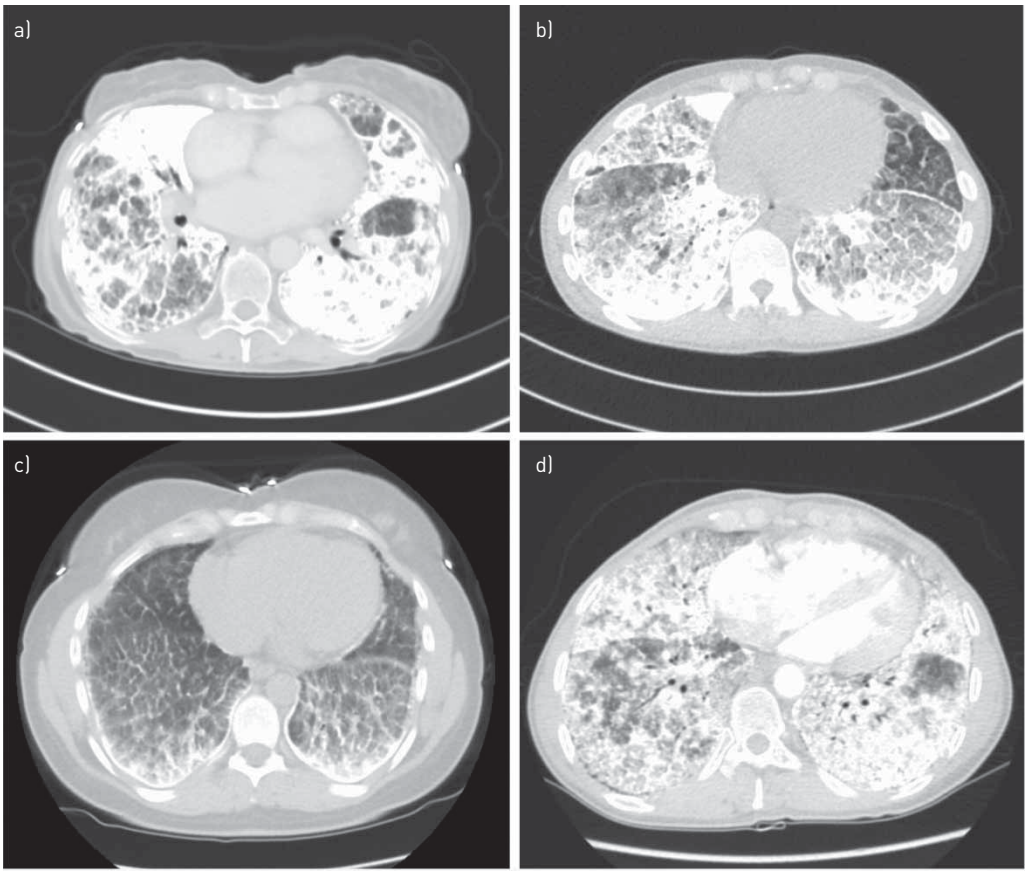

FIGURE 2 Computed tomography (CT) scans in pulmonary alveolar microlithiasis (PAM) patients showing different degrees of calcification with numerous sand-like calcifications, calcified interlobular septa and consolidations throughout the lungs with a subpleural localisation. Patient 12 (a) and patient 13 (b) are homozygous for the same genetic variant in *SLC34A2* and present with similar degrees of calcification in the lungs. Different levels of severity of lung involvement are seen in patient 1 (c) (mild clinical disease) and patient 7 (d) (severe clinical disease).

The majority of patients reported symptoms typical for PAM, most commonly dyspnoea. The typical radiographic appearance of hyperdense infiltrates was seen in all patients on chest radiography, computed tomography (CT) scan and/or high-resolution computed tomography (HRCT) scan (figure 2). Pulmonary function tests (PFTs) revealed a restrictive pattern with impaired total lung capacity (TLC) for five out of 10 patients and decreased D_{LCO} for 10 out of 11 patients. The diagnosis was confirmed by histopathological examination of a lung biopsy (either a transbronchial biopsy or a surgical lung biopsy) in eight out of nine patients. In the majority of patients, blood levels of calcium and phosphate were within normal levels. Kidney and gallbladder stones were reported in three patients, one of which was additionally found to have calcifications in the gastric ventricular wall and was diagnosed in adolescence with a significant aortic valve stenosis (AS). Considerable comorbidity was reported in eight patients (see supplementary table E3).

