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Genes involved in innate immunity associated with asbestos-related fibrotic changes

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ABSTRACT

Objectives To determine whether genetic polymorphisms in several candidate genes related to innate immunity and protease–antiprotease balance modify individual susceptibility to develop asbestos-related fibrotic pleuropulmonary changes.

Methods Sixteen polymorphisms from nine genes (NLRP3, CARD8, TNF, TGFB1, GC, MMP1, MMP9, MMP12 and TIMP2) were genotyped from 951 Finnish asbestos-exposed workers. The genotype/haplotype data were compared to signs of fibrosis and pleural thickenings using linear and logistic regression analysis adjusted for potential confounders.

Results A functional polymorphism (Q705K; rs35829419) in the NLRP3 gene was associated with interstitial lung fibrosis (p=0.013), and the TGFB1 rs2241718 SNP with visceral pleural fibrosis (VPF) (p=0.044). In stratified analysis, the carriage of at least one NLRP3 variant allele conferred a 2.5-fold increased risk for pathological interstitial lung fibrosis (OR 2.44, 95% CI 0.97 to 6.14). Conversely, the carriage of at least one TGFB1 rs2241718 variant allele protected against VPF (OR 0.62, 95% CI 0.39 to 0.98). The TIMP2 rs2277698 SNP and a haplotype consisting of the TGFB1 rs1800469 and rs1800470 SNPs were associated with the degree of pleural thickening calcification (p=0.037 and p=0.035), and the CARD8 rs2043211 SNP with the greatest thickness of pleural plaques (p=0.015).

Conclusions Our results support the hypothesis that the NLRP3 inflammasome is important in the development of fibrotic lung disease by associating the NLRP3 rs35829419 variant allele with increased risk of asbestos-related interstitial lung fibrosis, and the TGFB1 rs2241718 variant allele with decreased risk of asbestos-related VPF. Polymorphisms in CARD8 and TIMP2 are proposed to modify the development and/or calcification of pleural thickenings.

INTRODUCTION

Asbestos has been mined and used widely for various insulating and construction purposes because of the thermochemical and electrical resistance, high tensile strength, and the flexibility of these naturally occurring fibrous silicate minerals. Worldwide, the most common type of asbestos used commercially is chrysotile (<90%). In Finland, however, approximately 40% of all asbestos used has been anthophyllite as it was previously produced domestically.[1]

Asbestos usage peaked in the mid 1970s, after which the health hazards of asbestos were recognized and the demand for asbestos started to decline in the USA and in Europe.[1] In Finland, asbestos spraying was

What this paper adds

▶ There is growing evidence that an altered innate immunity response involving the NLRP3 inflammasome complex may be an important mediator of fibrotic lung diseases related to occupational exposures; however, there are very few data on the association between the genes involved in innate immunity and pulmonary fibrosis.

▶ In this study, polymorphisms in NLRP3 and TGFB1 were shown to modify the risk for fibrotic lung changes among asbestos-exposed workers.

▶ Our results support the hypothesis that the NLRP3 inflammasome complex is important in the development of fibrotic lung disease related to asbestos exposure.

forbidden in 1976 and a complete asbestos ban was introduced in 1994. Subsequently, 54 other countries have banned or restricted the use of asbestos.[2] Despite these constraints, asbestos-induced pulmonary and pleural diseases are still a significant health concern due to the enormous amounts of asbestos mined and used since the early 1900s, and the long latency period of most asbestos-associated conditions.[3]

The major diseases caused by asbestos are mesothelioma and lung cancer.[4] In addition, asbestosis, which is interstitial lung fibrosis caused by the deposition of asbestos fibres in the lungs, is an important asbestos-related disease that greatly contributes to the mortality caused by asbestos exposure.

The WHO has recently estimated that 107 000 deaths are caused annually by asbestos-related diseases.[4] Based on an analysis of all deaths caused by mesothelioma and asbestosis reported to the WHO for 1994–2010, it is calculated that approximately 1300 people die worldwide each year from asbestosis, the majority in the Americas (597) and Europe (535).[5] However, countries such as China, India and Russia are not included in this estimate as they did not supply national data.

