A new variant in the *ZCCHC8* gene: diverse clinical phenotypes and expression in the lung

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The novel c.586G>A variant in the *ZCCHC8* gene is associated with pulmonary fibrosis and diverse other short telomere-related phenotypes. This finding underlines the importance of including *ZCCHC8* in diagnostic gene panels for these diseases. https://bit.ly/48Ypk16

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Abstract

Introduction Pulmonary fibrosis is a severe disease which can be familial. A genetic cause can only be found in ∼40% of families. Searching for shared novel genetic variants may aid the discovery of new genetic causes of disease.

Methods Whole-exome sequencing was performed in 152 unrelated patients with a suspected genetic cause of pulmonary fibrosis from the St Antonius interstitial lung disease biobank. Variants of interest were selected by filtering for novel, potentially deleterious variants that were present in at least three unrelated pulmonary fibrosis patients.

Results The novel c.586G>A p.(E196K) variant in the *ZCCHC8* gene was observed in three unrelated patients: two familial patients and one sporadic patient, who was later genealogically linked to one of the families. The variant was identified in nine additional relatives with pulmonary fibrosis and other telomere-related phenotypes, such as pulmonary arterial venous malformations, emphysema, myelodysplastic syndrome, acute myeloid leukaemia and dyskeratosis congenita. One family showed incomplete segregation, with absence of the variant in one pulmonary fibrosis patient who carried a *PARN* variant. The majority of *ZCCHC8* variant carriers showed short telomeres in blood. ZCCHC8 protein was located in different lung cell types, including alveolar type 2 (AT2) pneumocytes, the culprit cells in pulmonary fibrosis. AT2 cells showed telomere shortening and increased DNA damage, which was comparable to patients with sporadic pulmonary fibrosis and those with pulmonary fibrosis carrying a telomere-related gene variant, respectively.

Discussion The *ZCCHC8* c.586G>A variant confirms the involvement of ZCCHC8 in pulmonary fibrosis and short-telomere syndromes and underlines the importance of including the *ZCCHC8* gene in diagnostic gene panels for these diseases.

Introduction

Pulmonary fibrosis is an interstitial lung disease (ILD) characterised by progressive scarring of the lung and reduced survival. The most severe form of pulmonary fibrosis is idiopathic pulmonary fibrosis (IPF), with a median survival of 3–5 years after diagnosis [1]. The cause of IPF is unknown, but genetics are known to play an important role in disease development. Approximately 20% of IPF patients have familial

pulmonary fibrosis (FPF) [2, 3]. FPF is usually inherited in an autosomal dominant way. A genetic cause can also be suspected in sporadic cases based on early age of onset or disease phenotype. Causal variants are mostly found in telomere-related genes (TRGs), such as *TERT*, *RTEL1*, *PARN* and *TERC* [4, 5]. A rare variant in the *ZCCHC8* gene, which plays a role in the maturation of telomerase RNA (*TR*), has been discovered in one family with FPF [6].

Variants in TRGs can cause short-telomere syndromes (STS) that may manifest as diverse phenotypes of pulmonary fibrosis, as well as a spectrum of extrapulmonary phenotypes which are usually haematological, liver-related or mucocutaneous [7–9]. Pulmonary fibrosis is often the first manifestation of disease in STS families and telomere length in both blood and lungs is usually shortened in these patients [7, 10, 11].

Although increasing numbers of genes are being linked to pulmonary fibrosis, a causal variant can only be identified in ∼40% of the probands with FPF [4, 5], indicating that our knowledge of the genetic involvement in pulmonary fibrosis is still vastly incomplete.

The St Antonius ILD Center of Excellence is a tertiary referral centre for ILD with a nationwide biobank and data registry with a special interest in pulmonary fibrosis. As such, the biobank contains data and material from a significant proportion of pulmonary fibrosis patients across the Netherlands. Searching for shared variants among a group of Dutch pulmonary fibrosis patients may be a promising strategy to identify new variants involved in disease development [12, 13].

We therefore performed whole-exome sequencing (WES) on a cohort of patients with pulmonary fibrosis and searched, without *a priori* selection of genes, for novel, potentially deleterious genetic variants that are shared between three or more unrelated patients. In this study, we identified a novel likely pathogenic variant in the *ZCCHC8* gene (c.586G>A p.(E196K)). We describe patient clinical characteristics and investigated ZCCHC8 RNA and protein expression, telomere length and the level of DNA damage in patient specimens.

Methods

Patients and samples

WES was performed on 152 unrelated subjects with pulmonary fibrosis and no known genetic cause from the St Antonius ILD biobank (included between 2005 and 2020). Patients were selected for WES because they had FPF (n=104; 68%), sporadic pulmonary fibrosis with a diagnosis at age <55 years (n=9; 6%) or because there was a suspicion of STS (n=4; 3%). Additionally, a group of sporadic IPF patients, which had been sequenced as part of a previous study [10] was included (n=35; 23%).

Demographics, clinical characteristics, blood samples, skin biopsies and residual tissue were collected from patients carrying the novel *ZCCHC8* c.586G>A variant and from a subset of their relatives. Variants of interest were screened in a larger independent cohort of patients with pulmonary fibrosis (n=1808), patients with non-PF (nonpulmonary fibrosis) ILD (n=605, mostly sarcoidosis) and healthy controls (n=561) from the ILD biobank. Material from patients with sporadic IPF (sIPF), pulmonary fibrosis patients carrying a rare *TERT* variant (PF-*TERT*) and carriers of the *ZCCHC8* c.557C>T variant (the same variant as previously reported [6]) was included for comparison in expression studies. Sample collection is described in supplementary method 1.

Subjects or their legal representative provided written informed consent. The study was approved by the Medical Research Ethics Committees United (MEC-U) of the St Antonius Hospital (approval number R05-08A) and Erasmus MC (approval number MEC-2018-1518).

