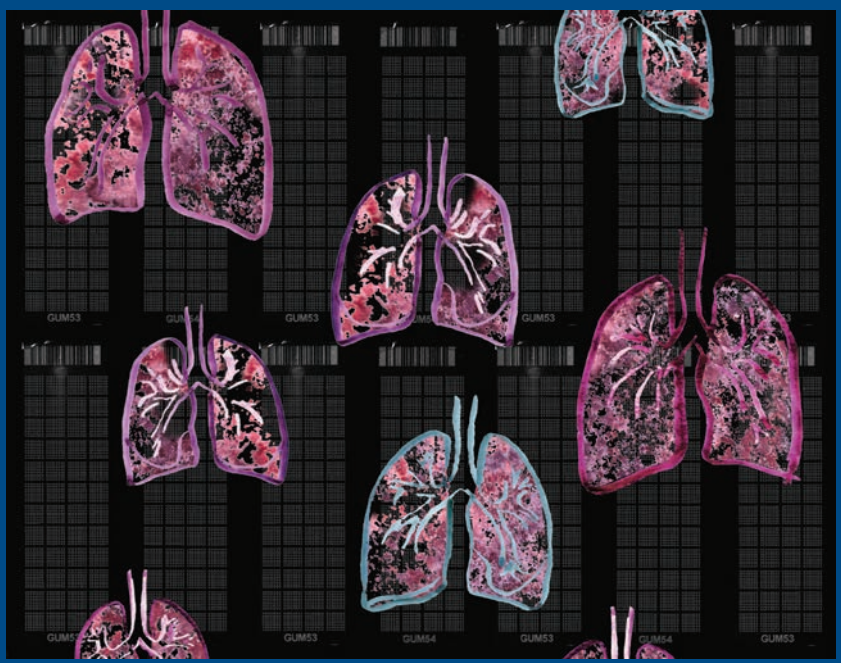
Mari Kukkonen

Genetic susceptibility to asbestos and tobacco smoke related non-malignant pleuropulmonary changes



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Helsinki 2013

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ACADEMIC DISSERTATION

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ABSTRACT IN FINNISH – TIIVISTELMÄ

Asbestialtistuksen tiedetään aiheuttavan useita keuhkosairauksia, kuten keuhkokudoksen arpeutumista (keuhkofibroosi) ja keuhkopussin syöpää (mesotelioma). Tarkkaa mekanismia, jolla asbestikuidut vaurioittavat keuhkoja, ei tunneta, mutta siihen uskotaan liittyvän reaktiivisten happiyhdisteiden, sytokiinien ja muiden tekijöiden välittämä jatkuva tulehdustila sekä epätasapaino keuhkojen tukikudosten proteiineja hajottavien entsyymien ja niitä hillitsevien tekijöiden välillä.

Tupakansavu sisältää happiradikaaleja ja tuhansia kemikaaleja, jotka voivat asbestin tavoin johtaa keuhkokudoksessa krooniseen tulehdukseen ja proteiini- ja DNA-tason muutoksiin. Tupakoinnin tiedetäänkin lisäävän keuhkosyövän ja useiden muiden syöpien riskiä ja altistavan myös keuhkoahtaumataudille (Chronic obstructive pulmonary disease; COPD) ja keuhkolaajentumalle (emfyseema).

Tässä väitöskirjatyössä tutkittiin useiden vierasaineiden muokkaamiseen (EPHX1, GSTM1, GSTM3, GSTP1, GSTT1 ja NAT2), luonnolliseen immuniteettiin (NLRP3 ja CARD8), tulehdukseen (TNF, TGFB1 ja GC) ja tukikudosten hajottamiseen (MMP1, MMP9, MMP12, SERPINE2 ja TIMP2) liittyvien proteiinien geenien monimuotoisuuden merkitystä hyvänlaatuisten keuhkomuutosten synnyssä. Tutkittavat geenit ja monimuotoisuuskohdat valittiin aiemman kirjallisuuden perusteella. Osa geenianalyyseista suoritettiin perinteisemmillä, geelielektroforeesiin ja entsyymirestriktioon perustuvilla erottelumenetelmillä, osa fluoresenssileimaukseen (TaqMan) perustuvilla reaaliaikaisilla PCR-menetelmillä (kuoppalevy- ja siruformaatit) ja osa lusiferaasi-valoreaktioon perustuvalla pyrosekvensointimenetelmällä.

Tutkimusaineisto koostui kahdesta otoksesta suomalaisia asbestille ja tupakansavulle altistuneita rakennustyöntekijöitä, joille oli tehty huolelliset kliiniset ja radiologiset tutkimukset; keuhkojen tilavuus- ja virtausmittaukset oli suoritettu spirometrialla, keuhkokudoksen kaasujenvaihtokyky oli mitattu diffuusiokapasiteettitutkimuksella ja keuhkopussin ja keuhkokudoksen muutokset oli määritetty korkean resoluution tietokonetomografialla (High resolution computed tomography; HRCT).

Tutkimuksessa havaittiin vierasaineiden muokkaamiseen osallistuvan GSTT1 entsyymin geenin puutoksen altistavan sekä vaikealle keuhkokudoksen fibroosille että emfyseemalle ja kaasujenvaihtokapasiteetin alenemiselle. Nämä yhteydet olivat tilastollisesti merkitseviä sekä koko tutkimuspopulaatiossa että molemmissa alkuperäisissä tutkimusotoksissa. Keuhkokudoksen fibroosille altistava muoto löydettiin lisäksi luonnolliseen immuunivasteeseen osallistuvan, nk. inflammasomikompleksin keskeisen osan, NLRP3:n, geenistä. Toisen kompleksin osan, CARD8:n geenin, tietty muoto taas liitettiin keuhkopussin plakkien paksuuteen.

Tulehdusvälittäjäaine TGFB1:n geenin tietyn muodon puolestaan havaittiin suojaavan keuhkopussin fibroosilta, ja tietty kahden genotyypin yhdistelmä, haplotyyppi, yhdistettiin keuhkopussin plakkien kalkkeutumisasteeseen.

Keuhkoemfyseeman kehittymiseen liittyviä muotoja löydettiin lisäksi *SERPINE2*, *MMP9*, *TIMP2*, *TNF* ja *TGFB1* geeneistä. Proteiinien hajottamista rajoittavan SERPINE2:n geenin tietyn muodon ja haplotyypin havaittiin altistavan panlobulaarisen emfyseematyypin kehittymiselle. Tämä yhteys oli merkitsevä sekä koko tutkimuspopulaatiossa että molemmissa tutkimusotoksissa erikseen.

Proteiineja hajottavan MMP9:n ja tulehdussytokiini TGFB1:n geenin tiettyjen muotojen todettiin suojaavan sentrilobulaariselta, läheisesti tupakointiin liittyvältä emfyseematyypiltä, kun taas proteiinien hajottamista säätelevän TIMP2:n ja tulehdussytokiini TNF:n geenin tiettyjen muotojen havaittiin lisäävän paraseptaalisen emfyseeman kehittymisen riskiä. *TIMP2*:n geenimuoto oli lisäksi yhteydessä FEV_1/FVC ja MEF50 keuhkotoimintamuuttujiin, joiden aleneminen viittaa emfyseemalle tyypilliseen pienten keuhkoputkien ahtautumiseen. Vierasaineiden muokkaamiseen osallistuvan, emfyseemaan ja alentuneeseen keuhkotoimintaan aikaisemminkin liitetyn GSTM3:n geenin tietyn

muodon havaittiin niin ikään olevan yhteydessä FEV_1/FVC ja MEF50 keuhkotoimintamuuttujiin, joista MEF50 oli merkitsevästi alentunut kyseisen geenimuodon kantajilla.

Löydösten perusteella vierasaineiden muokkaamiseen, tukikudosten hajottamiseen, luonnolliseen immuniteettiin ja tulehdukseen osallistuvien proteiinien perinnöllisellä muuntelulla on vaikutusta asbesti- ja tupakka-altistumiseen liittyvien emfyseema- ja fibroosimuutosten sekä keuhkotoiminnan häiriöiden kehittymiseen ja muutosten vaikeusasteeseen. Lisäksi havainnot emfyseeman suhteen selventävät rakenteellisesti erilaisten emfyseematyyppien syntymiseen johtavia tekijöitä. Koska useimpien sairauksien taustalla vaikuttaa geneettisen muuntelun lisäksi monia muitakin tekijöitä, geneettisen informaation ekspressio-, proteiini- ja metaboliatason muutoksiin yhdistävän systeemibiologisen lähestymistavan uskotaan tulevaisuudessa vievän lähemmäksi sairauksien syntymekanismien selviämistä, hoitoa ja ehkäisemistä.

ABSTRACT

The adverse pulmonary effects of inhaling asbestos fibers are well characterized; in addition to malignant diseases, they have been shown to promote the development of several non-malignant lesions of the lungs and pleura. Although the exact mechanisms by which the inhalation of asbestos fibers leads to lung tissue injury are still unclear, they may involve a persistent inflammatory response mediated by reactive oxygen species (ROS), cytokines, growth factors, and pro-inflammatory mediators, as well as changes in the proteolytic balance of the lungs.

Inhalation of tobacco smoke exposes the lungs to thousands of chemicals many of which evoke oxidative stress, DNA-damage, chronic inflammation, and alterations in the protease-antiprotease balance. This, in turn, may induce carcinogenesis and promote the development of non-malignant disorders, such as chronic obstructive pulmonary disease (COPD) and emphysema.

Since oxidative stress, inflammation, and proteolytic imbalance are potential consequences of exposures to both asbestos fibers and tobacco smoke, the proteins involved in these pathways have been implicated in the pathogenesis of lung diseases related to these exposures. Moreover, genetic variations affecting the expression or functional properties of these proteins are believed to partly account for the inter-individual differences in morbidity and disease progression between patients with similar exposure histories.

In this work, role of several polymorphisms in genes encoding proteins involved in xenobiotic metabolism (EPHX1, GSTM1, GSTM3, GSTP1, GSTT1, and NAT2), protease-antiprotease balance (MMP1, MMP9, MMP12, SERPINE2, and TIMP2), inflammation (TNF, TGFB1, and GC), and innate immunity (NLRP3 and CARD8) was examined in

relation to asbestos and smoking related non-malignant pleural and pulmonary changes in two clinically and radiologically examined cohorts of Finnish construction workers. The studied genes and polymorphisms were selected based on the published literature which indicated that they might be potential candidate modifiers of the abovementioned lung diseases. Different sophisticated techniques were employed in the genotyping analysis including multiplex PCR, PCR-based restriction fragment length polymorphism (PCR-RFLP), pyrosequencing, TaqMan® allelic discrimination, and OpenArray®.

The *GSTT1* gene deletion was found to increase the risk for interstitial lung fibrosis, pulmonary emphysema, and decreased diffusing capacity for carbon monoxide (DL_{CO} and DL_{CO}/VA). These associations were significant in the whole study population and in both of the study cohorts separately. The risk for interstitial lung fibrosis was also found to be increased by a certain genotype of the innate immunity related *NLRP3* gene, protein product of which is an important part of the NLRP3 inflammasome complex. A truncating polymorphism in the gene of another member of the complex, *CARD8*, was found to be associated with the greatest thickness of pleural plaques. Moreover, both a polymorphism and a haplotype of the inflammatory cytokine *TGFB1* gene were found to be associated with visceral pleural fibrosis and calcification degree of pleural plaques.

Polymorphisms associated with pulmonary emphysema were also found in *SERPINE2*, *MMP9*, *TIMP2*, *TNF* and *TGFB1* genes. Moreover, a certain genotype and a haplotype in the serine protease inhibitor *SERPINE2* gene were found to predispose to panlobular emphysema. These associations were significant in the whole study population and individually in both of the study cohorts. Genotypes in metalloproteinase *MMP9* and inflammatory cytokine *TGFB1* genes, on the other hand, were found to protect from centrilobular emphysema, disease type closely related to smoking. In addition, the risk for paraseptal emphysema was increased by certain genotype of metalloproteinase inhibitor *TIMP2* and inflammatory cytokine *TNF* genes.

The studied *TIMP2* polymorphism was also associated to decreased FEV_1/FVC and MEF50, indicative of peripheral obstruction typical of smoking related emphysema and COPD. Similarly, a promoter polymorphism in xenobiotic metabolizing enzyme GSTM3 gene was associated with FEV_1/FVC-ratio and MEF50; stratified analysis revealed

significantly reduced MEF50 among individuals with certain *GSTM3* genotype.

Our results indicate that polymorphisms of genes involved in xenobiotic metabolism, protease-antiprotease balance, inflammation, and innate immunity are important modifiers of the risk of developing non-malignant pleural and pulmonary changes and lung function impairment related to asbestos and tobacco smoke exposure. The findings concerning emphysema also shed light on the etiology of different emphysema subtypes. Due to the complex nature of biological systems, however, genetic variation is expected to define the disease course in concert with other factors such as epigenetic changes and life course events. The advent of a systems biology approach allows the integration of different "omics" and these factors and in the future, will hopefully increase the understanding of the human diseasome to a whole new level.

CONTENTS

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications:

I Kukkonen MK, Hämäläinen S, Kaleva S, Vehmas T, Huuskonen MS, Oksa P, Vainio H, Piirilä P, Hirvonen A. Genetic susceptibility to asbestos related fibrotic pleuropulmonary changes. *Eur Respir J*. 2011; **38**(3):672–8.

II Kukkonen MK, Hämäläinen S, Kaleva S, Vehmas T, Huuskonen MS, Oksa P, Vainio H, Piirilä P, Hirvonen A. Genetic polymorphisms of xenobiotic metabolizing enzymes influence the risk of pulmonary emphysema. *Pharmacogenet Genomics*. 2011; **21**(12):876–83.

III Kukkonen MK, Tiili E, Hämäläinen S, Vehmas T, Oksa P, Piirilä P, Hirvonen A. *SERPINE2* haplotype as a risk factor for panlobular type of emphysema. *BMC Med Genet*. 2011; **12**:157.

IV Kukkonen MK, Tiili E, Vehmas T, Oksa P, Piirilä P, Hirvonen A. Association of genes of protease-antiprotease balance pathway to lung function and emphysema subtypes. *BMC Pulm Med*. 2013; **13**:36

V Kukkonen MK, Vehmas T, Piirilä P, Hirvonen A. Genes involved in innate immunity associated with asbestos related fibrotic changes. *Occup Environ Med.* 2013; Oct 4 (Online First). Doi: 10.1136/oemed-2013-101555.

The publications are referred to in the text by their Roman numerals.

ABBREVIATIONS

AAT	Alpha-1 antitrypsin
AM	Alveolar macrophage
ASBE	Patient group recruited in 1996–1997
ASSE	Patient group recruited in 2003–2004
CARD8	Caspase recruitment domain containing protein 8
CI	Confidence intervals
COPD	Chronic obstructive pulmonary disease
CT	Computed tomography
CYP	Cytochrome P450
DL_{CO}	Single breath diffusing capacity for carbon monoxide
DL_{CO}/VA	Diffusing capacity related to alveolar volume
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
EPHX	Epoxide hydrolase
FEV_1	Forced expiratory volume in one second
FVC	Forced vital capacity
GC	Group specific component
GST	Glutathione S-transferase
GWA	Genome-wide association study
HRCT	High resolution computed tomography
HWE	Hardy-Weinberg equilibrium
IL	Interleukin
IPF	Idiopathic pulmonary fibrosis
1KGP	1000 Genomes Population
LD	Linkage disequilibrium
LAA%	Per cent of lung low attenuation area
MAF	Minor allele frequency

MEF50	Maximal expiratory flow where 50% of FVC remains exhaled
MMP	Matrix metalloproteinase
NAT	N-acetyltransferase
NLRP3	NLR family, pyrin domain containing protein 3
OR	Odds ratio
PAH	Polycyclic aromatic hydrocarbon
PCR	Polymerase chain reaction
PN1	Protease nexin 1
PMN	Polymorphonuclear monocyte
PY	Pack years of smoking
PYCARD	PYD and CARD domain containing protein
RFLP	Restriction fragment length polymorphism
ROS	Reactive oxygen species
SD	Standard deviation
SERPINA1	Serpin peptidase inhibitor, clade A, member 1
SERPINE2	Serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 2
SNP	Single nucleotide polymorphism
TGFB1	Transforming growth factor beta 1
Th	T-helper cell
TIMP	Tissue inhibitor of metalloproteinases
TLC	Total lung capacity
TNF	Tumor necrosis factor
XME	Xenobiotic metabolizing enzyme

1 INTRODUCTION

The pulmonary effects of inhalation of asbestos fibers are well characterized; they are known not only to increase the risk for bronchogenic carcinoma and malignant mesothelioma, but also to promote the development of several non-malignant lesions of the lungs and the pleura [1]. Despite the current national bans and restrictions, asbestos induced pulmonary and pleural diseases still represent a significant health concern due to the huge amounts of asbestos that have been mined and used in different insulating and construction purposes since the early 1900s, combined with the long latency period of most asbestos associated conditions [2].