The exact mechanism by which the inhalation of asbestos fibres leads to lung tissue injury is still unclear, but it may involve a persistent inflammatory response mediated by reactive oxygen species (ROS), cytokines, growth factors, and pro-inflammatory factors, as well as changes in the proteolytic balance of the lungs.[6–9] Genes involved in these pathways are potential candidate modifiers of individual susceptibility to develop lung fibrosis and other asbestos-associated disorders.

Asbestos is known to induce the production of IL-1β and TNF from alveolar macrophages, and these cytokines are therefore believed to play an essential role in the early inflammatory response following asbestos exposure.[10] Furthermore, TNF enhances the expression of TGFB1,[11] a multifunctional cytokine, which may lead to immune suppression and lung fibrogenesis.[12 13] Polymorphisms in both the *TNF* and *TGFB1* genes have previously been associated with the development of asbestosis.[14 15]

TGFB1 can down-regulate collagen degradation through matrix metalloproteinases (MMPs) and their inhibitors (TIMPs), which play an essential role in tissue repair and remodelling, and have been proposed to contribute to the development of pulmonary fibrosis.[8] Differential levels of several MMPs and TIMPs, such as MMP1, MMP9, MMP12 and TIMP2, have been detected in different fibrotic lung diseases.[16 17]

Asbestos-induced secretion of IL-1β from alveolar macrophages is mediated through the NLRP3 inflammasome, which identifies asbestos as a threat via ROS.[18] The NLRP3 inflammasome complex consists of several components, such as NLRP3, ASC and CARD8 (also known as TUCAN), genetic variation of which may affect the function of the complex. Certain polymorphisms in the *NLRP3* and *CARD8* genes have been proposed to be associated with IL-1β production and severe inflammation.[19] Animal studies also suggest that NLRP3 could play a role in the development of asbestosis.[18 20]

GC (group specific component, also known as vitamin D-binding protein, VDBP) is a multifunctional serum protein which participates in several immunologically important functions such as macrophage activation.[21] It has been proposed that GC is involved in chronic lung inflammation, and its gene polymorphisms have been studied in relation to several pulmonary disorders, such as chronic obstructive pulmonary disease (COPD), lung cancer and asthma.[22]

We investigated the significance of nine potential candidate genes in the development of interstitial lung fibrosis, visceral pleural fibrosis (VPF) and pleural plaques detected with high resolution CT (HRCT) among 951 Finnish Caucasian asbestos-exposed workers. The 16 gene polymorphisms (*NLRP3*: rs35829419 and rs10925027; *CARD8*: rs2043211, rs1062808 and rs2288877; *MMP1*: rs1799750; *MMP9*: rs3918242; *MMP12*: rs652438; *TIMP2*: rs2277698; *TNF*: rs1799724 and rs1800629; *TGFB1*: rs1800469, rs1800470 and rs2241718; and *GC*: rs7041 and rs4588) were chosen based on previous association studies suggesting that they were potential modifiers of the development of fibrotic pleuropulmonary changes.

METHODS
Study population
This study combines data from two previous studies, the ASBE and the ASSE, which sought to detect early occupational chest diseases among asbestos-exposed workers.

The ASBE study was conducted in 1996–1997. In 1990–1992, a total of 18 943 asbestos-exposed workers participated in a comprehensive screening study aiming to prevent asbestos-related risks. The screening identified 2857 subjects with asbestos-related occupational disease,[23 24] and 642 of these workers who lived in the Helsinki area, had a previous asbestosis diagnosis regardless of smoking history, or had pleural plaques (without an asbestosis diagnosis) and a smoking history of ≥10 pack years, were invited to participate in ASBE.[25 26] The subjects willing to participate (n=602) formed the ASBE study group. The mean age of ASBE participants was 63 (38–81) years and most of them were men (n=592).

The ASSE study, conducted in 2003–2004, included asbestos-exposed subjects from two sources.[27] The study group mainly consisted of workers with asbestosis and/or asbestos-related pleural findings who had visited occupational medicine clinics in Helsinki and Tampere for clinical follow-up[27] and were willing to participate in ASSE. The study group also included asbestos-exposed participants from the original screening study conducted in 1990–1992[23 24]; all heavily exposed subjects (exposure index >70)[28] living in three geographical areas (Helsinki, Tampere and Turku) were invited to participate in ASSE. A total of 758 subjects were invited and the 633 who were willing to participate formed the ASSE study group. The mean age of ASSE participants was 65 (45–87) years and most of them were men (n=627).