Variant selection

WES was performed by Novogene (Hong Kong, China) according to standard protocol. In short, variants were filtered so that remaining variants 1) passed confidence filters; 2) were absent in population databases; 3) were predicted deleterious; and 4) were present (heterozygous) in three or more unrelated subjects (supplementary method 2). Details on DNA isolation, WES and targeted genotyping are provided in supplementary methods 3 and 4.

Immunofluorescence images and quantification of ZCCHC8 in the lung

Lung biopsy tissue from control subjects, pulmonary fibrosis patients carrying the *ZCCHC8* c.586G>A variant (PF-*ZCCHC8*), sIPF and PF-*TERT* patients was co-stained for ZCCHC8, 4′,6-diamidino-2-phenylindole (DAPI) for nuclear staining and the cell markers pro-surfactant protein (SP)-C for alveolar type 2 (AT2) cells or CC10 for club cells (supplementary method 5). ZCCHC8 protein

quantification in AT2 cells was performed in controls (n=3), sIPF patients (n=3), PF-*TERT* patients (n=3) and PF-*ZCCHC8* patients (n=2). Expression was quantitatively analysed using the Telometer plugin of ImageJ (supplementary method 6).

ZCCHC8 protein expression in skin fibroblasts

Skin fibroblasts were cultured from patients with the *ZCCHC8* c.586G>A variant (n=4), carriers of the *ZCCHC8* c.557C>T variant (n=4), relatives who did not carry the variant (n=5) and unrelated healthy controls (n=4) (supplementary method 7). ZCCHC8 protein expression was measured using Western blotting (supplementary method 8).

ZCCHC8 *RNA expression in blood*

Relative *ZCCHC8* RNA expression in whole blood was quantified using quantitative PCR on patients carrying *ZCCHC8* c.586G>A (n=3), carriers of the *ZCCHC8* c.557C>T variant (n=4), relatives who did not carry the variant (n=7) and unrelated healthy controls (n=7) (supplementary method 9).

Quantification of telomere length and DNA damage in lung tissue

Diagnostic lung biopsy tissue was obtained from two PF-*ZCCHC8* patients. Cell-type specific telomere length and γH2AX as a marker for DNA damage were measured in AT2 cells, club cells and myofibroblasts, as described previously [14] (supplementary method 10). Data were compared to previously published data from controls (n=5), sIPF (n=10) and PF-*TERT* patients (n=6) [15].

Telomere length measurement

Telomere length in blood was measured by relative telomere to single copy gene (T/S) ratio (supplementary method 11). Telomere length was additionally measured in a subset of samples by Repeat Diagnostics (Aachen, Germany) using flow-fluorescence *in situ* hybridisation (FISH), as described previously [16].

Statistical analysis

Statistical significance was analysed using Prism (version 8.3; GraphPad Software, San Diego, CA, USA) using the Mann–Whitney U-test or Kruskal–Wallis test. In case of a significant result ($p<0.05$) on the Kruskal–Wallis test, Dunn's multiple comparisons test was applied.

Results

Identified variants

WES analysis of 152 biobank samples revealed three unrelated patients who carried the *ZCCHC8* c.586G>A variant, which was absent in population databases. The variant was predicted to cause an amino acid substitution p.(E196K) and is potentially damaging based on *in silico* predictions (table 1). *ZCCHC8* c.586G>A was initially found in two probands with familial disease and one patient with sporadic disease (arrows in figure 1).

In family 1, the *ZCCHC8* variant segregated with disease in three relatives. Furthermore, genealogical research linked the sporadic patient (Fam2.V.1) to family 1 through a common ancestor four generations earlier. In family 3, the proband, two siblings and five paternal cousins carried the *ZCCHC8* variant and were diagnosed with pulmonary fibrosis and/or bone marrow failure or emphysema. Previous screening in pulmonary fibrosis related genes in the proband, prior to the first report of *ZCCHC8*, and family analysis, including WES in Fam3.III.4, 8, 9 and 11 had yielded no results. Remarkably, a paternal cousin (Fam3.III.11) with IPF did not carry the *ZCCHC8* c.586G>A variant, but carried the ultra-rare *PARN* c.1045C>T p.(R349W) variant, which was absent in the other described relatives and predicted to be deleterious (table 1). Targeted genotyping showed that the *ZCCHC8* c.586G>A and *PARN* c.1045C>T variant were absent in 1808 other pulmonary fibrosis patients and a control group consisting of 561 healthy controls and 605 non-PF ILD patients.

Clinical characteristics

The male proband of family 1 (Fam1.IV.1) was diagnosed with IPF at the age of 46 years and died at 47 years (table 2). High-resolution computed tomography (HRCT) was consistent with a usual interstitial pneumonia (UIP) pattern. The pathology pattern after autopsy was UIP with superimposed diffuse alveolar damage. The proband's father died from pulmonary fibrosis. Two paternal cousins with IPF also carried the variant. The distantly related sporadic patient was a young man (Fam2.V.1) who had a disease phenotype consistent with STS. He presented with myelodysplastic syndrome (MDS) and aplastic anaemia at the age of 17 years and was diagnosed with dyskeratosis congenita (DC) at 27 years. Pulmonary arteriovenous malformations (PAVMs) were found at the age of 21 years (supplementary results 1), and

TABLE 1 New variants identified in the *ZCCHC8* and *PARN* genes

	Transcript	Nucleotide change	Amino acid change	dbSNP	gnomAD percentage	GenomeNL	SIFT	PolyPhen-2	CADD	Mutation Taster2021[#]	ACMG classification
ZCCHC8	NM_017612.5	c.586G>A	p.(E196K)		Not present	Not present	Damaging	Possibly damaging	32	Benign	Uncertain significance[¶]
PARN	NM_002582.4	c.1045C>T	p.(R349W)	rs754368658	⩽0.001%	Not present	Tolerated	Possibly damaging	32	Deleterious	Uncertain significance