Tobacco smoke, which is known to contain thousands of harmful chemicals, is also a major source of subjective and environmental exposure and a truly global health burden being responsible for several millions of deaths yearly all around the world [3]. In addition to causing lung cancer, tobacco smoke has been shown to increase the risk for other malignancies and promote the development of cardiovascular and respiratory disorders [4–6].

Both tobacco smoke and asbestos fibers can trigger the release of oxygen radicals, proteolytic enzymes and toxic mediators by interacting with inflammatory and other target cells [7, 8]. In the lungs, free radicals and foreign compounds are inactivated by different antioxidants and groups of xenobiotic metabolizing enzymes (XMEs) intended to detoxify and eliminate these harmful agents [9, 10]. Nonetheless, even though there are effective defense mechanisms, these agents may still induce oxidative stress, alter the protease-antiprotease balance, induce innate and adaptive immune responses, and lead to the presence of persistent inflammation eventually causing a lung injury [11, 12].

Although asbestos exposure and tobacco smoke play an important role in the development of several lung diseases, there is huge variation

in morbidity, disease progression, and clinical manifestations between individuals with similar exposure histories. This variation is believed to originate from individual susceptibilities, which in turn are due to genetic (alterations in DNA sequence) and/or epigenetic (modifications of DNA that do not alter the sequence) variation, life course events, and ultimately to the complex interplay between the environment and these factors [13].

In this thesis, the effects of genetic variation on the risk and severity of asbestos and smoking related non-malignant pleural and pulmonary changes and lung function impairment were examined in Finnish construction workers. The studied polymorphic genes encode for proteins involved in several pathways potentially linked to asbestos and tobacco smoke exposure: xenobiotic metabolism (EPHX1, GSTM1, GSTM3, GSTP1, GSTT1, and NAT2), proteolytic balance (MMP1, MMP9, MMP12, SERPINE2, and TIMP2), inflammation (TNF, TGFB1, and GC), and innate immunity (NLRP3 and CARD8).

2 REVIEW OF THE LITERATURE

2.1 Asbestos and the lungs

2.1.1 Characteristics and use of asbestos

The term asbestos refers to a group of naturally occurring fibrous silicate minerals utilized in many industrial applications because of their thermochemical and electrical resistance, high tensile strength, and flexibility. There are several commercially used, chemically and physically different asbestos types, *e.g.,* the serpentine mineral, chrysotile (white asbestos), and the amphibole minerals; crocidolite (blue asbestos), amosite (brown asbestos), anthophyllite, tremolite, and actinolite [14].

Chrysotile fibers are extremely thin and flexible; they are composed of hydrated, magnesium-containing phyllosilicate-sheets that have been rolled up to form hollow cylinders. Bundles of chrysotile fibers can have variable length and are often curvilinear. The amphibole minerals are needle-like, crystalline structures typically less hydrated and less flexible than the chrysotile fibers. The core structures of amphiboles amosite and crocidolite contain a considerable amount (27–30%) of iron that can be redox activated, whereas in chrysotile a smaller portion (1–6%) of redox-active iron is located on the surface of the mineral [14, 15].

Asbestos has been used all around the world for more than 3000 years in thousands of products, such as textiles, plastics, cement products, brakes, engine gaskets and insulation for boilers, steam pipes, and electrical wiring. Asbestos usage peaked in the middle of the 1970s, when 4.8 megatons of asbestos were produced and used in industrial applications [16]. As the health hazards of asbestos became to emerge, the demand for asbestos started to decline in the United States as well as in many European countries.

Worldwide, most of the commercially consumed asbestos type (< 90%) has been chrysotile, followed by crocidolite, amosite, and anthophyllite [16]. In Finland, however, approximately 40% of all the asbestos used has been anthophyllite due to its domestic production: a total of 350 000 tons of anthophyllite were mined in Paakkila between 1918 and 1975 [17]. It has also been estimated that over 200 000 Finnish workers have been exposed to asbestos, and that more than 200 000 tons of asbestos are still to be found in hundreds of thousands of buildings [18].

In Finland, asbestos spraying was forbidden in 1976 and a complete asbestos ban was introduced in 1994 (Finnish Government decision 1380/1994). Subsequently, 54 other countries have banned or restricted the use of asbestos [19]. Nonetheless, the global use of asbestos has remained rather stable during 2003–2007, the main consumers being China, India, and Russia [20].

2.1.2 Mechanisms of injury

Although asbestos can enter the body also via skin contact and ingestion, it is the inhalation of fibers that is essentially responsible for all present asbestos-related adverse health effects [21]. The toxicity of inhaled asbestos depends on multiple factors, *e.g.,* the physical and chemical properties of the mineral since they will affect the biodurability and surface reactivity of the fiber, cumulative dose, time that has been elapsed since the initial exposure, and genetic background of the host [7, 11].

Chrysotile has greatly reduced biopersistence compared to the amphiboles; the soluble magnesium molecules on the outside of the chrysotile structures promote the breakdown of the fibers in the lung tissue, and the clearance of inhaled chrysotile can occur within a period of few weeks. The amphiboles, in contrast, are generally more resistant to fragmentation and degradation in the lungs and their biological clearance is usually measured in decades [7, 14].

Once inhaled and having reached the alveolar region, the asbestos fibers are surrounded and internalized by alveolar macrophages (AM), which triggers an inflammatory reaction and the release of reactive oxygen species (ROS) from the activated AMs. However, the longer fibers cannot be completely engulfed by the macrophages, and so called “frustrated phagocytosis” takes place. This leads to the formation of asbestos bodies,

fibers coated with mucopolysaccharides and iron-rich proteins (see Figure 1), alongside with the release of cytokines and growth factors and the generation of ROS and reactive nitrogen species (RNS) [2, 11]. There is recent evidence suggesting that ROS can also be generated from the mitochondria of inflammatory and other target cells, such as alveolar epithelial cells (AECs) [22, 23]. Another source of ROS and RNS following asbestos exposure is the reactions occurring on the surface of the mineral fibers, which are mainly attributed to the presence of redox active iron [24, 25].

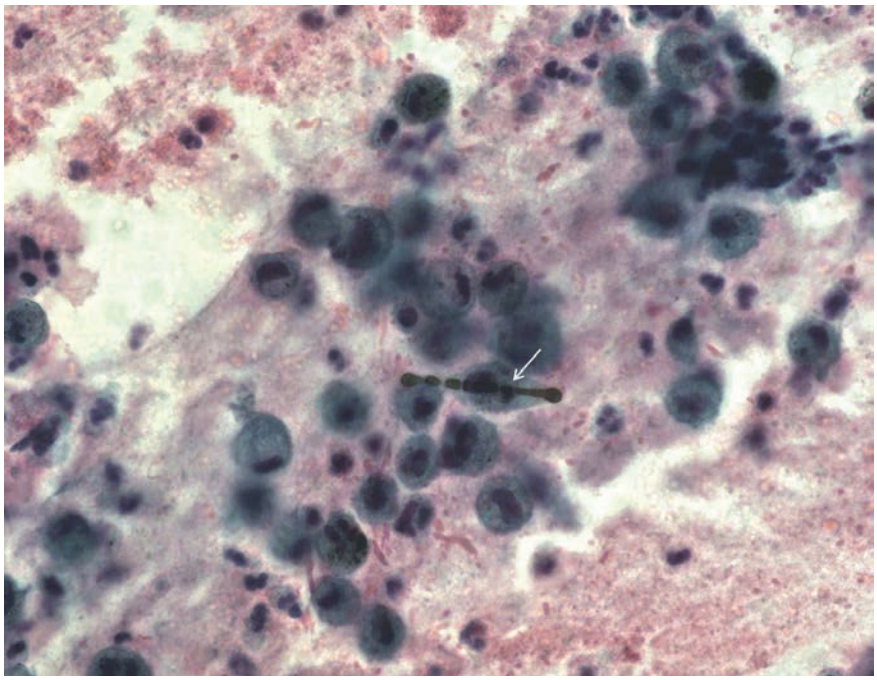

Figure 1. Papanicolaou-stained bronchial sample with asbestos-body inside a macrophage. Picture from the archive of Finnish Institute of Occupational Health by courtesy of Sauli Savukoski.

ROS and RNS are considered as important mediators of the carcinogenicity and toxicity of asbestos fibers since these radicals can induce DNA-damage and activate several signaling pathways leading to cell

proliferation, cell death, and inflammation [26]. The combination of DNA-damage and ROS can induce apoptosis of AECs, which seems to be an important factor for myofibroblast differentiation, collagen deposition, and ultimately for the development of pulmonary fibrosis [11, 27].

Asbestos and silica can also activate NLRP3 inflammasome complex through fiber phagocytosis and ROS originating from the mitochondria [28–30]. Activation of the NLRP3 complex leads to increased interleukin 1β (IL-1β) production and an inflammatory response, and this may be an important mediator of asbestos-related fibrosis and cancers. Asbestos has also been shown to alter the expression of tumor suppressor p53, a protein which has been implicated in the pathophysiology of pulmonary fibrosis and asbestos associated malignancies [31–33].

Growth factors and cytokines released from phagocytic macrophages include platelet derived growth factor (PDGF), transforming growth factor β (TGFB1), and tumor necrosis factor α (TNF). PDGF stimulates fibroblasts to migrate and proliferate, and subsequently TGFB1 activates them to produce collagen and other matrix proteins [34]. TNF, on the other hand, regulates TGFB1 production. Together these signaling molecules can trigger a fibrogenic response after the exposure to asbestos fibers [26].

Tobacco smoke has been shown to amplify the toxic effects of asbestos, promoting the development of asbestos-associated diseases [35]. It seems that at least in the case of lung cancer, the relationship between these exposures is synergistic [36]. This has been speculated to be due to the impaired clearance of asbestos from the lungs of smokers, *i.e.*, smokers have an increased fiber burden [37].

2.1.3 Non-malignant disorders associated with asbestos exposure

In addition to bronchogenic carcinoma and malignant mesothelioma (which were not topics of this thesis), asbestos is known to promote the development of several non-malignant lesions of the lungs and the pleura [1].

Asbestosis refers to interstitial lung fibrosis caused by the inhalation and deposition of asbestos fibers in the lungs. It is characterized by the accumulation of the extracellular matrix (ECM) and inflammatory cells

in the alveolar interstitial tissue. Asbestosis is usually associated with dyspnea, dry cough, bilateral crackles on auscultation, and changes in lung function [38]. Although restrictive impairment and decreased diffusing capacity are typical among asbestosis patients [39], mixed restrictive-obstructive pattern may also be observed [38].

Asbestosis resembles closely some other inflammatory and fibrotic lung disorders such as idiopathic pulmonary fibrosis (IPF), which complicates diagnosis. Although a chest radiograph showing the characteristic signs of asbestosis in the presence of an appropriate exposure history has previously been considered as adequate to allow the diagnosis of the disease [38], high resolution computed tomography (HRCT) is a more sensitive and specific technique for revealing the parenchymal and pleural changes which are characteristic of asbestosis and asbestos exposure [40, 41]. The typical HRCT findings in asbestosis include subpleural dot-like or curvilinear opacities, interlobular septal thickening, parenchymal bands, and in more advanced fibrosis, honeycombing (see figure 2) [42]. Figure 3 shows a CT image from a patient with massive fibrosis.

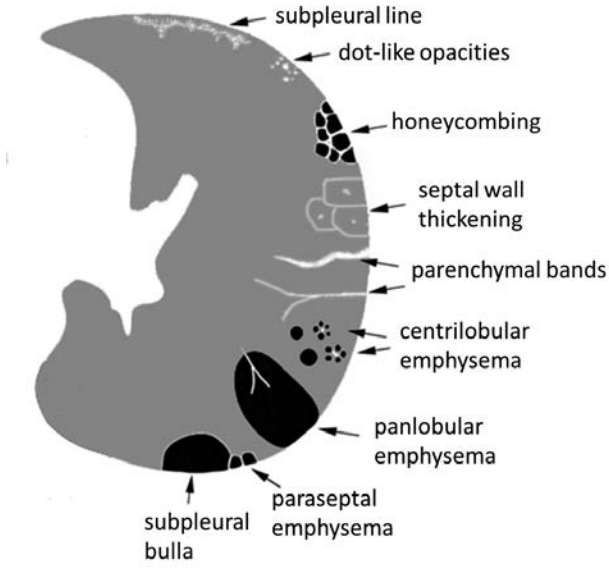

Figure 2. HRCT findings associated to asbestosis and emphysema. Modified from [43].

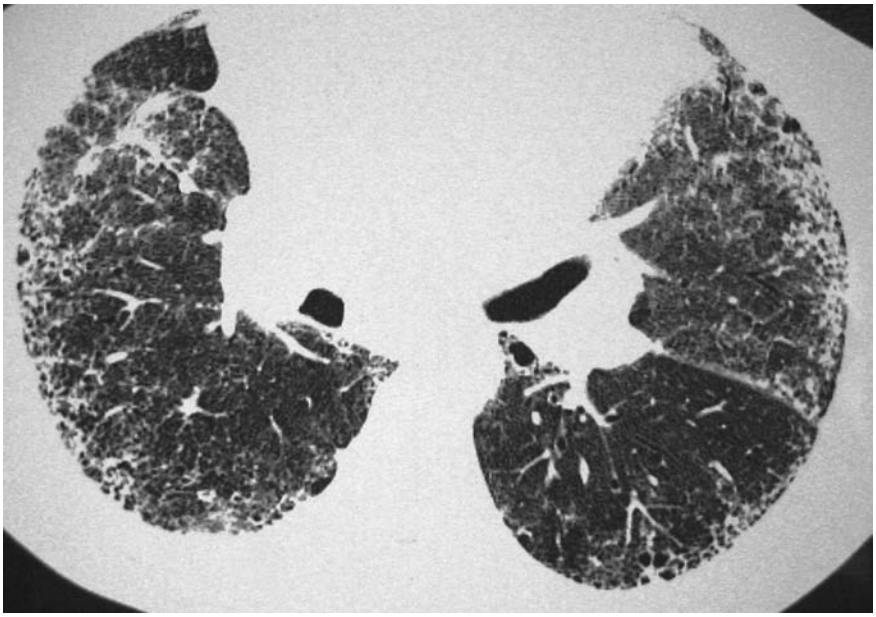

Figure 3. Massive fibrosis in both lungs of an asbestosis patient. Picture from the archive of Finnish Institute of Occupational Health by courtesy of Tapio Vehmas.

Asbestos exposure may also result in a thickening of the pleura due to collagen deposition. This thickening can be widespread diffuse fibrosis, extending from the visceral to the parietal pleura or parenchyma, or it can occur as discrete plaques limited to the parietal pleura [21]. The thickened visceral pleura may also fold to form rounded atelectasis. Circumscribed plaques are usually bilateral and may calcify over time. They are the most common manifestation of the inhalation of asbestos, but rarely occur in a timespan of less than 20 years from the initial exposure [38].

HRCT is very useful in detecting pleural plaques and distinguishing them from extra-pleural fat [38, 44]. Although pleural plaques are considered as relatively innocuous, they may be associated with a restrictive ventilator pattern, the clinical significance of which is unknown [39, 45]. The number and extent of pleural plaques have also been associated with coronary heart disease and increased all-cause mortality [46–48]. Figure 4 shows a CT image from a patient with calcified bilateral pleural plaques.