Treatment and course of disease

In five patients, specific treatments for PAM had been attempted (table 1). Patient 12 was put on a low phosphate diet (LPD) for about 2 years and, after 4 months, their serum phosphate level had decreased from 1.26 to 1.00 $mmol \cdot L^{-1}$; however, no clinical benefits were reported. PAM was progressive in nine patients and stable in four (with similar follow-up times in the two groups, p=0.26). One patient died shortly after inclusion in the study due to respiratory failure and two of the patients have previously been described in detail [2, 37]. Both were reported symptomatic with decreased lung function and without treatment benefits. Since then, slow clinical progression has been observed in both patients.

Severity of disease

Based on the clinical disease severity score, six patients were categorised as having mild disease, two as having moderate disease and five as having severe disease (table 2 and supplementary table E4). Six of the seven patients with either moderate or severe disease were current or former smokers. A correlation was

TABLE 2 Variants in *SLC34A2* identified in the patients

Patient ID	Nucleotide change	Exon	Protein change	Pathogenicity variant class[#]	Variant severity[¶]	Clinical disease severity[+]	Reference
Eight novel allelic variants in *SLC34A2* identified in PAM patients							
1[§]	c.316G>A	4	p.Gly106Arg	I	Moderate	Mild	
	c.1238G>A	11	p.Trp413Ter	II			
2	c.560G>A	6	p.Gly187Glu	III	Moderate	Mild	
3	c.646G>T	7	p.Gly216Ter	II	Severe	Severe	
4	c.906G>A	8	p.Trp302Ter	II	Moderate	Mild	
5	c.1136G>A	10	p.Cys379Tyr	III	Mild	Mild	
6	c.1238G>A	11	p.Trp413Ter	II	Moderate	Not applicable	
7[f]	c.1327delC	11	p.Leu443Ter	II	Severe	Severe[##]	
8	c.1327delC	11	p.Leu443Ter	II	Severe	Severe	
9	c.1333+1G>A	Intron 11	p.?	II	Severe	Moderate	
Two recurrent allelic variants in *SLC34A2* identified in PAM patients							
10[¶¶]	c.1390G>C	12	p.Gly464Arg	I	Moderate	Mild	[16][++]
11	c.1390G>C	12	p.Gly464Arg	I	Moderate	Mild	[16][++]
12	c.1402_1404delACC	12	p.Thr468del	I	Moderate	Severe	
13	c.1402_1404delACC	12	p.Thr468del	I	Moderate	Severe	[2]
14	c.1402_1404delACC	12	p.Thr468del	I	Moderate	Moderate	[2]

PAM: pulmonary alveolar microlithiasis. [#]: based on the American College of Medical Genetics and Genomics (ACMG) and the Association for Molecular Pathology (AMP) criteria [35] (where I: pathogenic, II: likely pathogenic, III: uncertain significance, IV: likely benign and V: benign); [¶]: predicted effect on NaPi-2b function based on variant type and localisation within the protein. Variants causing premature truncation or with a presumed crucial localisation within the protein scored one point each. Missense variants and small in-frame deletions scored zero points (where mild: 0 points, moderate: 1 point and severe: 2 points); [+]: disease severity was graded into three groups: mild, moderate and severe, based on a composite measure of the patient's symptoms, lung function, limitation of life due to PAM, signs of advanced disease and disease course (nine parameters in total); [§]: compound heterozygous allele state (all other patients had variants in a homozygous allele state); [f]: sister of patient 8; [##]: deceased; [¶¶]: brother of patient 11; [++]: Izumi *et al.* [16] presented the variant c.1390G>C in heterozygous state together with c.1048+1G>A in a PAM patient (*SLC34A2* DNA reference sequence: Ensembl Transcript ID ENST00000382051.7 (GRCh38.p12 assembly)).

found between smoking and disease severity in all patients (Spearman rank correlation coefficient=0.762, p=0.003) but this was not statistically significant on excluding the children (Spearman rank correlation coefficient=0.617, p=0.057) (see supplementary tables E5 and E6).