Sorting Intolerant From Tolerant (SIFT), PolyPhen-2, Combined Annotation Dependent Depletion (CADD) and American College of Medical Genetics and Genomics (ACMG) scores as displayed by the QCI software. *ZCCHC8* c.586G>A was also not present in the ExAc and 1000 Genomes databases. dbSNP: Single Nucleotide Polymorphism Database; [#]: accessed through web-based software at www.genecascade.org/MutationTaster2021/; [¶]: classification changed to "likely pathogenic" based on the findings in this study.

acroangiodermatitis 2 years later. At the age of 22 years, the patient was screened for hereditary haemorrhagic telangiectasia (HHT), but typical HHT lesions were not observed. Testing for a HHT gene panel revealed heterozygosity for the likely benign *ENG* c.572G>A p.(G191D) variant. At the age of 25 years, liver computed tomography was performed to assess the possibility of hepatopulmonary syndrome (HPS). Some degree of portal venous shunting was observed, but there were no indications for an Abernethy syndrome. No signs of cirrhosis or fibrosis were mentioned at that time, but liver fibrosis and abnormal liver biochemical and function tests were observed at the age of 32 years. The most recent chest HRCT at 32 years showed minor reticulations. Telomere length in blood was below the 1st percentile. The patient's father's blood telomere length was also below the first percentile, but his chest HRCT at the age of 61 years showed no abnormalities.

The 61-year-old male proband of family 3 (Fam3.III.9) visited our outpatient clinic with a chronic cough. The initial HRCT showed fibrotic abnormalities with bronchiectasis and reticulation in an apicobasal gradient consistent with a probable UIP pattern and an IPF diagnosis. He had been diagnosed previously with MDS and rheumatoid arthritis at the ages of 55 and 59 years, respectively. Disease course showed progressive fibrotic disease and HRCT evolved into a UIP pattern. The patient died at 67 years. Two siblings also presented with the STS phenotypes IPF and bone marrow failure. Four paternal cousins had IPF, whereas HRCT of one female cousin, aged 68 years, displayed signs of emphysema and familial interstitial lung abnormalities (FILA) [17]. Furthermore, two paternal cousins died of acute myeloid leukaemia. Details on haematological phenotypes are described in supplementary method 12 and supplementary results 2. Clinical characteristics of nine *ZCCHC8* c.586G>A carriers with ILD are summarised in table 3.

One proband and three asymptomatic relatives who are heterozygous for the *ZCCHC8* c.557C>T p.P186L variant that was previously described by Gable *et al.* [6] were included in expression studies as a comparison. The male proband of the family had pulmonary fibrosis at the age of 62 years with the HRCT showing a fibrotic nonspecific interstitial pneumonia pattern. In addition, he had a disease history of liver cirrhosis.

Telomere length in blood

Two *ZCCHC8* c.586G>A carriers, one *ZCCHC8* c.557C>T carrier and the carrier of the *PARN* variant had a telomere length <1st percentile. Four patients had a telomere length in blood between the 1st and 10th percentile (figure 1c). Additionally, in three *ZCCHC8* c.586G>A carriers and one *ZCCHC8* c.557C>T carrier, telomere length was measured using flow-FISH (supplementary results 5). Whereas both methods showed analogous results for Fam3.III.9 (telomere length <10th percentile) and the patient with the *ZCCHC8* c.557C>T variant (telomere length <1st percentile), Fam3.III.1 and Fam3.III.4 had shorter telomeres with flow-FISH in samples obtained from later time points.

ZCCHC8 is expressed in alveolar type 2 cells

In lung tissue from controls, sIPF and PF-*TERT* patients, and patients carrying the *ZCCHC8* c.586G>A variant, ZCCHC8 protein was observed in AT2 cells and in proSP-C-negative cells in the alveoli. Club cells, the CC10-positive cells located in the bronchioles, were also positive for ZCCHC8 (figure 2).

ZCCHC8 quantification

No differences were observed in ZCCHC8 protein levels in AT2 cells between controls, sIPF, PF-*TERT* and PF-*ZCCHC8* patients by immunofluorescence staining techniques (figure 3a).