As 178 of the subjects recruited in 2003–2004 had already participated in the first study conducted in 1996–1997, they were excluded from the second patient group in the present study before the data were combined.

In the combined study population, blood samples were available for 1021 subjects. However, 25 subjects were excluded because of missing smoking information and 45 because of insufficient asbestos exposure data. Thus, the final study group consisted of 951 subjects (935 males, 16 females).

Approval for the study was obtained from the local ethics committees (the Ethics Committee of the Finnish Institute of Occupational Health and the Ethics Committee for Research in Occupational Health and Safety, Hospital District of Helsinki and Uusimaa) according to the legislation in force at the time of the original study. All subjects gave informed consent to participate in the study.

Exposure assessment
The ASBE participants were personally interviewed by an occupational physician using a standardised questionnaire including questions on smoking habits and occupational history. They were construction workers who had installed heat and fire insulation or asbestos-containing walls and ceiling panels, used asbestos paints, putties and fillers, dismantled asbestos-containing materials, or cleaned areas where asbestos was present. They had a mean duration of asbestos exposure of 26.1±9.7 years and a mean of 23.7 ±15.0 pack-years of smoking. Most were ex-smokers (70%) or current smokers (27%), and only 3% had never smoked.

The ASSE participants filled in a self-administered questionnaire modified from the Finnish Environment and Asthma Study Questionnaire.[29] The final questionnaire included queries on demographic characteristics, respiratory symptoms and diseases, smoking exposure, and occupational exposures with a focus on asbestos. Most of the ASSE participants were construction workers, cleaners or plumbers. The ASSE participants had a mean duration of asbestos exposure of 20.8±11.4 years, and a mean of 16.1±18.2 pack-years of smoking. Most were ex-smokers (54%) or current smokers (17%), and 29% had never smoked.

Radiological examinations
The lungs of study subjects were imaged prone in full inspiration with four different scanners: in 1996–1997 the Picker PO 2000 (Picker International, Cleveland, Ohio, USA) device was used, while in 2003–2004 the Siemens Somatom Balance (Siemens Medical, Erlangen, Germany) was used in Helsinki, the Siemens Somatom Plus 4 (Siemens Medical) in Tampere, and the GE LightSpeed 16 Advantage (GE Healthcare, Milwaukee, Wisconsin, USA) in Turku. The HRCT images were printed as hard copies and analysed blindly by two (2003–2004) or three (1996–1997) radiologists.

The radiologists visually scored the signs of interstitial lung fibrosis using an arbitrary semiquantitative scale from 0 to 5

including one subclass between each of the five classes: 0 (normal finding), 1 (subnormal finding), 2 (mild fibrosis), 3 (moderate fibrosis), 4 (severe fibrosis) and 5 (extreme fibrosis).[25]

VPF includes diffuse pleural thickening, rounded atelectasis and parenchymal bands.[30] A VPF variable was constructed by adding up scores for parenchymal bands (scale 0–5), adherences at the diaphragm and sinuses as well as other adherences (scales 0–3) and rounded atelectasis (score 0–3 for up to 2 atelectases) in the ASBE study material.[31] This sum score was then dichotomised to match the frequency distribution of the dichotomous VPF variable used in the ASSE study. Several signs of pleural changes were also recorded: the extent (cm^2) and greatest thickness of pleural thickenings (ILO 'width': 0=no plaques, 1=≤5 mm, 2=5–10 mm, 3=10 mm) and their degree of calcification (0=no, 1=sparse, 2=a considerable part of the pleural thickening, 3=nearly all). The methods, including intra- and inter-reader agreement, have been described in more detail previously.[25]

Genotyping analyses

DNA was extracted mechanically (Thermo KingFisher system; Thermo Fisher Scientific, Erembodegem, Belgium) from whole blood using a BioSprint 15 DNA Blood Kit (Qiagen, Hilden, Germany) and stored at −20°C until use.

Two *NLRP3* SNPs (rs35829419 and rs10925027), three *CARD8* SNPs (rs2043211, rs1062808 and rs2288877), two *TNF* SNPs (rs1799724 and rs1800629), two *TGFB1* SNPs (rs1800469 and rs2241718), two *GC* SNPs (rs7041 and rs4588), one *MMP12* SNP (rs652438) and one *TIMP2* SNP (rs2277698) were genotyped using the OpenArray system (BioTrove, Woburn, Massachusetts, USA), a next-generation quantitative PCR platform based on TaqMan chemistry.