Genetic findings

All 14 patients presented with rare variants in *SLC34A2*, including eight novel allelic variants (see table 2 and figures 3 and 4). The variants comprised four nonsense, four missense and one splice site variant. In addition, one variant previously described by our group [2], a three nucleotide deletion in exon 12 (c.1402_1404delACC), was found in an unrelated patient. All patients were homozygous for the identified variants, except for patient 1, who was compound heterozygous for a missense variant in exon 4 (c.316G>A) and a nonsense variant in exon 11 (c.1238G>A). The c.1238G>A variant was additionally found in a homozygous state in patient 6. The mother of patient 2 was found to be a carrier of the same variant as her child and the parents and a sibling of patients 10 and 11 were identified as carriers. None of the carriers had clinical signs or symptoms of PAM.

Eight out of 10 patients were homozygous for an additional variant in *SLC34A2* located in exon 13, c.1901A>G, a common variant (rs6448389) with a high but variable allele frequency (81–100%) in different populations. Another additional variant located in exon 9, c.936T>G, a moderately rare variant (rs112461275) with a reported allele frequency of up to 7% in some African subpopulations [26], was detected in homozygous form in the French patient originating in Morocco (patient 4). None of these variants were considered as possibly causative for PAM due to their high frequency in the general population.

Classification of the variants

Variant severity was classified as mild (n=1), moderate (n=6) or severe (n=3), reflecting the predicted effect on NaPi-2b function based on type and localisation within the protein. Two variants in a compound

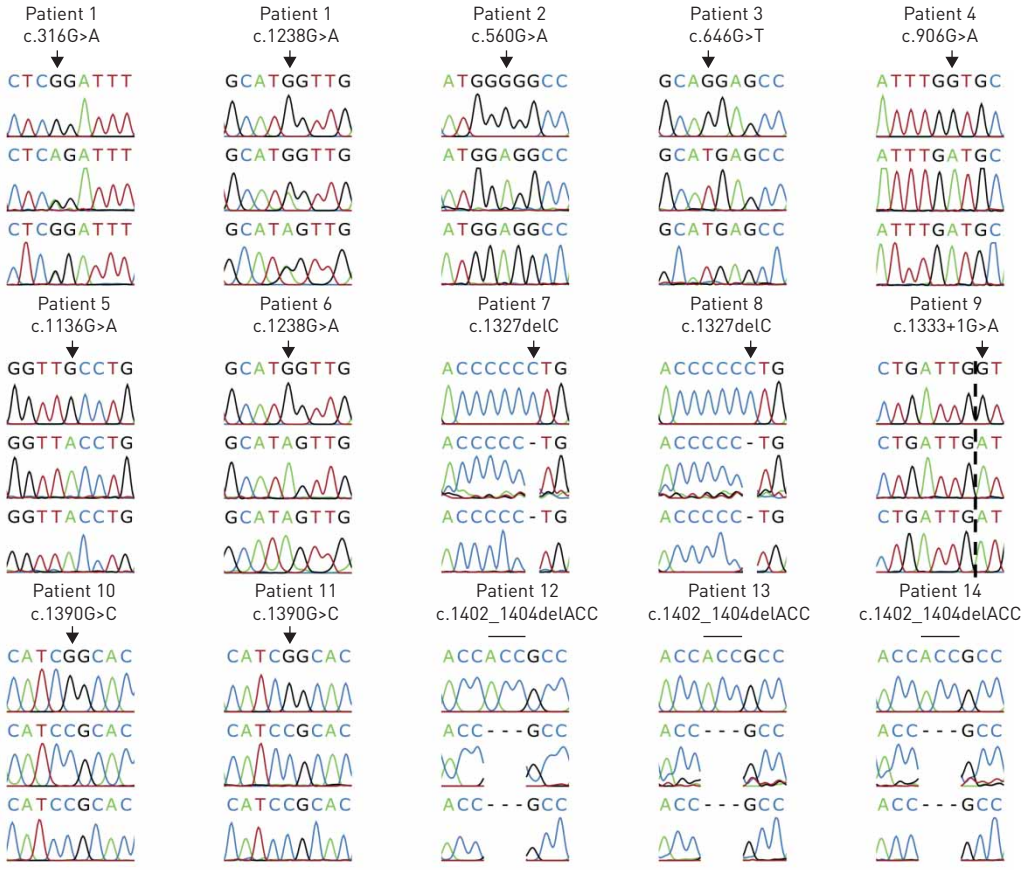

FIGURE 3 DNA sequence analysis of selected regions of *SLC34A2* in patients 1–14. Two different allelic variants are found in patient 1 (a compound heterozygous state), while all other variants are present in homozygous form. Top row: *SLC34A2* DNA reference sequence (Ensembl Transcript ID ENST00000382051.7 (GRCh38.p12 assembly)). Middle row: forward sequence. Bottom row: reverse sequence. The dotted line represents the border between exon 11 and intron 11. The arrows and bars indicate the locations of the variants.