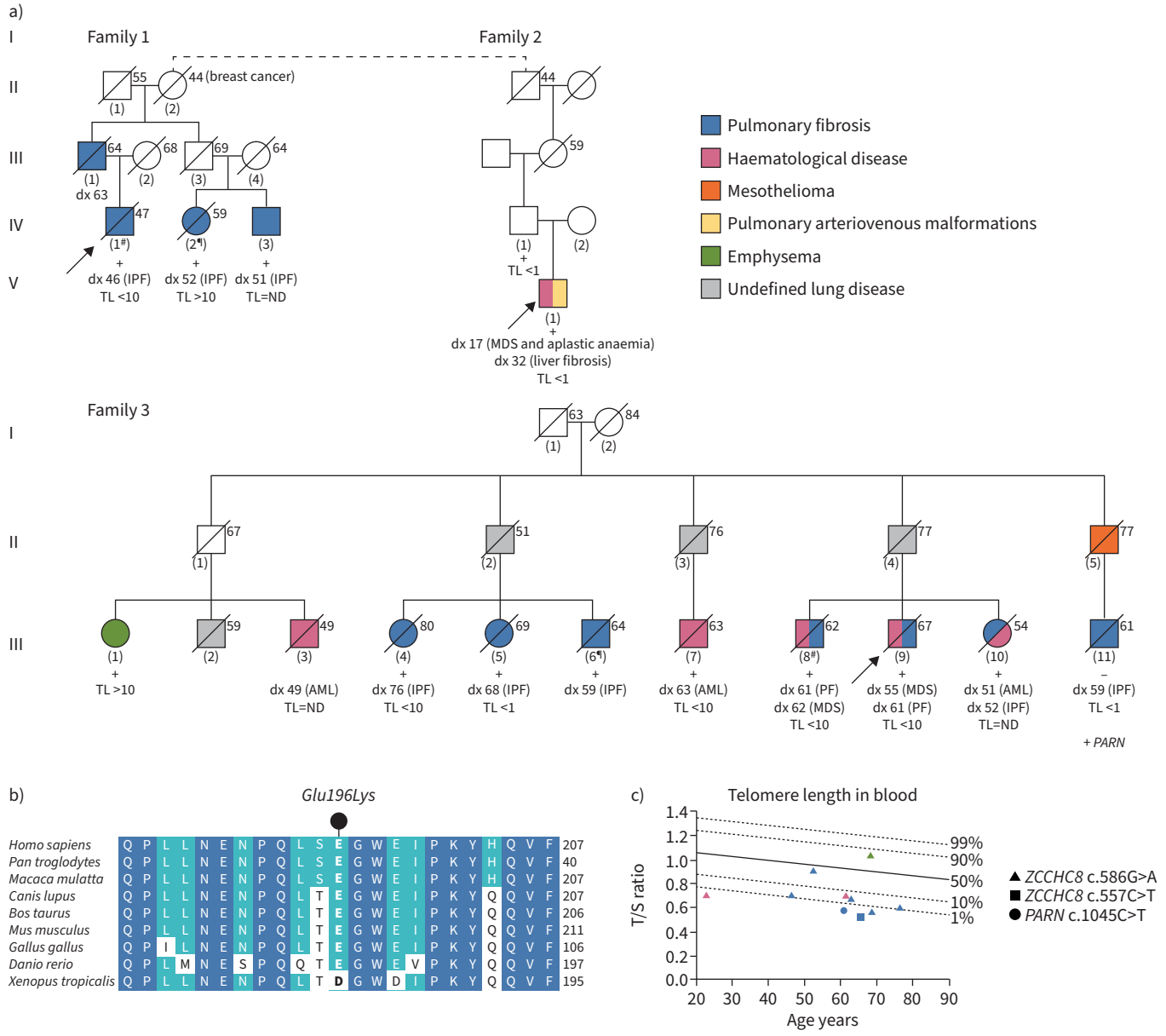

FIGURE 1 a) Condensed pedigrees of family 1; a sporadic patient, later named family 2; and family 3. Arrows indicate probands. Roman numerals indicate the generation. Genealogical research linked family 1 to the sporadic patient (dashed lines) through a common ancestor (generation I). Age at time of death is indicated at the upper right of each symbol. +: heterozygosity for *ZCCHC8* c.586G>A; –: wild type for this variant; dx: age at time of diagnosis; IPF: idiopathic pulmonary fibrosis; TL: telomere length in blood; <1: <1st percentile for age; <10: <10th percentile for age; >10: >10th percentile for age; ND: not determined; MDS: myelodysplastic syndrome; AML: acute myeloid leukaemia. In family 3, subject III.11 does not carry the *ZCCHC8* c.586G>A variant, but does carry *PARN* c.1045C>T. Smoking status "never": Fam1.IV.1, Fam1.IV.2, Fam2.IV.1, Fam2.V.1, Fam3.III.4, Fam3.III.10; "former": Fam1.IV.3, Fam3.III.1, Fam3.III.5, Fam3.III.7, Fam3.III.8, Fam3.III.9, Fam3.III.11. #: autopsy tissue available; ¶: lung biopsy tissue available. b) *ZCCHC8* E196K conservation across nine species. Darker shading indicates a more conserved residue (*Homo sapiens* used as reference). c) Telomere length in blood plotted against age from patients carrying the novel *ZCCHC8* c.586G>A or *PARN* c.1045C>T or the *ZCCHC8* c.557C>T variant. Symbol coloration indicates the initial telomere-related presentation, being haematological disease, pulmonary fibrosis or emphysema. Lines indicate percentiles of healthy control subjects. T/S ratio: relative telomere to single copy gene ratio.

In skin fibroblasts, relative ZCCHC8 protein levels did not differ significantly between controls, carriers of the *ZCCHC8* c.586G>A or c.557C>T variant, and relatives without either variant (p=0.20) (figure 3b, supplementary results 3). However, when comparing all *ZCCHC8* variant carriers (n=8) *versus* all noncarriers (n=9), we found significantly reduced protein expression in the carriers (p=0.046). A trend towards increased *ZCCHC8* RNA expression in blood was present in the c.557C>T carriers (p=0.08) (figure 3c).

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TABLE 2 Clinical characteristics of *ZCCHC8* c.586G>A or *PARN* c.1045C>T variant carriers

	Relationship to proband	*ZCCHC8* c.586	*PARN* c.1045	Sex	Age at ILD diagnosis (death) years	HRCT pattern	ILD diagnosis	T/S ratio <10th percentile for age	Notes and features of STS
Fam1.IV.1	Proband	GA	CC	Male	46 (47)	UIP	IPF	Yes	
Fam1.IV.2	Cousin	GA	CC	Female	52 (59)	Probable UIP	IPF	No	Osteoporosis
Fam1.IV.3	Cousin	GA	ND	Male	51	UIP	IPF	ND	
Fam2.V.1	Proband	GA	CC	Male		Indeterminate/ early UIP	No ILD diagnosis yet	Yes	Age 17: MDS and aplastic anaemia Age 21: pulmonary arteriovenous malformations Age 23: acroangiodermatitis Age 27: DC Age 32: liver fibrosis Age at last contact: 33 years
Fam2.IV.1	Father	GA	ND	Male		Normal	No ILD diagnosis	Yes	Age 25: Completely grey hair Age at last screening HRCT: 61 years
Fam3.III.1	Cousin	GA	CC	Female		Emphysema and FILA	No ILD diagnosis yet	No	Age at last contact: 68 years
Fam3.III.4	Cousin	GA	CC	Female	76 (80)	UIP	IPF	Yes	
Fam3.III.5	Cousin	GA	CC	Female	68 (69)	Indeterminate for UIP	Working diagnosis IPF	Yes	Age 25: Completely grey hair Age 62: Osteoporosis Age 65: Hodgkin lymphoma
Fam3.III.6	Cousin	GA	ND	Male	59 (64)	UIP	IPF	ND	
Fam3.III.7	Cousin	GA	ND	Male	(63)		No pulmonary fibrosis	Yes	Age 63: AML
Fam3.III.8	Brother	GA	CC	Male	61 (62)	Probable UIP	IPF	Yes	Age 62: MDS Upon autopsy: Pericentral liver necrosis and mild infiltrate in the portal fields
Fam3.III.9	Proband	GA	CC	Male	61 (67)	UIP	IPF	Yes	Age 55: MDS Age 59: Rheumatoid arthritis
Fam3.III.10	Sister	GA	ND	Female	52 (54)	Indeterminate for UIP	Working diagnosis IPF	ND	Age 51: MDS, AML Strong suspicion of DC Age 52: Stem cell transplant
Fam3.III.11	Cousin	GG	CT	Male	59 (61)	UIP	IPF	Yes	