The *TGFB1* rs1800470 SNP was genotyped using an allelic discrimination assay on the ABI 7500 Real-Time PCR system (Applied Biosystems, Foster City, California, USA) with TaqMan probes.[32] The *MMP1* rs1799750 SNP was analysed with a pyrosequencing method based on an assay from the PyroMark Assay Database (Qiagen), and the *MMP9* rs3918242 SNP was genotyped using a PCR-RFLP-based method essentially according to Joos *et al.*[33] A detailed description of the genotyping methods is presented in online supplement 1.

For quality control, two independent readers interpreted the results and a random selection of 10% of all samples was re-tested. No discrepancies were discovered in the replicate tests for rs35829419, rs10925027, rs1062808, rs2288877, rs7041, rs4588, rs652438, rs1800470, rs1799750 or rs3918242. Minor error rates were detected for OpenArray assays rs2043211 (1%), rs1799724 (1%), rs1800629 (2%), rs2241718 (2%), rs2277698 (2%) and rs1800469 (3%).

To verify the reliability of the OpenArray platform, a random selection of 15% of samples was re-analysed for rs1800629 and rs1799724 SNPs using an RFLP-based method[34] (rs1800629) and a pyrosequencing-based method (rs1799724) with 100% concordant results with the earlier estimates.

Statistical analysis

Our study (n=951) has 80% power to detect ORs from 1.57 to 2.63 depending on the minor allele frequency (4–43%). The calculations, based on a two-sided α of 0.05, were performed using standard methods.

Linkage disequilibrium (LD) among the studied SNPs was examined using HaploView V.4.2.[35] When moderate or strong linkage was detected ($r^2 > 0.5$), haplotypes consisting of the SNPs in question were statistically reconstructed from population genotype data with the Markov chain method for haplotype assignments using the PHASE program (V.2.1).[36] The associations of the haplotypes with pulmonary parameters were then examined as with the single SNPs.

The χ^2 test with a cut-off p value of 0.05 was used to check for deviation from Hardy–Weinberg equilibrium (HWE).

The associations between genotypes/haplotypes, fibrosis and pleural plaques were evaluated using a general linear model, while logistic regression analysis was used to evaluate potential confounders and to further study the risk for fibrotic changes and pleural plaques and their severity with a certain genotype. Covariates used in the analysis were: sex, age, pack years of smoking and years of asbestos exposure.

For further analysis, the cases were divided according to the existence and severity of radiological signs. The signs of interstitial lung fibrosis (radiological score >0) were considered subnormal if the radiological score was <2 and pathological if the score was ≥2. The degree of calcification of pleural plaques was categorised as mild if calcification was sparse and high if a considerable part or nearly all of the pleural thickenings were calcified.

All of the data analyses were performed using SPSS V.18.0 (SPSS, Chicago, Illinois, USA).

RESULTS

The demographics and HRCT characteristics of the asbestos-exposed workers are summarised in table 1. All the genotype distributions of the studied gene polymorphisms were in HWE (p>0.09), except for the *CARD8* rs1062808 and rs2288877 SNPs (p<0.01), which were therefore excluded from further analysis.

The *NLRP3* rs35829419 SNP was associated with interstitial lung fibrosis (p=0.013) and the *TGFB1* rs2241718 SNP with VPF (p=0.044). The *CARD8* rs2043211 SNP was associated with the greatest of pleural plaques thickness (p=0.015) and

Table 1 Characteristics of the asbestos-exposed workers

	Mean (SD) or n (%)
Age, years	63.2 (7.3)
Male	935 (98.3%)
Smoking history	
Never smoker	135 (14.2%)
Ex-smoker	595 (62.6%)
Current smoker	221 (23.2%)
Pack years	20.4 (16.7)
Years of asbestos exposure	23.9 (10.7)
Interstitial lung fibrosis	
RS=0	201 (21.1%)
0<RS<1	434 (45.6%)
1≤RS<2	234 (24.6%)
1≤RS<2	72 (7.6%)
RS≥3	10 (1.1%)
Visceral pleural fibrosis (n=941)	131 (13.9%)
Yes	131 (13.9%)
No	810 (86.1%)
Pleural plaques	
Greatest thickness (n=949)	1.88 (0.67)
Extent, cm^2 (n=947)	104.4 (72.5)
Calcification (n=939)	1.39 (0.94)

n=951 except as noted.
RS, radiological score.