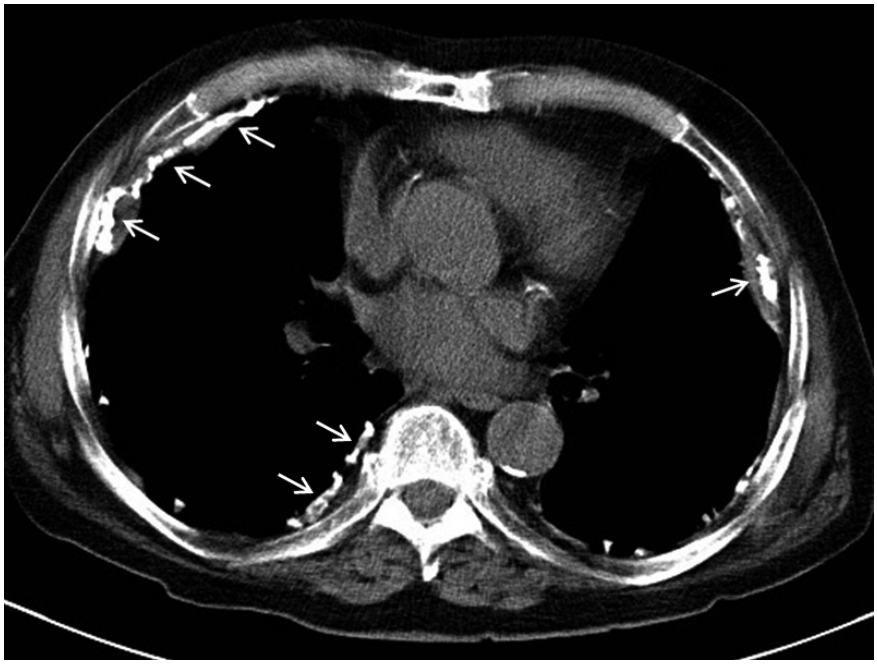

Figure 4. Calcified bilateral pleural plaques. Picture from the archive of Finnish Institute of Occupational Health by courtesy of Tapio Vehmas.

2.2 Tobacco smoke and the lungs

Tobacco smoking is a major source of environmental exposure and a huge global health burden. Worldwide, approximately 43% of men and 10% of women smoke, and it has been estimated that tobacco is responsible for over 5 million deaths yearly [3]. Cancer is one of the main causes of death in smokers, and approximately 90% of lung cancers are attributable to smoking [4]. In addition, the risks of respiratory tract, urinary bladder, pancreas, kidney, stomach, liver, esophagus, bone marrow, and cervix cancers are all increased in smokers. Aside from malignancies, tobacco smoke is an essential promoter of cardiovascular and chronic respiratory diseases [5, 6].

2.2.1 Biotransformation of tobacco smoke compounds

Tobacco smoke is a complex mixture of over 5000 chemical compounds, including carcinogens such as polyaromatic hydrocarbons (PAHs), *N*-nitrosamines, aromatic amines, and benzene, as well as co-carcinogens, tumor promoters, toxicants, irritants, free radicals, and inflammatory agents [6, 49]. The inhalation of tobacco smoke exposes the respiratory tract to these agents making it the primary target site for their toxic effects.

In the lungs, as well as in other organs, most foreign compounds (*i.e.*, the xenobiotics) are metabolized by phase I and phase II enzymes intending ultimately to achieve detoxification and elimination of the xenobiotic [9]. A schematic presentation illustrating the metabolism of xenobiotic compounds is shown in Figure 5.

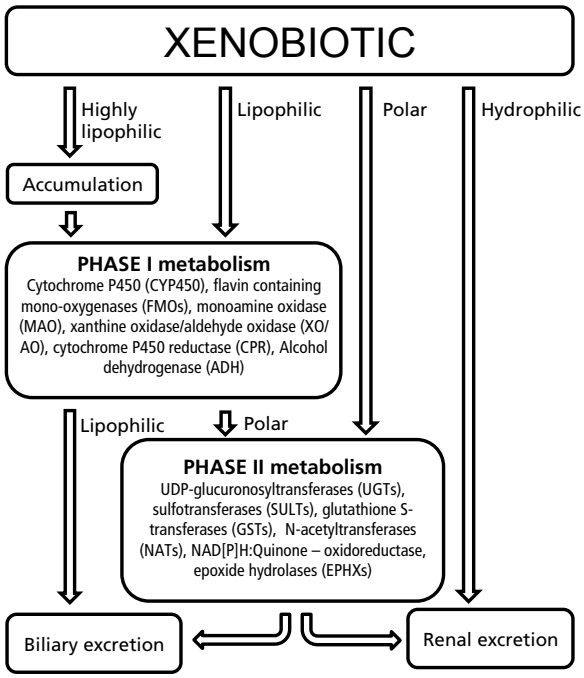

Figure 5. Schematic presentation of the metabolism of xenobiotics. Figure based on [9, 50].

Phase I enzymes include the cytochrome P450 (CYP) superfamily, flavin containing mono-oxygenases (FMOs), monoamine oxidase (MAO), and xanthine oxidase/aldehyde oxidase (XO/AO). Phase I reactions, such as oxidation, epoxidation and dehydrogenation, can introduce or unmask a functional group and this can even lead to bioactivation of an inactive compound. These potentially active and/or toxic metabolites are then modified by phase II metabolizing or conjugating enzymes, such as UDP-glucuronosyltransferases (UGTs), sulfotransferases (SULTs), glutathione *S*-transferases (GSTs), epoxide hydrolases (EPHXs), and *N*-acetyltransferases (NATs). Phase II reactions generally result in the deactivation of the toxic compound as well as an increase in its hydrophilicity thereby facilitating its excretion from the body [50]. The expression of phase I and II enzymes may be enhanced by the exposure to toxic agents [51].

2.2.2 Oxidative stress

Tobacco smoke is an plentiful source of oxidants; the gaseous phase alone contains approximately 10^{15} radicals per puff, mostly short-lived oxidants such as superoxide anion (O_2^-) and nitric oxide (NO), the latter of which reacts quickly to form peroxynitrite. The water-soluble tar phase contains more stable and organic radicals, such as phenol and semiquinone radicals, which can react with O_2^- to form the hydroxyl radical ($^{\cdot}OH$) and hydrogen peroxide (H_2O_2) [52, 53]. In addition to the exposure to these toxic compounds in the smoke, reactive oxidants can arise endogenously from inflammatory, epithelial, endothelial, and mesenchymal cells within tissues [54].

The amount of oxidants in the lungs is normally controlled by the many antioxidant defense mechanisms which are present in pulmonary tissue, *e.g.,* glutathione (GSH), GSH peroxidase, catalases, and superoxide dismutases (SODs) [10]. Oxidants in tobacco smoke can activate the nuclear factor erythroid 2-related factor 2 (Nrf2), a transcription factor which regulates the expression of many genes involved in the antioxidant response [55]. A shift from the antioxidant balance in favor of the oxidants creates oxidative stress [56]. Excess ROS, in turn, can evoke direct damage on DNA, proteins, lipids, and other components of the lung matrix, interfere with elastin synthesis and repair, inactivate

anti-proteases and activate proteases leading to a proteolytic imbalance, and trigger different inflammatory pathways [57, 58]. These adverse effects of oxidative stress are involved in the development of tobacco smoke associated malignancies as well as non-malignant lung diseases such as asthma, chronic obstructive pulmonary disease (COPD), and lung fibrosis [59–61].

2.2.3 Inflammatory response

The airways and lungs have evolved effective host defense mechanisms against pathogenic microbes and other environmental agents. These include innate defense mechanism, such as the epithelial lining, mucociliary clearance, and cells that participate in innate immunity, as well as adaptive immune mechanisms [62].

The inhalation of tobacco smoke triggers an inflammatory response involving both innate and adaptive immune systems. This is characterized by recruitment of different inflammatory cells, such as neutrophils, macrophages and lymphocytes, into the lungs and by the release of numerous pro-inflammatory cytokines including TNF, IL-1, IL-6, and IL-8 [8, 58]. Figure 6 illustrates the effects of tobacco smoke leading to inflammation and lung injury.

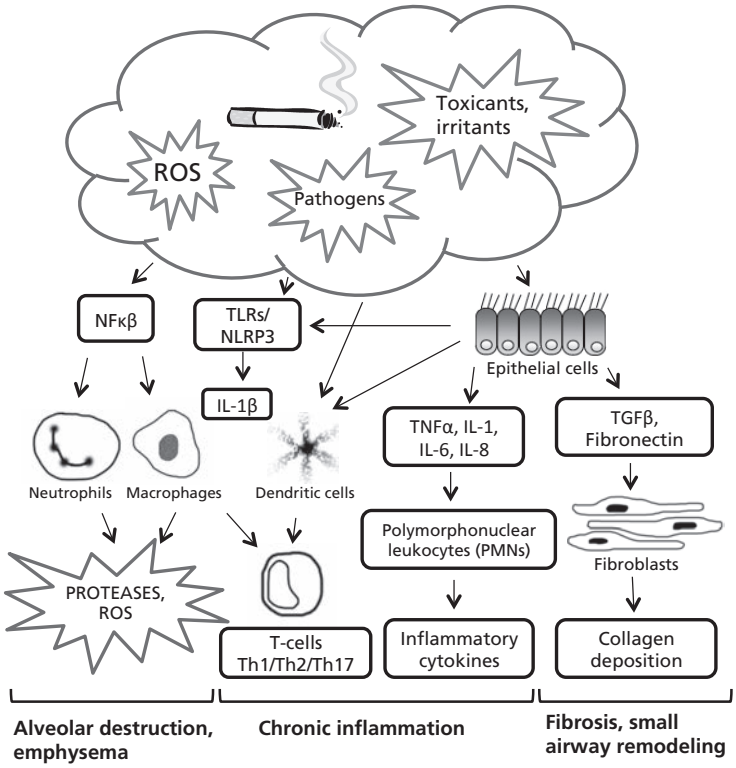

Figure 6. Simplified illustration on the effects of smoking leading to inflammation and lung injury. Modified from [63, 64].

Under normal conditions, the integrity of the tight junctions provides a barrier to airway epithelium by restricting the penetration of exogenous particles into the airway interstitium. The reactive components of tobacco smoke produce direct injuries, resulting in increased mucosal permeability. This is further reinforced by the ROS and other endogenous mediators released by the inflammatory cells [8]. Some of the normally intracellular molecules released after cellular damage can serve as danger-associated molecular patterns (DAMPs) which are identified by pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs) 4 and 2. These receptors may activate the NLRP3 inflammasome complex leading to increased IL-1β production and the generation of a non-specific inflammatory response [65].

The tobacco smoke induced injury also increases the number of macrophages and neutrophils [66], and stimulates the release of polymorphonuclear leukocytes (PMNs) from the bone marrow, followed by their accumulation into the lung microvasculature [67, 68]. These leukocytes then become attached to the endothelial cells of the vessels and are eventually able to enter the lungs due to the presence of airway irritation and injury. The attachment and penetration of inflammatory cells into the lungs is not completely understood, but is likely to be mediated via TNF and IL-1, which induce the expression of adhesion molecules, and IL-8, which serves as a chemoattractant to allow these cells to penetrate into the lungs [8]. The release of cytokines from epithelial and inflammatory cells is triggered by the activation of the transcription factor NFκB [66]. NFκB, a factor which can be activated by oxidative stress, seems to be an important regulator of tobacco smoke induced inflammation and lung tissue injury [8, 66].

Dendritic cells (DCs) link the innate and adaptive immune responses by capturing antigens released from damaged tissues and incoming pathogens and presenting them to naïve T-cells in the draining lymph nodes [69]. As a response to these antigens and other microenvironmental factors, T helper (Th) cells develop and differentiate into Th1, Th2, or Th17 cells [70]. Although Th1 and Th17 type inflammation has been associated with COPD and the Th2 type response with asthma, the role of tobacco smoke in T-cell balance remains controversial [8].

Tobacco smoke has also been associated with the development of several autoimmune diseases, such as rheumatoid arthritis and systemic lupus erythematosus. The exact mechanisms by which tobacco smoke contributes to autoimmunity have not yet been resolved [58].

2.2.4 Smoking related lung diseases: COPD and emphysema

COPD is a progressive, heterogeneous disorder characterized by inflammation of the airways, irreversible airway obstruction, and systemic effects [71, 72]. The typical pulmonary manifestations of COPD include chronic bronchitis, small airways disease, and emphysema, but it is also associated with severe extrapulmonary comorbidities such as cardiovascular disease, metabolic syndrome, skeletal muscle wasting, and osteoporosis

[73, 74]. Smoking is the main risk factor for COPD and it has been estimated that 15–20% of smokers will develop the disease [75, 76], although this value has been claimed to be an underestimation [77, 78].

At the structural level, the pulmonary changes related to COPD involve the central and peripheral airways, lung parenchyma, and the pulmonary vasculature [71]. The involvement of these compartments and the associated comorbidities, however, varies greatly between patients, and this reflects the heterogeneity of the disease. Currently, COPD is diagnosed based on the relevant symptoms (cough, sputum production, or dyspnea), history of exposure to the risk factors of the disease, and the presence of airflow obstruction (post-bronchodilator FEV_1/FVC ratio < 0.7) [79]. During recent years, the need for phenotyping the suspected COPD-patients has become evident [80, 81].

The destruction of the lung parenchyma is commonly referred to as pulmonary emphysema [82]. Emphysema is characterized by a progressive loss of alveoli, the gas-exchanging units of the lung, and a reduction in the pulmonary capillary bed resulting in marked airspace enlargement, reduced pulmonary circulation, and the loss of elastic recoil in the lungs [83]. Smoking associated emphysema can be classified into three major subtypes based on the morphology and anatomical distribution of the areas of lung destruction: centrilobular, paraseptal, and panlobular emphysema (see Figure 2) [84]. The extent and severity of the tissue destruction as well as the type of emphysema can be reliably detected with HRCT [82, 85].

Centrilobular (centriacinar) emphysema is the most common type of emphysema and closely associated to tobacco smoking [86, 87]. It is characterized by an enlargement of the centriacinar airspace, affecting mainly the proximal respiratory bronchioles. Centrilobular emphysema is predominantly distributed in the upper lung zones [88]. Paraseptal (distal acinar) emphysema involves the alveolar ducts and sacs in the subpleural regions adjacent to the interlobular septa [84]. It is commonly located in the dorsal surface of the upper lung, and may coexist with fibrosis and/or other emphysema types [89]. Figure 7 shows a CT image from a patient with extensive paraseptal and centrilobular emphysema.

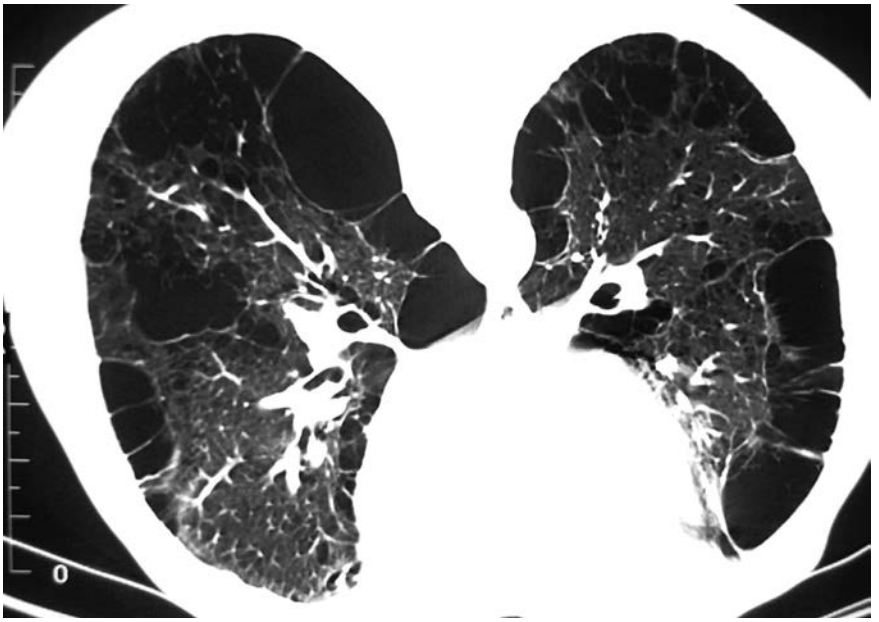

Figure 7. Centrilobular emphysema with extensive paraseptal emphysema in subpleural areas. Picture from the archive of Finnish Institute of Occupational Health by courtesy of Tapio Vehmas.