heterozygous state were given a combined severity score based on the highest predicted value of one of the variants (see table 2 and supplementary table E7). The variants were classified according to the ACMG criteria [35] as Class I (pathogenic (n=3)), Class II (likely pathogenic (n=5)) and Class III (uncertain significance (n=2)) (table 2).

Four out of 10 of the expected disease-causing allelic variants were absent in the GnomAD, ESP, 1000Genomes Project and dbSNP databases, and the remaining variants were present in very low frequency and only in the heterozygous state (see supplementary material). In addition, all variants were predicted to be "probably damaging", "deleterious" or "disease causing" by computational prediction tools (see supplementary table E8). Two of the variants (c.1390G>C and c.1402_1404delACC) have previously been described in PAM patients [2, 16]. In addition, a different nucleotide substitution (c.316G>C) leading to the same amino acid change as in patient 1 (p.Gly106Arg) was previously reported in a Turkish patient [3].

Phenotype and genotype

Four allelic variants (c.1238G>A, c.1327delC, c.1390G>C and c.1402_1404delACC) were found in more than one patient (patients 1, 6–8 and 10–14). Two different variants (c.1327delC and c.1390G>C) were present in both pairs of siblings (patients 7 and 8, as well as 10 and 11) and one variant (c.1402_1404delACC) was found in three unrelated patients (patients 12–14). Similar phenotypes were observed in the patients homozygous for the same variants (patients 7 and 8 (c.1327delC), 10 and 11 (c.1390G>C) and 12–14 (c.1402_1404delACC)) (table 2). Both adult sisters (patients 7 and 8) had severe clinical disease. Patient 7 later passed away due to respiratory complications and the other sister was successfully treated with lung transplantation. The young siblings (patients 10 and 11) both had mild clinical disease and were both without any symptoms since the age of four. As most of the patients in the cohort were found with different variants, it was impossible to establish overall specific genotype–