Table 2 Association between genetic polymorphisms and fibrotic pleuropulmonary changes

Phenotype	Gene	Polymorphism*	β Coefficient†	p Value
Interstitial lung fibrosis	NLRP3	rs35829419	0.078	0.013
Visceral pleural fibrosis	TGFB1	rs2241718	−0.660	0.044
Pleural plaques				
Greatest thickness	CARD8	rs2043211	0.076	0.015
Calcification	TIMP2	rs2277698	0.063	0.037

Covariates used in the analysis: sex, age, pack-years and years of asbestos exposure.
*Three levels of genotypes (the wild type homozygotes, heterozygotes, variant homozygotes) were included in the model.
†Standardised β coefficient.

the TIMP2 rs2277698 SNP with the degree of pleural plaque calcification (p=0.037) (table 2).

In stratified analysis, carriage of at least one NLRP3 rs35829419 variant allele conferred a 2.5-fold increased risk for pathological interstitial lung fibrosis (OR 2.44, 95% CI 0.97 to 6.14), although this association was only borderline significant. In addition, the carriage of at least one TIMP2 rs2277698 variant allele conferred an almost 2-fold increased risk for a high degree of pleural plaque calcification (OR 1.90, 95% CI 1.09 to 3.33) (table 3). Conversely, the carriage of at least one TGFB1 rs2241718 variant allele was protective against VPF (OR 0.62, 95% CI 0.39 to 0.98) (table 4). No association between CARD8 rs2043211 SNP and the greatest thickness of pleural plaques was found in the stratified analysis.

In linkage analyses, significant LD was observed between the GC rs4588 and rs7041 SNPs (D′=1.00, r²=0.501). The TGFB1 rs1800469 and rs1800470 SNPs were also found to be linked to each other (D′=0.970, r²=0.738), but not to the third studied TGFB1 SNP (rs2241712) (D′<0.02, r²=0.00). The minor allele frequencies of the NLRP3 rs35829419 and TNF rs1799724 SNPs were too small for r² to detect LD (0.3% and 0.2%, respectively), despite the maximum D′s (D′=1.00, r²=0.032 for rs35829419; D′=1.00, r²=0.008 for rs1799724).

Next, a Bayesian method was used to reconstruct the TGFB1 and GC haplotypes from population genotype data, and the haplotypes' associations with pulmonary parameters were examined as with the single SNPs. The haplotype analysis identified three haplotypes for the GC rs4588 and rs7041 SNPs: the most

common haplotype was GC (wild type–wild type, 65.2%), followed by GA (13.7%) and TA (21.1%). No associations were seen between the GC haplotypes and the studied parameters.

For the TGFB1 rs1800469 and rs1800470 SNPs, four haplotypes were identified: GT (wild type–wild type, 60.5%), GC (24.9%), AT (10.3%) and AC (4.3%). Overall, the TGFB1 rs1800469–rs1800470 haplotype was found to be associated with the degree of pleural plaque calcification (p=0.035) (data not shown). In the stratified analysis, the GC and AT haplotypes conferred increased risks (OR 1.52, 95% CI 1.09 to 2.11 for GC; OR 1.95, 95% CI 1.18 to 3.22 for AT) for pleural plaque calcification compared with the most common haplotype, GT. The risks did not notably differ between mild and high degrees of calcification (table 5).

DISCUSSION

Although there is growing evidence of the involvement of the NLRP3 inflammasome in the development of fibrosis,[37][38] there are few data on the association between the genes involved in this inflammasome complex and pulmonary fibrosis. In the current study, we found a significant association between the NLRP3 rs35829419 SNP and asbestos-induced interstitial lung fibrosis. Moreover, a truncating polymorphism (C10X; rs2043211) in another member of the complex, CARD8, was associated with the greatest thickness of pleural plaques.