Panlobular (panacinar) emphysema involves the entire pulmonary lobule with a uniform dilatation of the air space from the respiratory bronchioles to the alveoli [88]. It is often distributed in the lower lung zones [84]. The bullae are sharply delineated areas of emphysema, at least 1 cm in diameter [90]. These structures can develop in association with any type of emphysema, but are most commonly seen with centrilobular and paraseptal disease. The term bullous emphysema is sometimes used when bullae become large enough to interfere with the respiratory function [88].

The airflow limitation characteristics of COPD results from thickening of the small airways and mucus plugging in the small conducting airways [91], promoted by peribronchiolar fibrosis, thus narrowing the lumen of bronchi and bronchioles [92]. The emphysematous destruction further contributes to a reduction in the expiratory flow affecting also the gas transfer capacity of the lungs [93, 94].

The abnormal regulation of the inflammatory response induced by tobacco smoke and/or other environmental pollutants is considered as a key mediator in the development of COPD and emphysema [12]. The inflammatory response has been shown to be enhanced in COPD patients and to persist even after smoking cessation [95]. In addition to chronic inflammation, alterations in cellular repair, apoptosis, protease-antiprotease balance, and oxidative stress have been implicated in the pathogenesis of COPD and emphysema [96, 97]. The exact mechanisms which lead to airway wall thickening and lung tissue destruction are not yet fully understood, but it is likely that they involve multifactorial processes interacting with genetic determinants [98, 99].

2.3 Individual susceptibility to asbestos and smoking related non-malignant lung diseases

Although asbestos and tobacco smoke play an important role in the development of various lung diseases, there is a great variation in morbidity, disease progression, and clinical manifestations between individuals with similar exposure histories. These differences are believed to originate from the complex interplay between genetic, epigenetic, and environmental factors [13].

2.3.1 Xenobiotic metabolizing enzyme genes

Since oxidative stress is a secondary effect from exposures to both asbestos fibers and tobacco smoke, genes involved in oxidative pathways are potential modifiers of the risk for developing lung diseases related to these exposures. The results from previous studies on *GST*, *EPHX1,* and *NAT2* polymorphisms and asbestos related non-malignant lung diseases are summarized in Table 1, and those of the meta-analyses on *GST* and *EPHX1* polymorphisms and COPD are shown in Table 2.

Table 1. Previous case-control studies on asbestos exposure associated non-malignant lung diseases and *GSTT1, GSTM1, GSTP1, EPHX1,* and *NAT2* polymorphisms.

Reference	Gene/ polymorphism	Phenotype	Case/ Control (n)	Nationality (ethnicity)	Main results
Jakobsson *et al.* 1994 [100]	*GSTM1* deletion	Asbestos-related chest X-ray abnormalities, lung function	78 cases	Sweden (Caucasian)	No significant associations found
Smith *et al.* 1994 [101]	*GSTM1* deletion,	Asbestos-related chest X-ray abnormalities	80/568	US (94.5% Caucasian)	*GSTM1* deletion a risk factor for asbestos associated parenchymal abnormalities (OR 2.1, 95% CI 1.1–3.7).
Hirvonen *et al.* 1996 [102]	*GSTM1* deletion, *GSTT1* deletion, *NAT2* slow/fast acetylator	Asbestosis and/or pleural plaques	52/69	Finland (Caucasian)	*GSTM1* deletion and *NAT2* slow acectylator genotype combination a risk factor for asbestos related non-malignant disorders (OR 4.1, 95% CI 1.1–17.2)
Kelsey *et al.* 1997 [103]	*GSTT1* deletion	Asbestos-related chest X-ray abnormalities	80/568	US (94.5% Caucasian)	No significant associations found
Horská *et al.* 2006 [104]	*GSTM1* deletion, *GSTT1* deletion, *GSTP1* Ile105Val, *EPHX1* low/high activity genotype	Asbestos-related diseases	27/34	Slovakia (Caucasian)	*GSTP1* 104Val protective against asbestosis (p = 0.044), *EPHX1* low activity genotype protective against fibrotic plaques (p = 0.045)
Franko *et al.* 2007 [105]	*GSTM1* deletion, *GSTT1* deletion	Asbestosis	262/265	Slovenia (Caucasian)	*GSTT1* deletion protective against asbestosis OR 0.61 (95% CI 0.40–0.94)

CI = Confidence intervals, OR = Odds Ratio

Table 2. Meta-analyses on associations between *GSTM1, GSTM3, GSTP1, GSTT1*, and *EPHX1* polymorphisms and COPD.

Reference	Gene	Polymorphism	Number of studies	Case/ Control (n)	Ethnicity	OR (95% CI) for homozygosity of the minor allele
Hu *et al.* 2008a [106]	*GSTM1*	deletion	12	1697/1867	Mixed	1.46 (1.16–1.83)
	GSTT1	deletion	8	983/1158	Mixed	1.06 (0.79–1.42)
Hu *et al.* 2008b [107]	*EPHX1*	rs1051740 (Tyr113His)	7 9	812/1349 1035/1106	Caucasian Asian	All: 1.59 (1.14–2.21), Caucasian 2.06 (0.96–4.43), Asian: 1.57 (1.24–1.98)
		rs2234922 (His139Arg)	7 8	812/1349 935/1006	Caucasian Asian	All: 0.90 (0.65–1.24), Caucasian 0.85 (0.54–1.35), Asian: 0.95 (0.61–1.48)
		low/high activity genotype	3 5	241/399 526/552	Caucasian Asian	All: 1.99 (1.19–3.33), Caucasian 3.40 (2.78–11.39), Asian: 1.27 (0.90–1.77); very slow vs. normal
Smolonska *et al.* 2009 [108]	*EPHX1*	rs1051740 (Tyr113His)	7 5	2017/3341 412/634	Caucasian Asian	All: 1.02 (0.91–1.36), Caucasian 1.02 (0.90–1.15), Asian: 1.02 (0.76–1.36)
		rs2234922 (His139*Arg*)	11 7	2347/4069 679/808	Caucasian Asian	All: 0.91 (0.83–1.01, Caucasian: 0.96 (0.85–1.07), Asian: 0.76 (0.61–0.96)
	GSTM1	deletion	9 5	1226/3563 551/700	Caucasian Asian	All: 1.30 (1.07–1.57), Caucasian: 1.32 (1.04–1.68), Asian: 1.24 (0.85-1.80)
	GSTT1	deletion	3 4	436/400 493/621	Caucasian Asian	All: 1.00 (0.82–1.22), Caucasian: 1.2 (0.63–2.29), Asian: 0.93 (0.73–1.19)

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Reference	Gene	Polymorphism	Number of studies	Case/ Control (n)	Ethnicity	OR (95% CI) for homozygosity of the minor allele
	GSTP1	rs1695 (Ile105Val)	6 6	821/2190 686/893	Caucasian Asian	All: 0.89 (0.73–1.10), Caucasian: 1.14 (0.96–1.35), Asian: 0.69 (0.56–0.86)
Castaldi *et al.* 2010 [109]	*GSTM1*	deletion	8	NA	NA	1.45 (1.09–1.92)
	GSTT1	deletion	5	NA	NA	0.92 (0.73–1.15)
	EPHX1	rs1051740 (Tyr113His)	15	NA	NA	1.11 (0.95–1.30)
		rs2234922 (His139Arg)	15	NA	NA	1.06 (0.91–1.23)
	GSTP1	rs1695 (Ile105Val)	10	NA	NA	1.09 (0.80–1.48)
Yan *et al.* 2010 [110]	*GSTP1*	rs1695 (Ile105Val)	10	1140/1263	Caucasian/ Asian	0.63 (0.43–0.94)
Zhong *et al.* 2010 [111]	*GSTP1*	rs1695 (Ile105Val)	6 9	938/1325 1276/1341	Caucasian Asian	All (recessive): 1.56 (1.23–1.97), Caucasian (recessive): 1.59 (1.20–2.11), Asian (recessive): 1.49 (0.98–2.27), Asian (dominant): 0.93 (0.68–1.27)
Lee *et al.* 2011 [112]	*EPHX1*	rs1051740 (Tyr113His)	10 8	6379/41816 1025/1154	Caucasian Asian	All: 1.38 (1.09–1.74), Caucasian: 1.40 (1.02–1.94), Asian: 1.38 (0.98–1.93)
		rs2234922 (His139Arg)	10 8	6565/41829 1024/1119	Caucasian Asian	All: 0.89 (0.78–1.02), Caucasian: 0.89 (0.77–1.03), Asian: 0.85 (0.53–1.36)
Xue *et al.* 2012 [113]	*GSTM1*	deletion	4 9	817/815 910/945	Caucasian Asian	All: 1.51 (1.17–1.95), Caucasian: 1.59 (1.09–2.32), Asian: 1.39 (0.98–1.96)
	GSTT1	deletion	1 8	217/160 836/919	Caucasian Asian	All: 1.05 (0.87–1.26), Caucasian: 0.91 (0.54–1.54), Asian: 1.07 (0.87–1.26)

CI = Confidence intervals, NA = not available, OR = Odds Ratio

GSTs consist of a superfamily of xenobiotic metabolizing enzymes involved in conjugation of glutathione with various electrophilic compounds and products of oxidative stress. A widely studied member of the family, GSTM1, is involved in the metabolism of diol-epoxide derivatives of PAHs and ROS [114]. GSTT1, on the other hand, can detoxify methylating agents, pesticides and many chemicals present in cigarette smoke [115]. The GSTP1 enzyme, which is the most abundantly expressed GST in human lungs, shares some substrate specificity with GSTM1, and is active in metabolizing many epoxides of PAHs including benzo(a)pyrene [116]. GSTM3, another member of the GST-family participating in the metabolism of PAHs, is also expressed in the lungs [116, 117].

The *GSTM1* null genotype leads to a complete lack of the enzyme activity. This gene deficiency has previously been connected to an increased risk of COPD [118, 119], and recent meta-analyses have clearly confirmed this association (Table 2) [106, 108, 109, 113]. *GSTM1* null genotype has also been shown to predispose smokers to emphysema and lung cancer [120], and to increase the risk for asbestos induced non-malignant pulmonary diseases (Table 1) [101].

GSTT1 null genotype has also been extensively studied concerning COPD, but meta-analyses have revealed that it does not significantly affect the risk of COPD (Table 2) [106, 108, 109, 113]. *GSTT1* deficiency has, on the other hand, been shown to protect against the development of asbestosis (Table 1) [105], and an interaction with the *GSTT1* deletion and smoking on lung function has also been observed [121].

In *GSTM3*, there are two functional polymorphisms, which are believed to affect the expression of the gene: a three-base pair deletion (**B* allele) that results in the formation of a recognition sequence for transcription factor YY1, and a promoter polymorphism (A→C) located 63 bp upstream the translation initiation site [122]. The expression level of GSTM3 has recently been associated with emphysema severity [123].

GSTP1 gene contains an amino acid changing polymorphism, Ile105Val, which has been shown to alter the catalytic activity of the enzyme [124, 125]. This polymorphism has been associated with asbestos-related pulmonary fibrosis, COPD, and impairment of lung function [126–128]. The results from a large meta-analysis indicated that *GSTP1* Val-allele could be a risk factor for COPD in Caucasian

but protective against COPD among Asian populations (Table 2) [108]. Another meta-analysis confirmed the disadvantageous effect of Val-allele among Caucasians, but failed to confirm the protective influence among Asians [111]. The *GSTP1* genotypes have also been associated to altered risk of CT defined emphysema [129].

NAT1 and NAT2 are involved in the metabolism of various xenobiotics including the aromatic and heterocyclic amines present in tobacco smoke and in the diet [130, 131]. The functional consequences of common *NAT2* gene polymorphisms are well known, dividing the individuals into rapid and slow NAT2 acetylators [131]. Our research group has previously found an association between the *NAT2* slow acetylator genotype and increased risk for both malignant (mesothelioma) and non-malignant (asbestosis and pleural plaques) pulmonary disorders in asbestos exposed workers (Table 1) [102, 132]. Polymorphisms affecting the function of NAT2 have also been associated with COPD [133].

EPHX1 is a critical biotransformation enzyme that plays a dual role in the activation and detoxification of exogenous chemicals, such as epoxides and PAHs [134]. EPHX1 is expressed in most tissues and cell types, including the bronchial epithelium [135]. The EPHX1 activity has been shown to be altered by two common gene polymorphisms, Tyr113His and His139Arg, predicting slow or fast EPHX1 activity phenotype [136]. The slow activity His-alleles and the putative slow EPHX1 activity phenotype have been associated with the development of COPD, emphysema, and impairment of lung function [118, 128, 137–143]. Other *EPHX1* polymorphisms have been connected to COPD-related traits including emphysema distribution, airway wall phenotype, and cardiopulmonary exercise capacity (Table 3) [129, 142, 144].

The promoting effect of slow *EPHX1* genotypes on the development of COPD could not be confirmed in two previous meta-analyses [108, 109], while the most recent meta-analysis indicated that the risk for COPD might be marginally increased by the His-alleles in Caucasian populations [112]. Another earlier meta-analysis, on the other hand, implied that the His-allele in codon 113 might be a risk factor for COPD in Asian populations, and the putative very slow phenotype in Caucasian populations (Table 2) [107].

Table 3. Previous association studies on COPD, COPD related traits, and *EPHX1* polymorphisms.

Reference	Polymorphism	Phenotype	Case/Control (n)	Nationality (ethnicity)	Main results
Smith & Harrison 1997 [137]	rs1051740 (Tyr113His) rs2234922 (His139Arg)	COPD and emphysema	68 (COPD)/ 94 (emphysema)/ 203	UK (Caucasian)	Homozygosity of Tyr113His minor (slow) allele risk factor for COPD (OR 3.5, 95% CI 1.5–8.0) and emphysema (OR 5.6, 95% CI 2.7–11.5). Putative slow phenotype risk factor for COPD (OR 4.1, 95% CI 1.8–9.7) and emphysema (OR 5.0, 95% CI 2.3–10.9)
Yim *et al.* 2000 [145]	rs1051740 (Tyr113His) rs2234922 (His139Arg)	COPD	83/76	Korea (Asian)	No significant associations found
Yoshikawa *et al.* 2000 [139]	rs1051740 (Tyr113His) rs2234922 (His139Arg)	COPD	40/140	Korea (Asian)	Homozygosity of Tyr113His minor (slow) allele risk factor for severe COPD compared to mild COPD cases (OR 2.9, 95% CI 1.1–7.4)
Sandford *et al.* 2001 [143]	rs1051740 (Tyr113His) rs2234922 (His139Arg)	Lung function decline of COPD patients	283 (rapid decliners)/ 308 (non-decliners)	Non-hispanic whites	A family history of COPD combined with putative very slow phenotype (His113-His139) risk factor for rapid decline of lung function (OR 4.9, 95% CI 1.1–34.9)
Budhi *et al.* 2003 [141]	rs1051740 (Tyr113His) rs2234922 (His139Arg)	Emphysema in smokers	63/172	Japan (Asian)	Putative very slow phenotype (His113-His139) risk factor for emphysema among ≥ 51 year old subjects alone (OR 2.5, 95% CI 1.01–6.5) and in combination with large amount of GT repeats in *HMOX* gene (OR 2.8, 95% CI 1.07–7.5)

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Table 3. Continues...