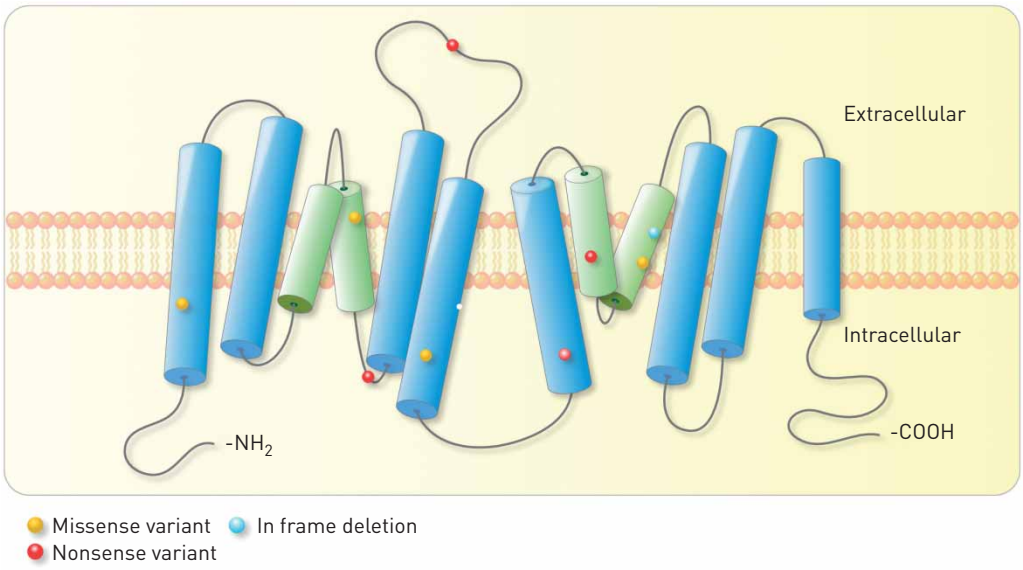

FIGURE 4 Allelic variants in *SLC34A2* presented in this report marked on a model of NaPi-2b. All variants, except for the splice site variant (c.1333+1G>A) are shown in the figure as dots. The transmembrane domains (TMDs) 3–4 and 8–9 (green) form the substrate coordination site. Important areas for electrogenicity, regulation and targeting are located in the area between TMDs 4–5, 10–11 and at the C-terminal region [38]. NaPi-2b belongs to the SLC34 transporter family along with NaPi-2a (*SLC34A1*) and NaPi-2c (*SLC34A3*). Significant sequence similarity is present in all SLC34 family members and the eukaryotic NaPi-2 isoforms are predicted to share the same transmembrane topology [39]. The model is made by superimposing human NaPi-2b on rat NaPi-2a predicted topology and is modified from Forster *et al.* [38] and Virkki *et al.* [40]. The protein sequences used for alignment in Clustal Omega version 1.2.4 [41] were Ensembl Transcript ID ENST00000382051.7 release 92 (Human (GRCh38.p12) assembly) and Ensembl Transcript ID ENSRNOT00000033749.5 (Rat (Rnor_6.0) assembly).

phenotype correlations. However, a correlation was found between disease severity and variant severity (Spearman rank correlation coefficient=0.629, p=0.021) (see supplementary table E9).

Discussion

In this study, we present the genetic analysis of *SLC34A2* in 14 PAM patients from six countries. All patients were either homozygous or compound heterozygous for variants in *SLC34A2* and eight allelic variants were novel. Both clinical course and age varied greatly among the patients. A correlation was found between disease severity and variant severity based on the variant type and localisation within NaPi-2b. Our results confirm the phenotypic variability of PAM, expand the spectrum of disease-causing variants in *SLC34A2* and suggest that disease severity may be associated with the severity of the variants.

PAM is considered to be a monogenic disorder [1]. Genetic variants in *SLC34A2* were found in all patients studied. This high detection rate is consistent with the recessive pathogenesis of PAM and it decreases the likeliness of locus heterogeneity. The variants in this report were very likely disease causing since 1) they have not been reported in homozygous form in the general population and all are rare; 2) they were all present in the patients in a homozygous or compound heterozygous state; and 3) they were all predicted to interfere with the structure and/or function of NaPi-2b.

In total, 10 genetic allelic variants were identified in the patients studied. To our knowledge, eight of the alterations identified have not previously been reported and their association with PAM is therefore unknown. Interestingly, four of the novel allelic variants (c.560G>A, c.646G>T, c.1327delC and c.1333 +1G>A) and two recurrent variants (c.1390G>C [16] and c.1402_1404delACC [2]) are located in functionally critical areas of the protein [38]. The novel splice site variant (c.1333+1G>A) is located at the donor splice site immediately after the 3' end of exon 11. It might thus lead to a splicing defect that causes either complete intron retention or activation of a cryptic splice site in the intron or in exon 12, leading to a frameshift which results in a premature stop codon.

Three variants in *SLC34A2* have previously been functionally investigated in cells [4, 42]. These studies revealed either a nonfunctional protein (in the case of a 19 amino acid indel and a splice site variant) or signs of reduced phosphate transport function (in the case of a p.Thr192Lys missense variant). Interestingly, the missense variant was located in a presumed functionally critical area of the NaPi-2b

protein [38]. One may therefore speculate that location of missense variants in a functionally critical region of the NaPi-2b protein might, in general, cause changes in structural conformation with possible disruption of its intracellular handling or changes in protein kinetics. This might give rise to proteins which have impaired ability (or are unable) to reach the membrane, thus leading to reduced or removed protein function. However, more functional cell studies are needed to investigate the possible functional impact of different variants.

A recurrent variant in exon 12 (c.1402_1404delACC) seems to be rather frequent in PAM, as it has been identified in three unrelated patients. The variant is located in a repetitive region of four ACCs, which may thus be predisposed to replication errors. Interestingly, the American patient with the variant in question originated from Italy and the variant has previously only been reported in Europeans. The c.1901A>G variant (rs6448389) is common and was therefore expected to be present in most of the patients. More surprising was the detection of the moderately rare variant rs112461275 in patient 4, who originated from Africa. It is noteworthy that this variant is reported with a higher frequency in Africans compared to other populations. However, it is so far unknown whether there exists an association between some specific rare single nucleotide polymorphism (SNP) and the disease itself.