The rs35829419 and rs2043211 SNPs have previously been linked to increased IL-1β production and severe inflammation.[19] The rs35829419 SNP changes amino acid (Q705K) in the NLRP3 protein, and recent evidence suggests that it is a gain-of-function mutation leading to a constantly active NLRP3 inflammasome and increased IL-1β levels.[39] This may induce fibrosis[37][38] and therefore agrees with our findings.

Interestingly, a recent study suggests an association between the variant allele of another NLRP3 SNP (rs1539019) and coal workers' pneumoconiosis (CWP).[40] CWP is a lethal fibrotic lung disease caused by inhalation of airborne coal mining dust, including crystalline silica.[41] Although the functional consequences of the rs1539019 SNP are not known, this finding is in agreement with our results and strengthens the hypothesis that the NLRP3 inflammasome complex is involved in the development of fibrotic lung diseases.

Genetic polymorphisms in TNF and TGFB1 have previously been associated with the development of asbestos-induced

Table 3 Distribution of NLRP3 and TIMP2 genotypes according to the existence and severity of radiological changes

Interstitial lung fibrosis

Genotype	No changes*	Any changes	OR (95% CI)†	Subnormal‡ changes	OR (95% CI)†	Pathological‡ changes	OR (95% CI)†
NLRP3 rs35829419							
Gln705Gln	182 (92.4)	661 (88.8)	1.0	589 (89.9)	1.0	72 (87.8)	1.0
Gln705Lys or Lys705Lys	15 (7.6)	83 (11.2)	1.57 (0.87 to 2.82)	73 (11.0)	1.47 (0.82 to 2.67)	10 (12.2)	2.44 (0.97 to 6.14)

Calcification of pleural plaques

Genotype	No calcification*	Calcification	OR (95% CI)†	Mild§ calcification	OR (95% CI)†	High§ calcification	OR (95% CI)†
TIMP2 rs2277698							
G853G	107 (83.6)	617 (77.1)	1.0	238 (79.1)	1.0	379 (76.0)	1.0
G853A or A853A	21 (16.4)	183 (22.9)	1.58 (0.95 to 2.65)	63 (20.9)	1.36 (0.78 to 2.38)	120 (24.0)	1.90 (1.09 to 3.33)

Data are presented as n (%) except as noted.
*Reference category.
†Adjusted for age, sex, pack-years and years of asbestos exposure.
‡The changes were considered as mild if the radiological score was <2 and severe if the radiological score was ≥2.
§The calcification was considered as mild if it was sparse and high if a considerable part/nearly all of the pleural plaques were calcified.

Table 4 Distribution of *TGFB1* genotypes according to the existence of visceral pleural fibrosis

Genotype	No changes*	Any changes	OR (95% CI)†
TGFB1 rs2277698			
G/G	577 (72.5)	104 (80.6)	1.0
G/A or A/A	219 (27.5)	25 (19.4)	0.62 (0.39 to 0.98)

Data are presented as n (%) except as noted.
*Reference category.
†Adjusted for age, sex, pack-years and years of asbestos exposure.

fibrosis in a German population.[14] [15] However, we could not replicate the findings concerning the *TNF* genotypes, possibly because of phenotypic heterogeneity. In the previous study, a strict diagnosis of asbestosis was used, whereas in the current study different fibrotic changes were studied separately. In addition, since the control subjects in the previous study were not exposed to asbestos, the analyses were not adjusted for asbestos exposure level.[14]

Regarding the studied *TGFB1* genotypes, the rs2241718 SNP was associated with asbestos-induced VPF, and two *TGFB1* haplotypes consisting of the rs1800469 and rs1800470 SNPs were associated with pleural plaque calcification. In stratified analysis, the variant allele of the *TGFB1* rs2241718 SNP was shown to be protective against VPF.

The *TGFB1* rs2241718 SNP is located in the non-coding area near the gene and has no known functional consequences. The promoter area SNP rs1800469 is in tight linkage with the third studied signal peptide SNP rs1800470 (Leu10Pro; formerly known as rs1982073), which has been associated with increased secretion and higher TGFB1 serum levels.[42] [43] Moreover, the F-SNP program predicts that rs1800470 is involved in splicing regulation.[44]

It is also possible that the causative variant is one of the several SNPs found in tight linkage ($r^2 > 0.9$ in the 1000 Genomes Project population) with the studied *TGFB1* SNPs, located in a completely different gene. However, since TGFB1 is a down-stream effector of the NLRP3-mediated innate immunity response, and also involved in the regulation of protease–antiprotease balance, it is highly likely that it has a role in the pathogenesis of external exposure and inflammation-related lung disease. The previous and current findings together imply that *TGFB1* is indeed involved in the development of asbestos-related fibrosis.