Reference	Polymorphism	Phenotype	Case/Control (n)	Nationality (ethnicity)	Main results
Cheng *et al*. 2004 [118]	rs1051740 (Tyr113His) rs2234922 (His139Arg)	COPD, COPD severity	184/212	Taiwan (Asian)	Homozygosity of Tyr113His minor (slow) allele risk factor for severe airflow limitation among COPD patients (OR 7.5, 95% CI 2.1–26.3)
Hersh *et al*. 2005 [140]	rs1051740 (Tyr113His) rs2234922 (His139Arg) + 6 SNPs	COPD, COPD severity, lung function	304/401 Family cohort: 127 probands/949 individuals	US (whites) Family cohort: US (98% whites)	His139Arg minor (fast) allele protective from COPD in the case-control population
Park *et al*. 2005 [138]	rs1051740 (Tyr113His) rs2234922 (His139Arg)	COPD	131/262	US (Caucasian)	Homozygosity of Tyr113His minor (slow) allele risk factor for COPD (OR 2.4, 95% CI 1.5–8.0)
Matheson *et al*. 2006 [146]	rs1051740 (Tyr113His) rs2234922 (His139Arg)	COPD, Lung function	72/220	Australia	No significant associations found
Brogger *et al*. 2006 [147]	rs1051740 (Tyr113His) rs2234922 (His139Arg)	COPD	244/248	Norway (Caucasian)	Homozygosity of Tyr113His minor (slow) allele risk protective against COPD (OR 0.5, p = 0.02). Adjustment did not change the OR, but the statistical significance was diminished (p = 0.06)
Hersh *et al*. 2006 [144]	rs1051740 (Tyr113His) rs2234922 (His139Arg) + 6 SNPs	COPD related phenotypes	304 cases	US (whites)	Rs1877724 associated with maximal output on cardiopulmonary exercise testing (p = 0.003). Rs868966, rs2292566 and rs2234922 (His139Arg) associated with diffusing capacity for carbon monoxide

Vibhuti *et al*. 2007 [128]	rs1051740 (Tyr113His) rs2234922 (His139Arg)	COPD, lung function, markers of oxidative stress	202/136	India (Asian)	Tyr113His minor (slow) allele (OR 1.77, 95% CI 1.30–2.41), His139Arg major (slow) allele (OR 1.48, 95% CI 1.03–2.13), and putative very slow phenotype (His113-His139; OR 3.26, 95% CI 1.73–6.15) risk factors for COPD
Demeo *et al*. 2007 [142]	rs1051740 (Tyr113His) rs2234922 (His139Arg) + 6 SNPs	CT assessed emphysema phenotypes	282/404	US (whites)	Rs2234922 (His139Arg) associated with CT assessed, upper lobe predominant emphysema distribution ($p = 0.005$) and radiologist score emphysema distribution ($p = 0.010$). Rs1051740 (Tyr113His), rs2260863, and rs360063 associated to CT assessed, upper lobe predominant emphysema distribution ($p < 0.049$)
Chappell *et al*. 2008 [148]	rs1051740 (Tyr113His) rs2234922 (His139Arg) +4 SNPs	COPD	1017/912	Europe (Caucasian)	No significant associations found
Zidsik *et al*. 2008 [149]	rs1051740 (Tyr113His) rs2234922 (His139Arg)	COPD	217/160	Slovakia (Caucasian)	Homozygosity of Tyr113His minor (slow) allele risk factor for COPD in combination with *GSTM1* gene deletion (OR 4.87, 95% CI 1.57–15.13)
Siedlinski *et al*. 2009 [150]	rs1051740 (Tyr113His) rs2234922 (His139Arg) + 3 SNPs	FEV_1 change in general population	1152	Germany (Caucasian)	No significant associations found

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Table 3. Continues...

Reference	Polymorphism	Phenotype	Case/Control (n)	Nationality (ethnicity)	Main results
Lakhdar *et al*. 2010 [151]	rs1051740 (Tyr113His) rs2234922 (His139Arg)	COPD, emphysema, chronic bronchitis	234/182	Tunisia	No significant associations found
Kim *et al*. 2011 [129]	rs1051740 (Tyr113His) rs2234922 (His139Arg) + 17 SNPs	CT-defined emphysema	379 cases	US (whites)	Rs2854450, rs3738042, and rs1009668 associated to per cent of lung area below -950 HU (LAA950; $p \leq 0.05$) and rs2854450 and rs3738042 associated with airway wall phenotype ($p < 0.02$)

CI = Confidence intervals, CT = Computed tomography, FEV1 = Forced expiratory volume in one second, OR = Odds Ratio, SNP = single nucleotide polymorphism

2.3.2 Genes involved in protease-antiprotease balance pathway

The imbalance between proteases and antiproteases is a widely accepted mechanism behind the lung tissue destruction leading to pulmonary emphysema [152, 153]. This theory has been gaining support since the discovery of genetic variants causing low serum levels of the serine protease inhibitor Alpha-1-antitrypsin (AAT), which has been shown to predispose to early panlobular emphysema [154]. AAT, encoded by the *SERPINA1* gene, has a major role in inactivating neutrophil elastase and other proteases thereby maintaining the protease-antiprotease balance and protecting lung tissue from destruction [155].

The gene of another member of the serpin-family, *SERPINE2* (serpin peptidase inhibitor, clade E [nexin, plasminogen activator inhibitor type 1] member 2) was identified as a COPD candidate gene in 2006 by gene expression analysis from murine and human lung tissues [156]. The protein product of *SERPINE2*, also known as protease nexin 1 (PN1), is mainly involved in coagulation and fibrinolysis with trypsin, thrombin, plasmin and urokinase as major substrates [157, 158]. In brain, it has been shown to control astrocyte proliferation and neurite outgrowth [159]. During recent years, the known functions of SERPINE2 has expanded, and a regulatory role has been proposed for it in cancer [160, 161].

In the first association study, several single nucleotide polymorphisms (SNPs) in *SERPINE2* gene were associated to COPD in family and case-control -based study populations [156]. Subsequently, various replication attempts have produced rather contradictory results (Table 4); the association between the *SERPINE2* SNPs and COPD has been replicated in two large family and case-control -based Caucasian populations [162] and in two Asian populations [163, 164], but it was failed to be replicated in one Caucasian [165] and in two Asian populations [166, 167]. Recently, *SERPINE2* SNPs have also been connected to pulmonary emphysema [129, 168].

In addition to the serine proteases, there are a number of proteolytic enzymes capable of degrading elastin and other matrix macromolecules, such as the matrix metalloproteinases (MMPs). Tissue inhibitors of metalloproteinases (TIMPs), in turn, bind MMPs and inhibit their ac-

tions [169]. MMPs and TIMPs play an essential role in tissue repair and remodeling, and there is increasing evidence that some of them may be important in airway inflammation, and ultimately in the development of emphysema and fibrosis [153, 170, 171]. It has been shown that transgenic mice over-expressing MMP1 or MMP9 develop pulmonary changes comparable to human emphysema [172, 173], and that mice lacking the *MMP12* gene are protected from emphysema despite the long term exposure to tobacco smoke [174]. Differential levels of MMP1, MMP9, MMP12, and TIMP2, have also been detected in different fibrotic lung diseases [175, 176]. The previous association studies and meta-analyses concerning *MMP1*, *MMP9*, *MMP12*, and *TIMP2* are shown in Tables 5 and 6, respectively.

Table 4. Previous association studies on COPD, emphysema, lung function, and *SERPINE2* polymorphisms

Reference	#SNPs	Phenotypes	Case/Control (n)	Nationality (ethnicity)	Main results
Demeo *et al.* 2006 [156]	48	COPD, COPD severity, lung function	304/441 Family cohort: 127 probands/949 individuals	US (whites) Family cohort: US (98% whites)	Rs7579646, rs840088, rs3795877, rs6747096, and 3795879 associated with lung function/ COPD severity in family cohort (p = 0.0008–0.02) and to COPD in case-control analysis (p = 0.016–0.0006)
Chappell *et al.* 2006 [165]	5	COPD	1018/119	European	No significant associations between the studied *SERPINE2* SNPs and COPD
Demeo *et al.* 2007 [142]	17	Emphysema distribution in COPD patients	282/441	US (whites)	No significant associations between the studied *SERPINE2* SNPs and emphysema distribution
Zhu *et al.* 2007 [162]	25	COPD, lung function	973/956 Family cohort: 635 pedigrees/1910 individuals	Norway (Caucasian) Family cohort: US/Europe	Rs6734100, rs729631, rs975278, rs7583463, and rs6748795 associated with COPD in family cohort (p = 0.0016–0.033) and to FEV_1/FVC in case-control analysis (p = 0.021–0.031)
Zhong *et al.* 2009 [166]	5	COPD	327/349	China (Asian)	No significant associations between the studied *SERPINE2* SNPs and COPD
Kim *et al.* 2009 [177]	64	BDR in severe COPD	389 cases Family cohort: 127 probands/949 individuals	US (whites) Family cohort: US (98% whites)	Rs6712954 (p = 0.04–0.09), rs7588220 (p = 0.004–0.01), and rs3795877 (p = 0.03–0.08) associated with BDR phenotype in case/ control analysis, but none replicated in the family cohort

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Table 4. Continues...

Reference	#SNPs	Phenotypes	Case/Control (n)	Nationality (ethnicity)	Main results
Cha *et al.* 2009 [163]	4	COPD	311/386	Korea	Rs16865421 (OR 0.66, 95% CI 0.45–0.97) and a haplotype consisting of rs16865421, rs7583463, rs729631, and rs6734100 (OR 0.58, 95% CI 0.38–0.89) associated with COPD
Fujimoto *et al.* 2010 [168]	2	Autopsy diagnosed emphysema	189/1146	Japan (Asian)	Rs975278 associated with emphysema (OR 1.54, 95% CI 1.02–2.30), prominently in smokers (OR 2.02, 95% CI 1.29–3.15)
Kim *et al.* 2011 [129]	64	CT-defined emphysema	379 cases	US (whites)	Rs6734100, rs729631, rs975278, rs6436449, and rs7608941 associated with per cent of lung area below -950 HU (LAA950; $p < 0.05$), rs643459 associated with airway wall thickness ($p = 0.03$)
Wang *et al.* 2011 [167]	3	COPD	409/411	China (Asian)	No significant associations between the studied *SERPINE2* SNPs and COPD
An *et al.* 2012 [164]		COPD, lung function	319/203	China (Asian)	Rs729631 and rs975278 associated with COPD, FEV_1, and FEV_1/FVC ($p < 0.05$). Association to COPD remained after Bonferroni correction

BDR = Bronchodilator responsiveness, CI = Confidence intervals, FEV1 = forced expiratory volume in one second, FVC = forced vital capacity, OR = Odds Ratio

Table 5. Previous association studies on COPD, emphysema, lung function, and *MMP1, MMP9, MMP12* and *TIMP2* polymorphisms.

Reference	Gene (#SNPs)	Phenotype	Case/ Control	Nationality (ethnicity)	Main results
Minematsu *et al.* 2001 [178]	MMP9 (1)	CT assessed emphysema	45/65 (non-emphysematous smokers) /94 (healthy non-smokers)	Japan (Asian)	*MMP9* rs3918242 minor allele associated with increased risk of smoking-induced emphysema (OR 2.69, 95% CI 1.16–6.23), lower FEV_1/VA (p = 0.02) and higher LAA% (p = 0.03)
Hirano *et al.* 2001 [179]	TIMP2 (2)	COPD	88/40	Japan (Asian)	*TIMP2* rs2277698 major allele (OR 5.4, 95% CI 2.3–12.6) and rs8179090 minor allele (OR 2.3, 95% CI 1.0–5.2) associated with increased risk of COPD
Joos *et al.* 2002 [180]	MMP1 (1) MMP9 (2) MMP12 (2)	Rate of decline in lung function	590 smokers: 284 fast decliners, 306 slow decliners	Non-hispanic whites	*MMP1* rs1899750 associated with fast rate of decline (p = 0.02), and *MMP1* (rs1899750)/ *MMP12* (rs652438) haplotypes associated with rate of decline of lung function (p = 0.0007). No significant associations between *MMP9* rs3918242 and rate of decline of lung function.
Zhou *et al.* 2004 [181]	MMP9 (1)	COPD	100/98	China (Asian)	*MMP9* rs3918242 minor allele associated with the risk of COPD
Hegab *et al.* 2004 [182]	TIMP2 (2)	COPD	106/72	Egypt	The genotype frequencies of rs2277698 differed significantly between COPD patients and controls (p = 0.029)
Ito *et al.* 2005 [183]	MMP9 (1)	COPD, lung function, emphysema findings	84/85	Japan (Asian)	Carriage of *MMP9* rs3918242 minor allele associated with higher LAA% (p = 0.04) and smaller mean CTv in the upper lung (p = 0.04)

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Table 5. Continues...

Reference	Gene (#SNPs)	Phenotype	Case/ Control	Nationality (ethnicity)	Main results
Tesfaigzi *et al.* 2006 [184]	MMP1 (1) MMP9 (3)	COPD, lung function	123/262	US (Non-Hispanic whites and Hispanics	*MMP9* rs17576 minor allele associated with increased risk for COPD among both Non-hispanic (OR 3.8, 95% CI 1.8–7.9) and Hispanic subjects (OR 3.5, 95% CI 1.5–8.1). Haplotype consisting of minor alleles of rs17576 and rs3918242, and long CA-repeat associated with increased risk of COPD (OR 1.7, 95% CI 1.1–2.7) among all subjects.
McAloon *et al.* 2009 [185]	MMP1 (6) MMP12 (4)	Emphysema severity, lung function, and gas transfer capacity	424 Alpha-1-antitrypsin deficiency (AATD) patients	UK (Caucasian)	*MMP*1 rs470358 minor allele associated with higher gas transfer (p = 0.025)
Hunninghake *et al.* 2009 [186]	MMP12 (2)	lung function in astma/COPD patient cohorts	417 (GACRS)/ 503 (CAMP)/ 109 (BAMSE)/ 127 (eoCOPD)/ 378(NETT)/ 311(Lovelace)/ 207 (NAS)	Mixed	*MMP12* rs2276109 minor allele associated with higher FEV_1 among asthmatic children and adult smokers with COPD or at risk for COPD (p = 2 x 10^{-6}). Rs2276109 major allele associated with increased risk of COPD in three COPD cohorts (p < 0.02)
Lee *et al.* 2010 [187]	MMP1 (1) MMP9 (1) MMP12 (1)	COPD	301/333	Korea (Asian)	*MMP9* rs3918242 minor allele associated with decreased risk of COPD (OR 0.69, 95% CI 0.45–0.98)

Haq *et al.* 2010 [188]	MMP1 (21) MMP9 (23) MMP12 (17)	COPD, lung function	977/876	Europe (Caucasians)	*MMP12* rs652438 associated with severe/very severe COPD (p = 0.08). A haplotype consisting of major alleles of rs652438 and rs652438 associated with decreased risk of COPD (OR 0.76, 95% CI 0.61–0.94) and lower FEV_1 (p = 0.013).
Haq *et al.* 2011 [189]	MMP12 (1)	CT assessed emphysema	1427 COPD patients	Mixed	*MMP12* rs652438 major allele dose-dependently associated with increased risk of emphysema (p = 0.016)

CI = Confidence intervals, CT = Computed tomography, FEV1 = forced expiratory volume in one second, LAA% = Per cent of lung low attenuation area, OR = Odds Ratio

Table 6. Meta-analyses on associations between *MMP9, TIMP2, TNF, TGFB1* and *COPD*

Reference	Gene	Polymorphism	No. of studies	Case/Control (n)	Ethnicity	OR (95% CI) for minor allele
Gingo *et al.* 2008 [190]	*TNF*	rs1800629 (G-308A)	16	610/1612	Mixed	1.28 (±0.03 SE); not significant
		rs361525 (G-238A)	4	1015/814	Caucasian	1.25 (±0.12 SE); not significant
Smolonska *et al.* 2009 [108]	*MMP9*	rs3918242 (C-1562T)	5	666/822	Mixed	1.45 (0.84–2.50)
	TGFB1	rs2241712 (A-10807G)	3	476/562	Mixed	0.73 (0.57–0.94)
		rs1800470 (T+29C)	5	3552/3659	Mixed	0.70 (0.58–0.84)
		rs1800469 (C-509T)	5	777/1806	Mixed	0.76 (0.54–1.08)
		rs6957 (A/G 3′UTR)	3	558/1668	Mixed	1.48 (1.13–1.93)
	TNF	rs1800629 (G-308A)	16 5	3269/3274 283/385	Caucasian Asian	All: 1.06 (0.95–1.17), Caucasian: 1.02 (0.92–1.14), Asian: 2.01 (1.21–3.31)
		rs361525 (G-238A)	5	1838/1719	Caucasian	1.10 (0.87–1.38)
		rs1800610 (G+489A)	6	1692/1596	Caucasian/ Asian	1.10 (0.92–1.33)
Castaldi *et al.* 2010 [109]	*MMP9*	rs3918242 (C-1562T)	4	NA	NA	0.69 (0.44–1.09)
	TIMP2	rs2277698 (G+303A)	3	NA	NA	0.59 (0.23–1.48)