ILD: interstitial lung disease; HRCT: high-resolution computed tomography; T/S ratio: relative telomere to single copy gene ratio; STS: short-telomere syndrome; UIP: usual interstitial pneumonia; IPF: idiopathic pulmonary fibrosis; ND: not done; MDS: myelodysplastic syndrome; DC: dyskeratosis congenita; FILA: familial interstitial lung abnormalities; AML: acute myeloid leukaemia.

TABLE 3 Summarised clinical characteristics of nine *ZCCHC8* c.586G>A carriers with interstitial lung disease

Age at diagnosis years	59.0±9
Male	5 (56)
Smoking ever	5 (71)
FVC % predicted (n=7)	85.9±19.2
D_{LCOc} % predicted (n=5)	56.7±18.4
RBC macrocytosis MCV ⩾98 (n=5)	3 (60)
Thrombocytopenia <150×10^9 cells·L^{-1} (n=5)	1 (20)
Aspartate aminotransferase >35 units·L^{-1} (n=6)	2 (33)
Alanine aminotransferase >45 units·L^{-1} (n=6)	1 (17)
Death	8 (89)
HRCT classification	
UIP	5 (56)
Probable UIP	2 (22)
Indeterminate for UIP	2 (22)
Diagnosis	
IPF	7 (78)
IPF working diagnosis	2 (22)
Telomere length (n=6)	
$T/S_{obs-exp}$	−0.232±0.089
T/S ratio <1st percentile for age	1 (17)
T/S ratio <10th percentile for age	5 (83)

Data are presented as mean±SD or n (%). Pulmonary function, haematological abnormalities and liver enzymes recorded closest to and within 3 months prior to or after diagnosis. FVC: forced vital capacity; D_{LCOc}: diffusing capacity of the lung for carbon monoxide corrected for haemoglobin; RBC: red blood cell; MCV: mean corpuscular volume; HRCT: high-resolution computed tomography; UIP: usual interstitial pneumonia; IPF: idiopathic pulmonary fibrosis; $T/S_{obs-exp}$: the difference between the observed relative telomere to single copy gene ratio and the expected T/S ratio at that age.

Telomere length in the lung

Telomere length in AT2 cells in diagnostic lung tissue of two Z*CCHC8* c.586G>A variant carriers was significantly shorter than in controls (p<0.0001), comparable to sIPF (p>0.99) and slightly longer than in PF-*TERT* patients (p<0.01) (figure 4a). Telomeres in club cells from PF-*ZCCHC8* patients were not shorter compared to controls (figure 4b). Telomere length was shorter in myofibroblasts from PF-*TERT* and PF-*ZCCHC8* patients compared to sIPF patients (figure 4c). Staining for the DNA damage marker γH2AX was relatively higher in PF-*ZCCHC8* patients compared to sIPF patients in all cell types and compared to PF-*TERT* patients in myofibroblasts and AT2 cells (supplementary results 4).

Discussion

In this study we identified the novel likely pathogenic c.586G>A variant in the telomere-related *ZCCHC8* gene. The variant was observed in 12 patients with an STS phenotype in two families and a sporadic case, who was later genealogically linked to one of the families. Previously, Gable *et al.* [6] identified the *ZCCHC8* c.557C>T variant in an FPF patient with bone marrow failure. For candidate genes to be convincingly implicated in disease, variants must be found in multiple unrelated individuals with a similar clinical presentation [18]. The current study adds a second variant in the *ZCCHC8* gene, thus accumulating important evidence for a gene–disease relationship.

Most carriers of the novel variant presented with pulmonary fibrosis (most commonly IPF) and/or bone marrow failure, characteristic of telomere-related disease. Several carriers had no ILD diagnosis. For example, one female patient (Fam3.III.1) with a history of smoking presented with emphysema and FILA. Emphysema is a rare pulmonary phenotype of TRG variants, especially in females [19]. The currently 33-year-old male patient (Fam2.V.1) did not have an ILD diagnosis either; however, interstitial lung abnormalities were observed on HRCT. He presented with a clinical phenotype of DC, aplastic anaemia and PAVMs. PAVMs can be a manifestation of HHT [20]. Our patient was heterozygous for *ENG* p.(G191D), a variant that was previously described as unlikely to be pathogenic for HHT [21]. Alternatively, pulmonary arteriovascular malformations may be provoked by vascular abnormalities in the liver, as suggested by Gorgy *et al.* [22], who described several relatively young patients with STS and HPS. Liver fibrosis as well as abnormal liver biochemical and function tests were observed in our patient, but an earlier screening for HPS had not been fully conclusive and HPS as (only) explanation for the degree of right-to-left shunt and PAVMs was deemed unlikely. Therefore, a relationship between the liver