The clinical variability in our patients is in agreement with previous findings [1]. Environmental factors like inflammation and smoking have been suggested to influence both the onset and the course of PAM and thereby partly explain the large clinical variability of the disease [2, 3, 19, 21]. In our study, no correlation between smoking and disease severity was found in the adult patients. We acknowledge that this could be due to a relatively small sample size.

Four patients in our cohort presented with calcifications at extrapulmonary sites and two patients (patients 9 and 13) had undergone a valve replacement due to AS. One of these patients (patient 13) was diagnosed with AS, renal stones and gallbladder stones, as well as calcifications in the gastric ventricle consistent with a multisystem disorder. Extrapulmonary calcifications in PAM might be caused by dysfunctional NaPi-2b in the tissues affected [1, 2, 37].

One patient was treated with lung transplantation, which is the only known effective treatment in PAM as systemic corticosteroids and therapeutic bronchoalveolar lavage (BAL) with saline are considered to be noneffective. A possible beneficial effect of bisphosphonates and sodium thiosulfate (STS) remains to be explored [1, 36]. Since variable degrees of fibrosis and inflammation of the pulmonary interstitium are accompanying features of calcification in advanced disease [43], treatment with antifibrotic agents could possibly be of relevance in some cases. To the best of our knowledge, antifibrotic treatment has so far not been reported in PAM.

A patient in our cohort was misclassified with sarcoidosis prior to the diagnosis of PAM; however, while it is uncommon, multiple micronodular calcifications can be seen in sarcoidosis [44]. Pulmonary calcification and ossification are associated with a variety of pulmonary and systemic diseases, such as metastatic calcification due to chronic renal failure, dystrophic calcification due to granulomatous disorders, DNA viruses, parasitic infections, pulmonary amyloidosis and various forms of pulmonary ossification (*e.g.* idiopathic dendriform diffuse pulmonary ossification) [44, 45]. Different imaging techniques are used to recognise specific patterns and if it is not conclusive, a biopsy is needed for the diagnosis. The histopathology of PAM is characteristic, in which concentric laminated calcium phosphate concretions are present inside the alveoli, whereas calcifications are found in the interstitial or vascular compartments in metastatic and dystrophic calcifications [44]. If histological investigation is not clear, the diagnosis of PAM may be confirmed by genetic evaluation of the *SLC34A2* gene.

No genotype–phenotype correlation has been described in patients with PAM [1]. However, only a few variants in a relatively small number of patients have so far been reported and the clinical data on patients is based on case reports with variable levels of detail. In our report, one of the variants was found in three apparently unrelated patients and all of them presented with moderate to severe disease. However, two of these patients are now in their sixties and the clinical severity might just reflect the slowly progressive nature of the disease (*i.e.* a survivor effect). On the other hand, the third patient was only an adolescent when significant symptoms appeared. Given most of the variants in this report were detected only once, no overall specific genotype–phenotype correlations were established. However, an interesting correlation between disease severity and variant severity was found.

We categorised the severity of disease based on a composite measure of different clinical parameters; however, we acknowledge this categorisation is subject to bias and standardised methods and validation are still lacking. Furthermore, we acknowledge the possibility of confounding due to age difference and variation in smoking status amongst the patients. The functional effects of variants in *SLC34A2* are not well investigated so far, and the classification and evaluation of different genotypes in this report are

therefore determined merely on a theoretical basis. Therefore, the findings in this study need to be confirmed and further addressed in larger patient series and functional studies. The main strength of this report is the presentation of both clinical and genetic data from a relatively large cohort of PAM patients from several countries. Furthermore, all genetic analyses were carried out in the same laboratory.

In conclusion, we detected eight novel allelic variants in *SLC34A2* in 14 PAM patients and variants were found in all patients studied. Our findings emphasise a significant role of *SLC34A2* in PAM and, in addition, expand the variant spectrum of *SLC34A2* in PAM patients. Data from this report suggest that an association may exist between severity of the phenotype and the variants. Functional experiments with variants in *SLC34A2* identified in the patients, combined with clinical studies, are warranted to understand the pathophysiology of PAM and to elucidate possible genotype–phenotype correlations. Our hope is that, in the future, improved knowledge may lead to specific pharmacological and nonpharmacological treatment strategies.

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