	TGFB1	rs1800469 (C-509T)	4	NA	NA	1.05 (0.70–1.58)
		rs1800470 (T+29C)	5	NA	NA	0.73 (0.64–0.83)
	TNF	rs1800630 (C-863A)	3	NA	NA	0.91 (0.67–1.24)
		rs1799724 (C-857T)	3	NA	NA	1.04 (0.77–1.40)
		rs1800629 (G-308A)	27	NA	NA	1.19 (1.01–1.40)
		rs361525 (G-238A)	5	NA	NA	0.82 (0.57–1.19)
		rs1799964 (T-1031C)	3	NA	NA	0.89 (0.67–1.17)
		rs1800610 (G+489A)	6	NA	NA	1.26 (0.96–1.66)
Gong *et al.* 2011 [191]	*TGFB1*	rs1800469 (C-509T)	7	487/1586 614/720	Caucasian Asian	All: 0.84 (0.66–1.07), Caucasian: 1.53 (1.03–2.27), Asian: 0.93 (0.64–1.37)
		rs1800470 (T+29C)	8	1267/2389	Caucasian/ Asian	All: 1.06 (0.70–1.60)
Zhan *et al.* 2011 [192]	*TNF*	rs1800629 (G-308A)	9 15	1288/1944 1092/1794	Caucasian Asian	All: 1.34 (1.17–1.52), Caucasian: 1.01 (0.86–1.19), Asian: 2.12 (1.76–2.73)
Zhang *et al.* 2011 [193]	*TNF*	rs1800629 (G-308A)	36	2380/3738	Caucasian/ Asian	All: 1.48 (1.24–1.77), Caucasian: 1.07 (0.91–1.25), Asian: 2.36 (1.84–3.02)
Chen *et al.* 2013 [194]	*MMP9*	rs3918242 (C-1562T)	3 8	538/638 1000/1088	Caucasian Asian	All: 1.46 (1.02–2.08), Caucasian: 1.07 (0.81–1.41), Asian: 1.66 (1.01–2.71); dominant model

CI = Confidence intervals, NA = not available, OR = Odds Ratio

2.3.3 Inflammation and innate immunity –related genes

Asbestos is known to induce the production of IL-1β and TNF from alveolar macrophages, and these cytokines are believed to play an essential role in the early inflammatory response following asbestos exposure [195]. Furthermore, TNF enhances the expression of TGFB1 [196], which may lead to immune suppression and lung fibrogenesis [197, 198]. Polymorphisms in both *TNF* and *TGFB1* genes have previously been associated with the development of asbestosis [199, 200].

TNF and TGFB1 also participate in down-regulation of collagen degradation through MMPs and TIMPs. Differences in the expression of TGFB1 and TNF cytokines have been shown to influence the pathogenesis of COPD in animal models, possibly via interactions with MMP9 and MMP12 [201–203]. Genetic association studies have connected several polymorphisms and/or haplotypes of *TGFB1* and *TNF* genes to the development of COPD [190, 204–206], and recent meta-analyses have confirmed some of these findings (Table 6) [108, 109, 190–193].

Asbestos-induced IL-1β secretion is mediated through the NLRP3 inflammasome, which senses asbestos as a danger signal via the produced ROS [28]. The NLRP3 inflammasome -complex consists of several components, such as NLRP3, PYCARD, and CARD8 (also known as TUCAN), the genetic variation of which may affect the function of the complex. Certain polymorphisms in *NLRP3* and *CARD8* genes have been proposed to associate with IL-1β production and severe inflammation [207]. There are also animal studies indicating that NLRP3 could play a role in the development of asbestosis [28, 29].

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 [208]. GC has been proposed to be involved in the chronic inflammation process of the lungs, and its gene polymorphisms have been extensively studied concerning several pulmonary disorders [209]. Certain *GC* polymorphisms have been associated with COPD (Table 7).

Table 7. Previous association studies on COPD, lung function, and *GC* polymorphisms.

Reference	SNPs	Phenotype	Case/Control	Nationality (ethnicity)	Main results
Kueppers *et al.* 1977 [210]	rs7041 (Glu432Asp) rs4588 (Thr436Lys)	COPD	114/114	US (whites)	Frequency of genotype Gc2-2 lower among COPD patients than in control group (0.01 vs. 0.05; p = 0.049)
Horne *et al.* 1990 [211]	rs7041 (Glu432Asp) rs4588 (Thr436Lys)	COPD	104/413	Canada	Homozygote Gc1F allele associated with higher risk of COPD (RR 4.8) and Homozygote Gc2 allele protective against COPD (RR 0.8)
Schellenberg *et al.* 1998 [212]	rs7041 (Glu432Asp) rs4588 (Thr436Lys)	COPD, lung elastic recoil, upstream airway resistance	75/64	Canada	Homozygote Gc2 allele protective against COPD (OR 0.17, 95% CI 0.03–0.83)
Ishii *et al.* 2001 [213]	rs7041 (Glu432Asp) rs4588 (Thr436Lys)	COPD	63/82	Japan (Asian)	Homozygote Gc1F allele associated with higher risk of COPD (OR 2.2, 95% CI 1.1–4.6)
Lu *et al.* 2004 [214]	rs7041 (Glu432Asp) rs4588 (Thr436Lys)	COPD	69/52	China (Asian)	Homozygote Gc1F allele associated with higher risk of COPD (OR 3.5, 95% CI 1.93–9.43)
Ito *et al.* 2004 [215]	rs7041 (Glu432Asp) rs4588 (Thr436Lys)	COPD, lung function decline, HRCT parameters	103/88	Japan (Asian)	Homozygote Gc1F allele associated with higher risk of COPD (OR 2.3, 95% CI 1.2–4.6), higher annual decline of FEV_1 ($dFEV_1$; p = 0.01), severe emphysema (> 60% of lung area below -960 HU; p = 0.01) and lower mean CT-score (p = 0.03).

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Table 7. Continues...

Reference	SNPs	Phenotype	Case/Control	Nationality (ethnicity)	Main results
Shen *et al.* 2010 [216]	rs7041 (Glu432Asp) rs4588 (Thr436Lys)	COPD	100/100	China (Asian)	Homozygote Gc1F allele associated with higher risk of COPD (OR 3.08, 95% CI 1.50–6.35) and Homozygote Gc2 allele protective against COPD (OR 0.22, 95% CI 0.06–0.77)
Janssens *et al.* 2010 [217]	rs7041 (Glu432Asp) rs4588 (Thr436Lys)	COPD, vitamin D (25-OHD) level	262/152	Belgium	Rs7041 (Glu432Asp) minor allele associated with higher risk of COPD (OR 2.11, 95% CI 1.20–3.71) and lower levels of serum 25-OHD ($p < 0.0001$)
Wood *et al.* 2011 [218]	rs7041 (Glu432Asp) rs4588 (Thr436Lys) +10 SNPs	COPD, COPD related phenotypes, serum vitamin D and Gc levels	471 (AATD) /364 (COPD) /107 (no COPD) 140/480 (replication)	UK (Caucasian)	Rs2070741 associated with airway bacterial colonization ($p = 0.04$) and bronchiectasis ($p = 0.01$). Rs7041 (Glu432Asp) associated with bronchiectasis and vitamin D concentration ($p = 0.01$). Homozygote Gc1F allele associated with increased risk for bronchiectasis (OR 15.1, 95% CI 1.02–2.22) and decreased risk for COPD (OR 0.79, 95% CI 0.65–0.99). The latter association also replicated in an independent cohort.
Bakke *et al.* 2011 [219]	rs7041 (Glu432Asp) rs4588 (Thr436Lys) +19 SNPs	COPD, lung function	953/956 Family cohort: 635 pedigrees/ 1910 individuals	Norway (Caucasian)	Rs17467825, rs2282680, rs4588 (Thr436Lys), and rs1155563 associated with decreased FEV_1 in case control analysis ($p < 0.026$), one of which (rs1155563) replicated in the family cohort ($p = 0.046$). Rs1155563 associated with decreased FEV_1/FVC in both case control analysis and family cohort ($p > 0.028$).

AATD = Alpha-1-antitrypsin deficiency, CI = Confidence intervals, CT = Computed tomography, FEV_1 = forced expiratory volume in one second, FVC = forced vital capacity, OR = Odds Ratio

2.3.4 Results from recent GWA studies

Genome-wide association (GWA) studies are being increasingly applied to identify the genetic determinants behind complex diseases. They are considered as an unbiased approach since tens, even hundreds of thousands of markers are examined throughout the genome to locate areas associated to disease. In recent years, GWA studies have accelerated the discovery of potential candidate genes for several lung diseases [220].

The first GWA study concerning COPD identified and replicated two major susceptibility loci containing the *CHRNA3* and *CHRNA5* (cholinergic receptor, nicotinic, alpha 3/5), *IREB2* (iron responsive element binding protein 2, involved in iron homeostasis) and *HHIP* (hedgehog interacting protein, known to have a role in lung development) genes [221]. The second GWA study confirmed these findings and identified a new locus containing the *FAM13A* gene (family with sequence similarity 13, member A) [222]. Since then, a third COPD susceptibility locus harboring the *RAB4 (RAB4A*, member of RAS oncogene family), *EGLN2* (*C. Elegans* homolog 2), *MIA* (melanoma inhibitory activity) and *CYP2A6* (cytochrome P450, subfamily IIA, polypeptide 6) genes has been discovered [223].

Subsequently, the association of these COPD susceptibility genes was analyzed against different COPD phenotypes, and the *CHRNA3/CHRNA5* locus was found to be associated with increased smoking intensity and HRCT-defined emphysema [224]. One GWA study conducted among COPD patients has also associated *BICD1* gene (bicaudal D1) locus to CT-defined emphysema [225].

Several GWA studies have evaluated the genetic components of lung function with the *HHIP* locus being among the most commonly replicated genomic areas [226–228]. One large meta-analysis found also a locus containing the *AGPHD1* (aminoglycide phosphotransferase domain containing protein 1), *IREB2*, and *CHRNA3/CHRNA5* genes to be associated with airflow obstruction [229]. Another recent GWA study associated two chromosomal areas with a decline in lung function, and identified differential expression of three genes, *TMEM26* (transmembrane protein 26), *ANK3* (ankyrin 3), and *FOXA1* (forkhead box A1), residing in close vicinity to the associated SNPs [230]. Other genes, including *TNS1* (tensin 1), *GSTCD* (glutathione S-transferase,

C-terminal domain containing), *AGER* (advanced glycosylation end product-specific receptor), *HTR4* (5-hydroxytryptamine [serotonin] receptor 4), *THSD4* (thrombospondin, type I, domain containing 4), *DNER* (delta/notch-like EGF-related receptor), *SOX9* (SRY-box containing gene 9), and *HLA-DQ* (major histocompatibility complex, class II, DQ alpha chain) genes have also been implicated in GWA studies on different lung function measures [228, 231].

To date, no GWA studies have been conducted investigating interstitial lung fibrosis or other asbestos associated non-malignant diseases. Instead, polymorphisms in *MUC5B* (mucin 5B) and *TERT* (telomerase reverse transcriptase) genes have been connected to idiopathic interstitial pneumonia (IIP) and idiopathic pulmonary fibrosis (IPF) in experiments using genome-wide applications [232, 233]. These associations have been replicated in another recent GWA study, which also found several novel IIP-associated loci, including *FAM13A* [234].

3 AIMS OF THE STUDY

The main objective of this thesis was to examine the role of several gene polymorphisms in the development of asbestos and tobacco smoke related non-malignant pleural and pulmonary changes among Finnish construction workers. More specifically, the studied polymorphisms were selected from genes involved in xenobiotic metabolism (*EPHX1*, *GSTM1*, *GSTM3*, *GSTP1*, *GSTT1*, and *NAT2*), protease-antiprotease balance (*MMP1*, *MMP9*, *MMP12*, *SERPINE2*, and *TIMP2*), inflammation (*TNF*, *TGFB1*, and *GC*), and innate immunity (*NLRP3* and *CARD8*), and their role examined in the development and severity of:

- asbestos related fibrotic changes in the visceral pleura and lung interstitium
- tobacco smoke related emphysematous changes of four subtypes (centrilobular, paraseptal, panlobular, and bullae), and
- impairment of lung function (FEV_1, FVC, FEV_1/FVC, MEF50, and TLC) and diffusing capacity (DL_{CO} and DL_{CO}/VA).

4 MATERIALS AND METHODS

4.1 Study populations

4.1.1 Finnish construction workers

This study combines data from two previous screening studies. The first study group (ASBE, n = 602) was recruited in 1996–1997 and consisted of asbestos exposed subjects who lived in the Helsinki area, had asbestosis or asbestos-related pleural plaques, and had a positive smoking history [235, 236]. The second study group (ASSE, n = 633) was recruited in 2003–2004 and consisted of asbestos exposed subjects from three geographic areas (Helsinki, Tampere, Turku), who had previously been diagnosed with an asbestos related occupational disease or had visited the clinics of occupational medicine in Helsinki, Turku, or Tampere for a clinical follow-up [237].

Altogether 178 of the subjects recruited in 2003–2004 had already participated in the first study. They were therefore excluded from the second patient group in the present study before combining of the data. In the combined study population, blood samples were available for 1021 subjects, five of whom were excluded because of missing background information. Thus, the final study population consisted of 1016 construction workers. In addition, the number of subjects in a particular study or analysis varied according to the number of successful genotyping analyses, the coverage of the clinical variable studied, and the confounders being used. Selected characteristics of the construction workers are shown in Table 8.

An approval for the study was obtained from the Ethical Committee of the Finnish Institute of Occupational Health (1995) and Ethics

Committee for Research in Occupational Health and Safety, Hospital District of Helsinki and Uusimaa (2003) according to the legislation at the time of the original study. All subjects gave informed consent to participate in the study.

Table 8. Selected characteristics of the construction workers.

	Mean (SD) or N (%)
Age, years	63.3 (7.3)
Male sex	1000 (98.4%)
Smoking history	
Never smoker	145 (14.3%)
Ex-smoker	638 (62.8%)
Current smoker	233 (20.7%)
Pack years of smoking (n = 996)	20.4 (16.9)
Years of asbestos exposure (n = 969)	23.9 (10.8)
Emphysema score (n = 372[1])	2.00 (2.36)
Centrilobular (n = 240)	1.26 (1.02)
Paraseptal (n = 181)	1.00 (0.89)
Panlobular (n = 172)	0.96 (0.88)
Bullae (n = 131)	0.70 (0.75)
Interstitial lung fibrosis (n = 792[1])	0.93 (0.66)
Visceral pleural fibrosis (n = 998)	135 (13.5%)
Pleural plaques	
Greatest thickness (n = 1012)	1.87 (0.67)
Extent, cm^2 (n = 1012)	103.1 (72.4)
Calcification (n = 1004)	1.37 (0.94)
Lung function[2]	
FEV_1 (n = 985)	83.8 (18.5)
FVC (n = 981)	89.3 (15.7)
FEV_1/FVC ratio (n = 925)	93.8 (12.4)
MEF50 (n = 983)	66.3 (29.6)
TLC (n = 965)	87.3 (13.4)
DL_{CO} (n = 970)	90.1 (20.1)
DL_{CO}/VA (n = 972)	98.2 (18.4)

n = 1016 except as noted
[1]Subjects with radiologic score > 0
[2]Percent of Finnish reference values [238]

4.1.2 Finnish population controls

In papers I and II, a demographic reference group was used in order to see whether the genotype frequencies in the case group differed from those in the general Finnish population. The recruitment of the demographic referents has been described in detail previously [239]. Briefly, the reference group consisted of 2155 Finnish Caucasians recruited from a health examination survey conducted by the Social Insurance Institution of Finland, Research and Development Centre. A random list, based on a population register was used to contact subjects living in south-western Finland. The recruitment system aimed at obtaining roughly equal numbers of subjects of both sexes in each of the five age strata. The age groups ending with seven (27-, 37-, 47-, 57-, and 67-years) were chosen in order to prevent the examination from coinciding with other health examinations usually organized at ages ending with zero or five. Forty-nine referents were excluded because they had been diagnosed with some form of malignant disease and one because of missing background information. Thus, the final demographic reference group consisted of 2105 subjects (1051 males and 1054 females). Selected characteristics of the demographic references are shown in Table 9.