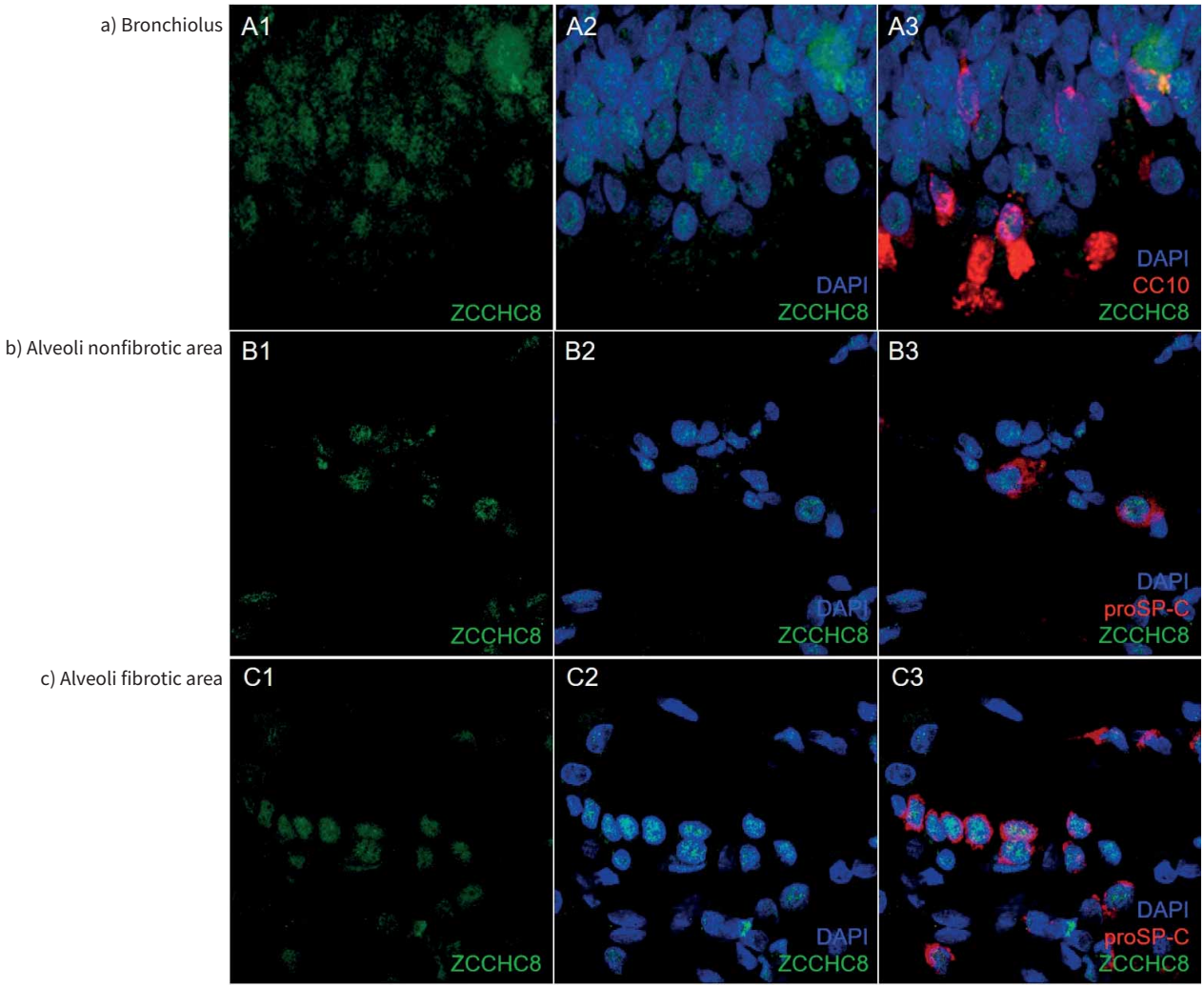

FIGURE 2 Example of ZCCHC8 protein expression in a) bronchioles and b) alveoli of non-fibrotic and c) fibrotic tissue of an idiopathic pulmonary fibrosis patient carrying a disease-associated *TERT* variant. Green: ZCCHC8 immunofluorescence staining; blue: 4′,6-diamidino-2-phenylindole (DAPI) DNA staining; A3 red: CC10 staining as a marker for club cells. B3,C3 red: pro-surfactant protein (SP)-C as a marker for alveolar type 2 cells.

disorder and the PAVMs in this patient cannot be established. Interestingly, the clinical presentation of our patient is in congruence with a previous study in which 12 relatively young patients (aged ⩽32 years) with a TRG variant were described [23]. These patients presented with DC, aplastic anaemia (in 50%) and PAVMs, hence establishing the latter as a phenotype of STS [23]. Thus, several clinical (pulmonary) manifestations were observed in the context of the novel *ZCCHC8* variant. These are in accordance with previously described phenotypes in the context of STS, but presentation may depend on environmental or additional genetic factors.

ZCCHC8 is located in the nucleoplasm and is part of the nuclear exosome targeting (NEXT) complex that targets RNAs, including *TR*, to the nuclear exosome for processing or degradation [6, 24]. Previously, Gable *et al*. [6] showed a reduction of mature *TR* in *ZCCHC8* knockdown cells and in lymphoblastoid cell lines from *ZCCHC8* c.557C>T variant carriers, consistent with a role for ZCCHC8 in mediating *TR* 3′-end targeting to the nuclear exosome and likely explaining the impaired telomere function and subsequent disease in these variant carriers. Their three carriers had telomere length in blood <1st percentile. We observed that STS phenotypes may also manifest in *ZCCHC8* c.586G>A variant carriers with telomere length >1st percentile and even >10th percentile. Interestingly, a mosaic (∼25%) c.-57A>C *TERT* promoter variant was observed in Fam1.IV.1. Somatic *TERT* promoter variants have been described previously in patients with germline variants in TRGs [25].