We also found an association between the *TIMP2* rs2277698 SNP and pleural thickenings; the rs2277698 variant allele was found to predispose to a high degree of pleural thickening calcification. The relationship between *TIMP2* and pleural plaques has not been explored before, but in an animal model, the overexpression of TIMP2 has been shown to inhibit atherosclerotic plaque development and destabilisation.[45] However, the mechanisms of the development of atherosclerotic plaques are probably very different from the development of pleural plaques.

The functional consequences of the synonymous rs2277698 SNP are unclear, but the F-SNP program predicts that it is highly likely to be involved in splicing regulation.[44] The rs2277698 SNP is also in strong linkage ($r^2 > 0.9$ in the 1000 Genomes Project population) with other potential causal SNPs, some of which (rs9889410 and rs11654470) are located in an area predicted to alter transcriptional regulation.[44]

One of the main strengths of our study is the very large, HRCT-characterised population, in which different asbestos-related abnormalities were recorded separately. Since different degrees of interstitial fibrosis were detected in 750 subjects, we

Table 5 Distribution of *TGFB1* haplotypes according to the degree of pleural plaque calcification

Haplotype	No calcification,* n (%)†	Calcification, n (%)†	OR (95% CI)‡	Mild§ calcification, n (%)†	OR (95% CI)‡	High§ calcification, n (%)†	OR (95% CI)‡
rs1800469–rs1800470							
GT	176 (67.7)	925 (57.2)	1.0	359 (58.7)	1.0	566 (56.3)	1.0
GC	59 (22.7)	454 (28.1)	1.52 (1.09 to 2.11)	165 (27.0)	1.42 (0.99 to 2.03)	289 (28.7)	1.49 (1.04 to 2.14)
AT	20 (7.7)	214 (13.2)	1.95 (1.18 to 3.22)	77 (12.6)	1.86 (1.09 to 3.16)	137 (13.6)	1.96 (1.15 to 3.36)
AC	5 (1.9)	25 (1.5)	0.96 (0.35 to 2.64)	11 (1.8)	1.13 (0.38 to 3.4)	14 (1.4)	0.66 (0.21 to 2.01)

*Reference category.
†Data are presented as number of chromosomes (%).
‡Adjusted for age, sex, pack-years and years of asbestos exposure.
§Calcification was considered as mild if it was sparse and high if a considerable part/nearly all of the pleural plaques were calcified.

were also able to compare the severity of these changes with the genotype data.

Our study also has potential limitations. First, since the patients were enrolled in three cities during two separate primary studies, four different CT scanners were used and seven radiologists participated in image reading. However, since the Finnish population is very homogenous and the three large cities where the patients were enrolled, are all located in southern Finland and very close to each other, we do not believe that geographical origin at the time of the examination has caused any significant bias in the data analysis. Moreover, any inconsistency in image reading causes inaccuracy and thus random noise affecting the results, leading to loss of power rather than systematic error. This increases the error variance in computations and the detected associations are therefore likely to be underestimated.

Second, the multiple comparisons performed increase the possibility of detecting false–positive associations. However, most of the methods correcting for multiple testing are very conservative, and, for example, it is unclear how many comparisons should be adjusted for.[46] In addition, based on previous findings, we had an a priori hypothesis for each polymorphism chosen, which reduces the need for correction. Nevertheless, these results should be considered with caution until replicated in another study population. The use of a large control population including asbestos-exposed subjects without pleural or pulmonary changes would also be beneficial in evaluating the reliability of our findings.

In summary, our results strengthen the hypothesis that the NLRP3 inflammasome is important in the development of fibrotic lung diseases. In particular, the *NLRP3* rs35829419 variant allele is proposed to increase the risk for asbestos-related interstitial lung fibrosis and the *TGFB1* rs2241718 variant allele to protect against asbestos-related VPF. In addition, polymorphisms in *CARD8* and *TIMP2* are proposed to modify the development and/or calcification of pleural thickenings.

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