Unfortunately, since neither asbestos exposure history nor clinical data were available for the population controls and since detailed data on smoking was obtained only from the current smokers, this group could only be used as demographic referents and not as a reference group in the statistical analyses.

Table 9. Selected characteristics of demographic referents.

	Mean (SD) or N (%)
Age, years	46.9 (14.0)
Male sex	1051 (49.9%)
Smoking history	
Never smoker	981 (46.6%)
Ex-smoker	506 (24.0%)
Occasional	133 (6.3%)
Current smoker	485 (23.0%)
Pack years[1]	21.4 (19.4)

[1]For current smokers only

4.2 Patient characterization

4.2.1 Exposure assessment

The ASBE-participants were personally interviewed by an occupational physician with 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. The mean years of asbestos exposure among ASBE-participants was 26.1±9.7, and mean pack-years 23.7±15.0. Most of them were ex-smokers (70%) or current smokers (27%), and only 3% of them had never smoked.

The ASSE-participants filled in a self-administered questionnaire modified from the Finnish Environment and Asthma Study Questionnaire [240]. The final questionnaire included questions on demographic characteristics, respiratory symptoms and diseases, smoking exposure, and occupational exposures with the focus on asbestos. Most of the ASSE-participants were construction workers, such as industrial, real estate and cleaning workers, or plumbers. The mean years of asbestos exposure among ASSE-participants was 20.8±11.4, and mean pack-years 16.1±18.2. Most of them were ex-smokers (54%) or current smokers (17%), and 29% of them had never smoked.

4.2.2 Radiological examinations

The lungs of the construction workers were imaged when they were prone in full inspiration with four different scanners: in 1996–1997 the Picker PO 2000 (Picker International, Cleveland, USA) device was used, whereas in 2003–2004 Siemens Somatom Balance (Siemens Medical, Erlangen, Germany) was used in Helsinki, Siemens Somatom Plus 4 (Siemens Medical) was used in Tampere, and GE Light-speed 16 Advantage (GE Healthcare, Milwaukee, WI, USA) was used in Turku. The HRCT images were printed as hard copies and analyzed blindly by two radiologists in consensus (ASSE 2003–2004), or by three radiologists separately (ASBE 1996–1997). In the latter case, average values were used in the analysis.

The radiologists scored visually the signs of interstitial lung fibrosis by using an arbitrary semiquantitative scale from 0 to 5 including one subclass between each class: 0 (normal finding), 1 (subnormal finding), 2 (mild fibrosis), 3 (moderate fibrosis), 4 (severe fibrosis), and 5 (extreme fibrosis) [241].

Visceral pleural fibrosis (VPF) variable was constructed by adding up the 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 atelectasis) considering the ASBE study material [242]. This sum score was then dichotomized to match the frequency distribution of the dichotomous VPF variable used in the ASSE study. Several signs of other pleural changes were also recorded: the extent (cm^2) and greatest thickness of pleural thickenings (ILO "width": 0 = no plaques, 1 ≤ 5mm, 2 = 5–10mm, 3 ≥ 10mm), and their degree of calcification (0 = no, 1 = sparse, 2 = a considerable part of the pleural thickenings, 3 = nearly all). The more detailed methods including the intra- and inter-reader consistencies of readings have been reported previously [241].

Emphysema was defined as sharply delineated low-density area(s) according to the criteria and reference images given by Webb *et al.* [243]. The signs of centrilobular, paraseptal, panlobular, and bullae-type emphysema were scored in both lungs by using a scale from 0 to 5: 0 (no changes), 1 (faint or subnormal abnormalities, in a single slice or few slices), 2 (slight abnormalities in some slices), 3 (clear abnormalities in several slices), 4 (score between 3 and 5), and 5 (abnormalities widely distributed in the whole lung, in all or most slices). These emphysema subtype scores were added up to create the emphysema sum score, its maximum being 20 per lung [236]. Mean scores of both lungs were used in the analysis. The intra- and inter-reader consistencies of readings have been reported previously [235].

4.2.3 Lung function examinations

Flow-volume spirometry was performed with a rolling-seal spirometer (Mijnhard BV, Bunnik, Holland) connected to a microcomputer (Medikro MR-3; Medikro, Kuopio, Finland), using Finnish reference values [238] and the standards of the European Respiratory Society (ERS) [244].

The following parameters were measured: forced vital capacity (FVC), forced expiratory volume in one second (FEV_1), the FEV_1/FVC ratio, and the maximal expiratory flow where 50% of FVC remains exhaled (MEF50).

The single breath diffusing capacity for carbon monoxide (DL_{CO}), specific diffusing capacity (diffusing capacity related to alveolar volume DL_{CO}/VA), and total lung capacity (TLC) with the helium single-breath dilution method were measured by using a Masterlab Transfer or a Compact Lab Transfer device (Erich Jaeger, Würzburg, Germany) according to ERS recommendations [245]. Correction of DL_{CO} was done according to the patient's actual hemoglobin levels.

The lung function variables were handled as percent of Finnish reference values [238] based on the distribution of values in the reference population. The FEV_1/FVC ratio was considered decreased if it was < 88% of predicted, FEV_1, FVC, and TLC values if they were < 80% of predicted, DL_{CO} and DL_{CO}/VA if they were < 74% of predicted, and MEF50 if it was < 62% of predicted [238].

4.3 Genotyping analysis

Multiplex PCR, PCR-based restriction fragment length polymorphism (PCR-RFLP), pyrosequencing, TaqMan® allelic discrimination, and OpenArray®-methodology were employed in the genotyping analysis, depending on the type and genetic context of the SNP in question and methods available at the time of the analysis. Table 10 shows all the studied genes, polymorphic sites, and the corresponding methodology used in genotyping.

Table 10. Genes, polymorphic sites, and the methods used in genotyping.

Gene	Polymorphism	Rs-number	Method	Reference/ID
CARD8	Ter*3Cys	rs2043211	OpenArray®	C__11708080_1_
	T/C	rs1062808	OpenArray®	C___3218826_10
	C/T	rs2288877	OpenArray®	C__15879993_10
EPHX1	Tyr113His	rs1051740	TaqMan®	[246]
	His139Arg	rs2234922	PCR-RFLP	[247]
GSTM1	large deletion	-	Multiplex PCR	[102, 248]
GSTM3	A-63C	rs1332018	TaqMan®	[249]
	---/AGG	rs1799735	PCR-RFLP	[250]
GSTP1	Ile105Val	rs1695	PCR-RFLP	[251]
GSTT1	large deletion	-	Multiplex PCR	[102, 248]
MMP1	-/G	rs1799750	Pyrosequencing	-
MMP9	C-1562T	rs3918242	PCR-RFLP	[180]
MMP12	Asn357Ser	rs652438	OpenArray®	C____785907_10
NLRP3	Lys705Gln	rs35829419	OpenArray®	C__25648615_10
	C/T	rs10925027	OpenArray®	C__30713882_10
NAT2	C282T	rs1041983	TaqMan®	[252]
	T341C	rs1801280	TaqMan®	[252]
SERPINE2	G/C	rs729631	OpenArray®	C____803914_10
	C/T	rs975278	OpenArray®	C___7614671_10
	C/G	rs6748795	OpenArray®	C___1677432_10
	C/T	rs840088	TaqMan®	C___7614655_10
TGFβ1	Leu10Pro	rs1800470	TaqMan®	[253]
	C/T	rs1800469	OpenArray®	C___8708473_10
	G/A	rs2241718	OpenArray®	C___7818377_1_
TIMP2	G/A	rs2277698	OpenArray®	C__15885241_10
TNF	C/T	rs1799724	OpenArray®	C__11918223_10
	G/A	rs1800629	OpenArray®	C___7514879_10
GC	Glu432Asp	rs7041	OpenArray®	C___3133594_30
	Thr436Lys	rs4588	OpenArray®	C___8278879_10

4.3.1 DNA extraction

DNA was extracted mechanically (Thermo King Fisher; Thermo Fisher Scientific, Erembodegem, Belgium) from whole blood by using BioSprint 15 DNA Blood Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer´s recommendations. The extracted DNA was stored at -20°C until use. One hundred ng of DNA was used in PCR-reactions for RFLP and OpenArray® analyses, 50 ng for Multiplex PCR, 30 ng for pyrosequencing analyses, and 20 ng for TaqMan® analyses.

4.3.2 Multiplex PCR

The presence (one or two copies of the gene) or absence of *GSTM1* and *GSTT1* genes was detected by using a multiplex PCR method as described earlier [102, 248]. Briefly, in addition to *GSTM1* and *GSTT1*-specific primer pairs, a primer pair specific for β-globin was added to the multiplex PCR-reaction. The β-globin specific fragment confirmed a successfully performed PCR-reaction, and the presence or absence of *GSTM1* and/or *GSTT1* specific fragments indicated the corresponding genotype.

4.3.3 PCR-RFLP

The 3-bp deletion of the *GSTM3* gene (rs1799735) was determined by using restriction enzyme digestion of the PCR-product with *Mnl*I [250]. The presence of the restriction site revealed the variant allele, *i.e.*, the 3-bp deletion.

The *GSTP1* exon 5 SNP (Ile105Val, rs1695) was analyzed by using PCR-RFLP method with *Sna*BI restriction enzyme digestion as described earlier [251]. The presence of the restriction site differentiated the variant allele from the wild type allele.

The *EPHX1* exon 4 SNP (His139Arg, rs2234922) was examined by using PCR-RFLP method with *Rsa*I digestion as described earlier [247]. The variant allele was identified through the presence of the restriction site.

The *MMP9* SNP (C-1562T, rs3918242) was genotyped by using a PCR-RFLP-based method essentially according to [180]. Briefly, the concentrations of the forward (5´-TTC GTG ACG CAA AGC AGA-3´)

and reverse (5′-AGC AGC CTC CCT CAC TCC T-3′) primers were 670 nM, and the cycling conditions were 95°C for 4 minutes, 34 cycles of 95°C for 30 seconds, 58°C for 30 seconds, and 72°C for 45 seconds followed by a final extension of 72°C for 5 minutes. The presence of the *Sph*I restriction site revealed the variant allele.

4.3.4 Pyrosequencing

The *MMP1* SNP (rs1799750) was analysed with a pyrosequencing-method based on an assay from PyroMark Assay Database (Qiagen). The PCR-primers were: forward primer 5′-biotin-CCC TTA TGG ATT CCT GTT TTC-3′and reverse primer 5′-CCC ATT CTT CTT ACC CTC TTG-3′. The primer concentrations in PCR reactions were 500 nM, and the cycling conditions were: 95°C for 5 minutes, 35 cycles of 95°C for 30 seconds, 54°C for 30 seconds, and 72°C for 30 seconds followed by a final extension of 72°C for 5 minutes.

The pyrosequencing was performed with PSQ™96MA (Qiagen) by using Pyromark Gold Q96 Reagents (Qiagen) according to the manufacturer's recommendations. Briefly, 40 µl of the PCR product was mixed with 37 µl of Binding buffer and 3 µl of Streptavidin Sepharose High Performance beads (GE Healthcare, Uppsala, Sweden). PCR products bound to the beads were collected and denatured to be single-stranded by treatment with 70% Ethanol, Denaturation Buffer, Washing Buffer, and mQ water in Pyrosequencing Washing Station. The sequencing primer 5′-GTA GTT AAA TAA TTA GAA AG-3′ was attached to the template by incubating for 2 minutes in 80°C in annealing buffer. The pyrosequencing run was conducted in the dispensation order of CAGC-TACTAGCA. The pyrograms were generated and analyzed with PSQ 96 SNP Software 1.1 (Qiagen).

4.3.5 TaqMan® Allelic discrimination assays

The *GSTM3* promoter area SNP (rs1332018), the *EPHX1* exon 3 SNP (Tyr113His, rs1051740), two *NAT2* SNPs (C282T, rs1041983; T341C, rs45532639), and *TGFB1* SNP (rs1800470) were genotyped by using TaqMan® allelic discrimination assays described earlier in detail [246, 249, 252, 253]. For the *SERPINE2* rs840088 SNP, a ready-made

TaqMan® SNP Genotyping Assay was purchased from Applied Biosystems (Foster City, CA, USA) (assay ID: C__7614655_10). The assays were performed according to the manufacturer´s recommendations with Applied Biosystems 7500 Real-Time PCR system by using TaqMan® probes. Sequence Detection Software 1.4 (Applied Biosystems) was used for the allele calling analysis.

4.3.6 OpenArray® assays

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), one *TIMP2* SNP (rs2277698), and three *SERPINE2* SNPs (rs729631, rs975278, and rs6748795) were genotyped by using the OpenArray-system (BioTrove Inc., Woburn, MA), a next-generation quantitative PCR platform based on TaqMan® chemistry. The assay IDs for TaqMan® SNP Genotyping Assays spotted on the array were C__25648615_10, C__30713882_10, C__11708080_1_, C___3218826_10, C__15879993_10, C__11918223_10, C___7514879_10, C___8708473_10, C___7818377_1_, C___3133594_30, C___8278879_10, C____785907_10, C__15885241_10, C__803914_10, C__7614671_10, and C__1677432_10, respectively. The plate format of 16 SNPs and 144 samples per array were used. The allele calling analysis was performed using OpenArray™ SNP Genotyping Analysis software (BioTrove Inc.).

4.3.7 Quality control

For the quality control, two independent readers interpreted the results and a random selection of 10% of all samples was re-tested. Minor error rates were detected for the OpenArray assays rs1799724 (1%), rs2043211 (1%), rs975278 (1%), rs6748795 (1%), rs1800629 (2%), rs2241718 (2%), rs2277698 (2%), and rs1800469 (3%).

In order to verify the reliability of OpenArray platform, a random selection of 15% of samples was re-analyzed for *TNF* rs1800629 and rs1799724 SNPs with 100% concordant results with the earlier esti-

mates. The re-analyses were conducted with a PCR-RFLP method [254] (rs1800629) and a pyrosequencing-based method (rs1799724), designed by using PyroMark Assay Design 1.0 -tool (Qiagen).

The primers and probes for the pyrosequencing protocol were as follows: forward primer: 5′-GGT AGG AGA ATG TCC AGG GCT ATG-3′, biotinylated reverse primer: 5′-biotin-ACT CCC TGG GGC CCT CTA-3′, and sequencing primer: 5′-TCG AGT ATG GGG ACC-3′. The primer concentrations in PCR reactions were 200 nM, and the cycling conditions were: 95°C for 5 minutes, 39 cycles of 95°C for 15 seconds, 56°C for 30 seconds, and 72°C for 15 seconds followed by a final extension of 72°C for 5 minutes. The pyrosequencing was performed with PSQ^{TM}96MA (Qiagen) by using Pyromark Gold Q96 Reagents (Qiagen) as described above for the analysis of the *MMP1* rs1799750 SNP.

4.4 Statistical and bioinformatics methods

4.4.1 Power calculations

The studies had variable powers to detect associations, depending on the minor allele frequency (MAF) and study size: Study I (n = 1008) had 80% power to detect odds ratios (ORs) from 1.54 to 1.80 (MAF 13–46%), Study II (n = 988) had 80% power to detect ORs from 1.45 to 1.66 (MAF 13–46%), Study III (n = 951) had 80% power to detect ORs from 1.66 to 1.72 (MAF 21–28%), Study IV (n = 951) had 80% power to detect ORs from 1.46 to 2.30 (MAF 4–42%), and Study V (n = 951) had 80% power to detect ORs from 1.57 to 2.63 (MAF 4–42%). The calculations, based on a two-sided alpha of 0.05, were performed by using standard methods.

4.4.2 Association analysis

The associations between genotypes/haplotypes, radiologic parameters, and lung function parameters (FEV_1, FVC, FEV_1/FVC, MEF50, DL_{CO}, DL_{CO}/VA, and TLC) were evaluated by using linear regression analysis. Logistic regression analysis was used to evaluate the potential confounders and to further study the risk for pleuropulmonary changes and their severity with certain genotype/haplotype.