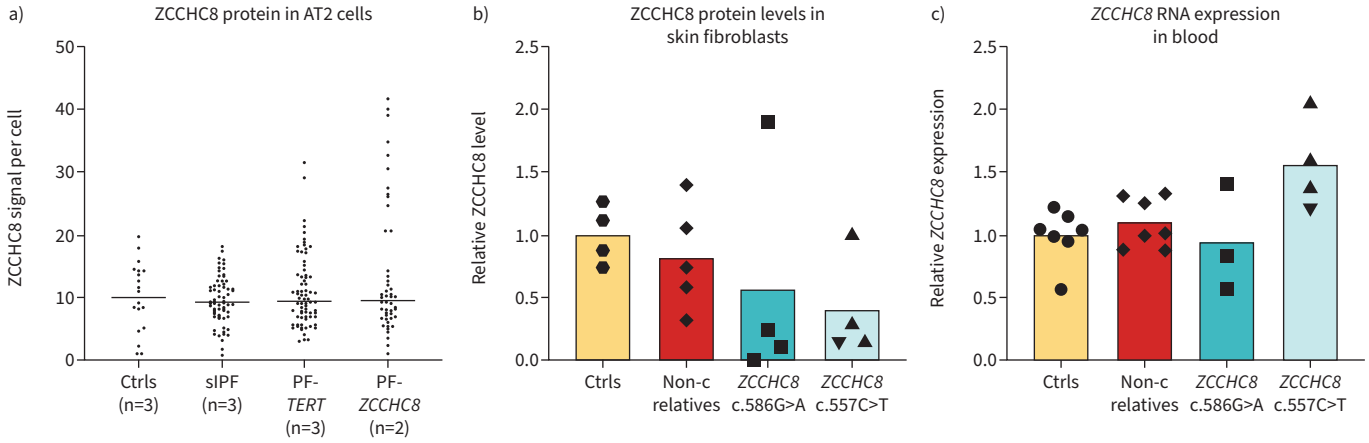

FIGURE 3 ZCCHC8 protein and RNA expression. a) Fluorescence *in situ* hybridisation quantification of ZCCHC8 protein expression levels in alveolar type 2 (AT2) cells as measured by relative fluorescence compared to 4′,6-diamidino-2-phenylindole (DAPI) staining of lung tissue of controls (Ctrls) and patients with sporadic IPF (sIPF), or pulmonary fibrosis with a variant in *TERT* (PF-*TERT*) or *ZCCHC8* c.586G>A (PF-*ZCCHC8*). Lines indicate the median. Each dot represents one AT2 cell. b) Western blot results of ZCCHC8 protein expression in primary skin fibroblast homogenates from Ctrls, non-carrier (non-c) relatives, symptomatic *ZCCHC8* c.586G>A and both asymptomatic (upward facing triangle) and symptomatic (downward facing triangle) *ZCCHC8* c.557C>T variant. Kruskal–Wallis test p=0.20. c) Quantitative PCR results for relative *ZCCHC8* RNA expression in whole-blood-derived samples. *ZCCHC8* expression was calculated compared to the household gene *ARF3* and expressed relatively to the unrelated healthy controls. Kruskal–Wallis test p=0.08. b,c) Levels are relative to the mean of controls; each symbol represents one individual.

Localisation of ZCCHC8 in the lung was not previously reported. We have demonstrated here that ZCCHC8 is present in AT2 cells and club cells. Both cell types have the capacity to self-renew and act as progenitor cells in their respective compartments [26, 27]. AT2 cells play a key role in pulmonary fibrosis development. To illustrate, in mice, depletion of the shelterin component TRF1 specifically in AT2 cells caused progressive and lethal interstitial lung fibrosis, whereas depletion in club cells, basal cells or fibroblasts did not [28, 29]. Telomere length in AT2 cells and club cells from *ZCCHC8* c.586G>A carriers was more comparable to sIPF patients, rather than *TERT* variant carriers. However, in myofibroblasts,

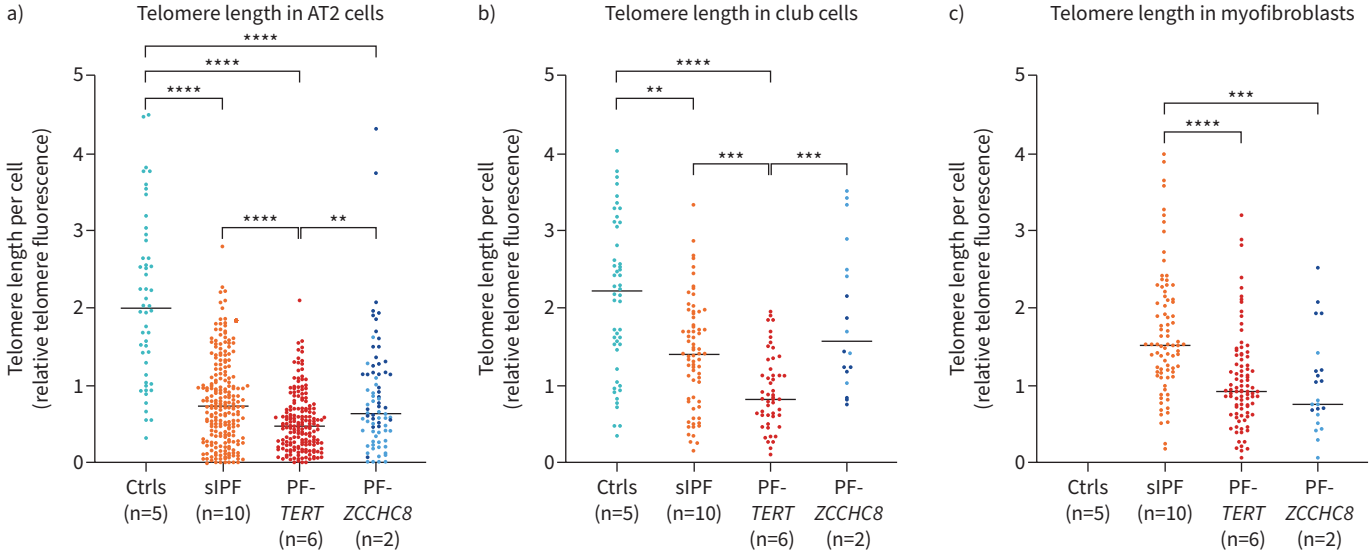

FIGURE 4 Cell-specific quantification of telomere length in lung tissue in control (n=5), sporadic IPF (sIPF; n=10) and pulmonary fibrosis with a variant in *TERT* (PF-*TERT*; n=6) or *ZCCHC8* c.586G>A (PF-*ZCCHC8*; n=2). Telomere length is measured using fluorescence *in situ* hybridisation in a) alveolar type 2 (AT2) cells, b) club cells and c) myofibroblasts. No telomere length is depicted for the control samples in (c), as myofibroblasts were not observed in control tissue. The two individuals included in the PF-*ZCCHC8* group are depicted with different shades of blue (light for Fam1.IV.2 and dark for Fam3.III.6). *: p<0.05; **: p<0.01; ***: p<0.001; ****: p<0.0001, calculated using Kruskal–Wallis with Dunn's multiple comparison tests.

shorter telomeres compared to sIPF patients and more profound γH2AX staining as a marker for DNA damage compared to both sIPF and PF-*TERT* patients were observed. This suggests that the consequence of TRG variants may vary by gene and cell type. Compensatory mechanisms for ZCCHC8 loss may exist in other cell types. Moreover, ZCCHC8 is involved in processes beyond telomere maintenance [6], which may be cell-specific.

The *ZCCHC8* c.586G>A variant described in our patients is predicted to cause the p.(E196K) amino acid change which lies in close proximity to the P186 residue that is affected in the patients described by Gable *et al.* [6]. Recently, Puno and Lima [30] showed that P186 is located on the interface (residues 176–216, hence also including E196) between ZCCHC8 and MTR4 and they hypothesise that the P186L alteration might impair NEXT assembly. However, whether this amino acid alteration actually impairs interaction or whether impaired interaction would be the leading cause of the observed phenotype, is not yet known. The reduced protein levels observed by Gable *et al.* [6] could also be an explanation: their *in vitro* work showed that *ZCCHC8* c.557C>T was associated with increased *ZCCHC8* mRNA expression, but reduced protein levels in lymphoblastoid cell lines. We observed similar *ZCCHC8* RNA levels in blood in the c.557C>T carriers, c.586G>A carriers and noncarriers. In skin fibroblasts, we found reduced ZCCHC8 protein levels in three out of four c.586G>A carriers and three out of four c.557C>T carriers. In the case of the c.586G>A variant, the fourth had a protein level far exceeding that of controls. Interestingly, these high levels were found in patient Fam3.III.4, who was diagnosed with IPF at the age of 76 years, which is the latest age at diagnosis among her siblings. Further studies are needed to determine if a compensatory mechanism underlies this observation and may inform on avenues to overcome deleterious effects of *ZCCHC8* variants. Functional experiments would also be necessary to test whether the amino acid alteration disrupts the protein's activity or interaction with other components of the NEXT complex.

Segregation analysis is a crucial tool in clinical genetics. It was complicated here because one member of family 3 with IPF and short telomeres in blood did not carry the *ZCCHC8* variant. However, the *PARN* p.(R349W) variant in this individual provided a plausible explanation for the observed disease phenotype. This variant was previously described in a patient who had an additional partial *PARN* deletion *in trans* and presented with severe bone marrow failure and neurological defects, but no pulmonary disease was mentioned (last follow-up at 21 years). No apparent phenotype was reported for that patient's heterozygous father (age unknown), who did have relatively short telomeres (between 1st and 10th percentile for age) [31]. PARN protein levels in the father's T-cells were 62% those of controls, suggesting a destabilising effect of the variant on the protein. In addition, functional experiments showed that the R349W variant severely impaired the protein's catalytic activity [31]. PARN is a deadenylase that trims the oligoadenylated tails of small nucleolar RNAs and small Cajal body RNAs containing an H/ACA box [32]. PARN also targets TERC and in line with this, Dhanraj *et al.* [31] showed that PARN-deficient patient cells contained increased levels of oligoadenylated TERC, but slightly reduced total TERC levels. This was associated with a telomere length in blood that was well below the 1st percentile for age. Hence, the monoallelic *PARN* p.(R349W) variant may cause a clinical phenotype particularly in combination with other factors, such as inherited short telomeres.

A limitation of our study is that we did not determine a functional effect of the variant on the protein. However, the *ZCCHC8* c.586G>A variant is predicted to be deleterious by *in silico* models and is absent in control databases. Most importantly, it was found in three seemingly unrelated patients with telomere disease and segregated with disease in 12 affected individuals. The variant has hence been classified as "likely pathogenic" in accordance with the American College of Medical Genetics and Genomics recommendations [33]. Second, the majority of the assays could be conducted in only two to four patients carrying the *ZCCHC8* c.586G>A variant. Interestingly, these samples already showed variable expression, telomere length and DNA-damage signals that may underline the phenotypic plasticity of variant carriers.

Conclusion

This study is the second to describe a likely pathogenic variant in the *ZCCHC8* gene that is associated within pulmonary fibrosis and STS. We show that the novel variant is associated with diverse STS phenotypes, including bone marrow failure, DC, PAVMs, liver fibrosis and emphysema. We further demonstrated that ZCCHC8 is expressed in the culprit cell of IPF, the AT2 cell. This study underlines the importance of an extensive cohort and the power of searching for shared variants to identify the cause of rare disease. Our findings confirm that *ZCCHC8* should be included in gene panels for patients suspected of monogenic pulmonary fibrosis or STS.

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Ethics statement: Subjects or their legal representative provided written informed consent. The study was approved by the Medical Research Ethics Committees United of the St Antonius Hospital (approval number R05-08A) and Erasmus MC (approval number MEC-2018-1518).

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