Covariates considered in the analyses included sex, age, pack years of smoking (PYs), years of asbestos exposure, height, and FEV_1: the covariate pattern was selected depending on the particular research question. In papers I–III, occasionally lacking covariate data was replaced by the group mean value (47 replacements for asbestos exposure, 20 replacements for smoking exposure, and 68 replacements for height)

The χ^2 analysis was used to test the deviation of the genotype distributions from the Hardy-Weinberg equilibrium (HWE).

All of the data analyses were performed by using the SPSS version 18.0 (SPSS Inc., Chicago, IL).

4.4.3 Population stratifications

For further analysis, the cases were divided according to the existence of the radiologic changes. In Studies I and V, the radiologic signs of fibrosis were considered mild/subnormal if the radiological score was < 2, and severe/pathological if the score was ≥ 2. In addition, the greatest thickness of pleural plaques was categorized as < 5 or ≥ 5 mm and their extent as ≤ 100 or > 100 cm^2. For Studies II–IV, the radiologic signs of emphysema were considered either mild/subnormal if the radiologic score was < 1 (< 2 in emphysema sum score), or severe/pathological if the radiologic score was ≥ 1 (≥ 2 in emphysema sum score). For the stratified analyses on smoking habits in study III, the subjects who had smoked less than 25 PYs were categorized as mild smokers, and subjects who had smoked for at least 25 PYs were considered as moderate/heavy smokers.

4.4.4 Phenotype construction

The two polymorphic loci of *NAT2* are considered to provide sufficient information for reliable prediction of the NAT2 phenotype in Caucasian populations [255]. Consequently, the subjects were categorized into fast and slow NAT2-acetylators according to the presence of wild-type and variant alleles in these two loci; subjects with two variant alleles were considered as slow acetylators, all others were included in the fast acetylator category [256]. Similarly, the *EPHX1* diplotypes were categorized in the putative phenotype groups (high, intermediate, and low activity) essentially according to [257].

4.4.5 Linkage disequilibrium and haplotype construction

For papers III–V, the linkage disequilibrium (LD) structure was examined by using HaploView program, version 4.2 [258]. When moderate or strong linkage ($r^2 > 0.5$) was detected, haplotypes consisting of the SNPs in question were statistically reconstructed from population genotype data with the Markov chain method for haplotype assignments by using the PHASE program (version 2.1) [259]. The associations of the haplotypes to pulmonary parameters were examined as with the single SNPs.

4.4.6 Database search

For papers IV and V, the F-SNP program [260], connected to main databases, was used to predict the function of the intronic SNPs studied, and the SNP Annotation and proxy search (SNAP) [261] was used to examine the LD of the studied SNPs in the 1000 Genomes Population (1KGP).

To further clarify the potential role of *SERPINE2* and its polymorphisms in the development of lung tissue destruction, the MADCOW-tool [262] was used to perform a microarray database search in order to locate genes that are co-expressed with *SERPINE2*. The genes in the resulting list were then classified by using the DAVID 6.7 functional annotation tool [263].

The eQTL Browser [264] was used to identify polymorphisms potentially associated to the expression of the studied genes from a microarray database.

5 RESULTS

5.1 Conformity of the genotype distribution with HWE in the study population

The genotype and putative phenotype frequencies of the studied polymorphisms in *GSTM1*, *GSTM3*, *GSTP1*, *GSTT1*, *EPHX1*, and *NAT2* genes were similar in the construction workers and the demographic references (Supplemental Table 1 in Papers I and II). In addition, these polymorphisms were in HWE among the demographic references ($p > 0.05$). The rest of the polymorphisms in *CARD8*, *MMP1*, *MMP9*, *MMP12*, *NLRP3*, *SERPINE2*, *TGFB1*, *TIMP2*, *TNF* and *GC* genes were studied only among the construction workers*;* almost all of them were in HWE ($p > 0.09$), except the *CARD8* rs1062808 and rs2288877 SNPs ($p < 0.01$). These SNPs were omitted from further analysis.

5.2 Linkage disequilibrium structures

Three of the studied *SERPINE2* SNPs (rs729631, rs975278, and rs6748795) were found to be in tight linkage disequilibrium ($r^2 \geq 0.980$). The haplotype block structure and pair-wise LD (D′) values for the studied *SERPINE2* SNPs are shown in Figure 8. Since examining several SNPs in almost complete linkage is not expected to offer any additional information, only one of these SNPs (rs729631) was chosen to be examined further in addition to the fourth *SERPINE2* SNP (rs840088), which was in moderate linkage with the other three SNPs ($D' = 0.500–0.551$, $r^2 = 0.027–0.031$).

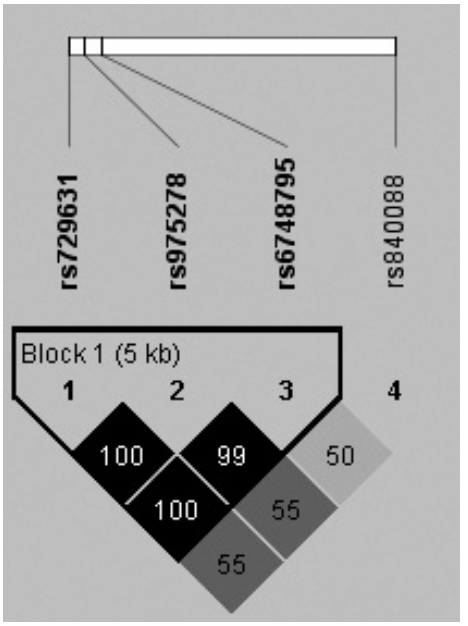

Figure 8. Linkage disequilibrium (LD) between the studied *SERPINE2* SNPs in the construction workers. Values of D´ are shown.

In addition, a moderate LD was observed between *GC* rs4588 and rs7041 SNPs (r^2 = 0.501) and *TGFB1* rs1800469 and rs1800470 SNPs (r^2 = 0.738). The haplotype block structure and D´ values for the *GC* and *TGFB1* SNPs are shown in Figure 9.

For *NLRP3* rs35829419 and *TNF* rs1799724 SNPs, the minor allele frequencies were too small (0.3 % and 0.2 %, respectively) for r^2 to detect LD, despite the maximum D´s (D´ = 1.00, r^2 = 0.032 for rs35829419; D´ = 1.00, r^2 = 0.008 for rs1799724).

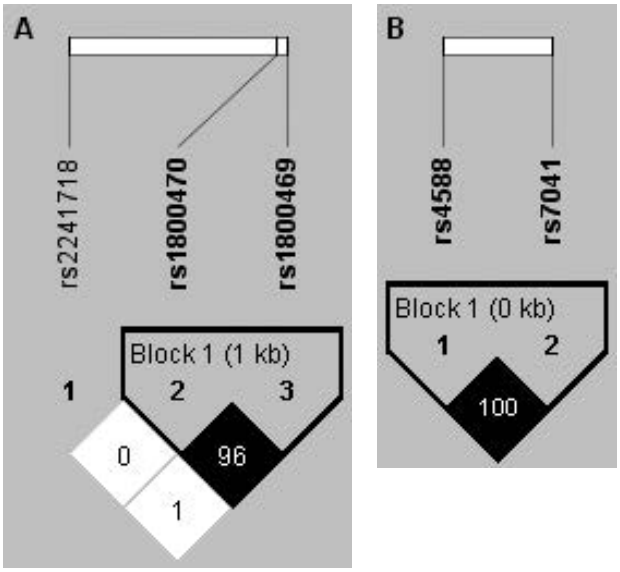

Figure 9. Linkage disequilibrium (LD) between the studied *TGFB1* SNPs (A) and *GC* SNPs (B) among the construction workers. Values of D´ are shown.

5.3 Reconstructed haplotypes

The haplotype analysis identified four different haplotypes for *SERPINE2* rs729631 and rs840088 SNPs. The most common of these was the GC (wild type-wild type, 55.0%) followed by GT (24.4%), CC (16.9%), and CT (3.7%). For *GC* rs4588 and rs7041 SNPs, three haplotypes were identified: the most common was GC (wild type-wild type, 65.2%), followed by TA (21.1%), and GA (13.7%). For *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%).

5.4 Genetic predisposition to fibrotic changes

Several significant associations were found between the studied polymorphisms and fibrotic pleuropulmonary changes in the construction workers (Table 11). The *GSTT1* deletion polymorphism and the *NLRP3* rs35829419 SNP were found to be associated with interstitial lung fibrosis (p = 0.003 and p = 0.013, respectively), and the *TGFB1* rs2241718 SNP with visceral pleural fibrosis (p = 0.044). In addition, the *GSTM1* deletion polymorphism and the *CARD8* rs2043211 SNP were found to be associated with the greatest thickness of pleural plaques (p = 0.009 and p = 0.015, respectively) and the *TIMP2* rs2277698 SNP with the calcification of pleural plaques (p = 0.037) (Table 11). No associations were found between the studied polymorphisms and the extent of the area covered by pleural plaques.

Table 11. Significant associations between gene polymorphisms and fibrotic pleuropulmonary changes.

Phenotype	Gene	Polymorphism	β[1]	p-value	Reference
Interstitial lung fibrosis	*GSTT1*	deletion	-0.09	0.003	Paper I
	NLRP3	rs35829419	0.078	0.013	Paper V
Visceral pleural fibrosis	*TGFB1*	rs2241718	-0.66	0.044	Paper V
Pleural plaques					
Greatest thickness	*GSTM1*	deletion	-0.008	0.009	Paper I
	CARD8	rs2043211	0.076	0.015	Paper V
Calcification	*TIMP2*	rs2277698	0.063	0.037	Paper V

[1] Standardized coefficient

In the stratified analysis, the *GSTT1* null genotype was found to pose a three-fold risk for pathological fibrotic changes (OR 3.12, 95% CI 1.51–6.43) compared to the wild type genotype. Similarly, the carriage of at least one *NLRP3* rs35829419 variant A-allele was found to pose an

almost 2.5–fold risk for developing pathological fibrotic changes (OR 2.44, 95% CI 0.97–6.14) although this association was only borderline statistically significant (Table 12). In contrast, the carriage of at least one *TGFB1* rs2241718 variant allele was found to reduce the risk for visceral pleural fibrosis (OR 0.62, 95% CI 0.39–0.98) (Table 13).

The *GSTM1* null genotype was found to slightly elevate the risk for ≥ 5-mm thick pleural plaques (OR 1.36, 95% CI 1.03–1.80) compared to the wild type genotype (Table 14), and the carriage of at least one *TIMP2* rs2277698 variant A-allele posed an almost two-fold risk for a high calcification degree in the pleural plaques (OR 1.90, 95% CI 1.09–3.33) (Table 15).

When the haplotypes were studied, the *TGFB1* rs1800469–rs1800470 haplotype was found to be associated with the calcification degree of pleural plaques (p = 0.035) (data not shown). In stratified analysis, the GC and AT haplotypes were found to increase the risk for calcification of the pleural plaques with ORs of 1.52 (95% CI 1.09–2.11) and 1.95 (95% CI 1.18–3.22), respectively, compared to the most common haplotype GT. The risks did not notably differ between the mild and high degree of calcification (Table 16).

Table 12. Distribution of *GSTT1* and *NLRP3* rs35829419 genotypes according to the existence and severity of interstitial lung fibrosis.

Genotype	No changes[1]	Any changes	OR (95% CI)	Subnormal changes	OR (95% CI)	Pathological changes	OR (95% CI)
GSTT1							
null	198 (89.2)	681 (86.6)	1.0	611 (87.9)	1.0	70 (76.9)	1.0
present	24 (10.8)	105 (13.4)	1.35 (0.83–2.18)	84 (12.1)	1.20 (0.73–1.95)	21 (23.1)	3.12 (1.51–6.43)
NLRP3							
Gln705Gln	182 (92.4)	661 (88.8)	1.0	589 (89.9)	1.0	72 (87.8)	1.0
Gln705Lys/ Lys705Lys	15 (7.6)	83 (11.2)	1.57 (0.87–2.82)	73 (11.0)	1.47 (0.82–2.67)	10 (12.2)	2.44 (0.97–6.14)

Data are presented as n (%), unless otherwise stated
[1] Reference category

Table 13. Distribution of *TGFB1* rs2277698 genotypes according to the existence of visceral pleural fibrosis.

Genotype	No changes[1]	Any changes	OR (95% CI)
TGFB1			
G/G	577 (72.5)	104 (80.6)	1.0
G/A or A/A	219 (27.5)	25 (19.4)	0.62 (0.39–0.98)

Data are presented as n (%), unless otherwise stated
[1] Reference category

Table 14. Distribution of *GSTM1* genotypes according to the thickness of pleural plaques.

Genotype	Greatest thickness		
	< 5 mm[1]	≥ 5 mm	OR (95% CI)
GSTM1			
null	181 (58.2)	353 (50.8)	1.0
present	130 (41.8)	342 (49.2)	1.36 (1.03–1.80)

Data are presented as n (%), unless otherwise stated
[1] Reference category

Table 15. Distribution of *TIMP2* rs2277698 genotypes according to the calcification degree of pleural plaques.

Genotype	No calcification[1]	Calcification	OR (95% CI)	Mild calcification	OR (95% CI)	High calcification	OR (95% CI)
TIMP2							
G853G	107 (83.6)	617 (77.1)	1.0	238 (79.1)	1.0	379 (76.0)	1.0
G853A/ A853A	21 (16.4)	183 (22.9)	1.58 (0.95–2.65)	63 (20.9)	1.36 (0.78–2.38)	120 (24.0)	1.90 (1.09–3.33)

Data are presented as n (%), unless otherwise stated
[1] Reference category

Table 16. Distribution of *TGFβ* haplotypes according to the calcification degree of pleural plaques.

TGFB1 Haplotype	No calcification[1]	Calcification	OR (95% CI)	Mild calcification	OR (95% CI)	High Calcification	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 (1.09–2.11)	165 (27.0)	1.42 (0.99–2.03)	289 (28.7)	1.49 (1.04–2.14)
AT	20 (7.7)	214 (13.2)	1.95 (1.18–3.22)	77 (12.6)	1.86 (1.09–3.16)	137 (13.6)	1.96 (1.15–3.36)
AC	5 (1.9)	25 (1.5)	0.96 (0.35–2.64)	11 (1.8)	1.13 (0.38–3.4)	14 (1.4)	0.66 (0.21–2.01)

Data are presented as number of chromosomes n (%), unless otherwise stated
[1] Reference category

5.5 Genetic predisposition to pulmonary emphysema

The significant associations between studied polymorphisms and emphysematous changes are shown in Table 17. Among construction workers, the *GSTT1* deletion polymorphism and *EPHX1* rs1051740 SNP were found to be associated with overall (p = 0.008 and p = 0.007, respectively), paraseptal (p = 0.015 and p = 0.039), panlobular (p = 0.031 and p = 0.013), and bullae-type emphysematous changes (p = 0.045 and p = 0.003). In addition, *TIMP2* rs2277698 SNP was found to be associated with overall (p = 0.022) and paraseptal emphysema (p = 0.010), *MMP9* rs3918242 SNP to centrilobular emphysema (p = 0.008), *TNF* rs1800629 SNP to paraseptal emphysema (p = 0.017), and *SERPINE2* rs729631 SNP to panlobular emphysema (p = 0.003) (Table 17).

Table 17. Significant associations between gene polymorphisms and emphysematous changes.

Phenotype	Gene	Polymorphism	β[1]	p-value	Reference
Emphysema, all	*GSTT1*	deletion	-0.080	0.008	Paper II
	EPHX1	rs1051740	-0.082	0.007	Paper II
	TIMP2	rs2277698	0.071	0.022	Paper IV
Centrilobular	*TGFB1*	rs2241718	-0.071	0.022	Paper IV
	MMP9	rs3918242	-0.082	0.008	Paper IV
Paraseptal	*GSTT1*	deletion	-0.075	0.015	Paper II
	EPHX1	rs1051740	-0.064	0.039	Paper II
	TNF	rs1800629	0.075	0.017	Paper IV
	TIMP2	rs2277698	0.081	0.010	Paper IV
Panlobular	*GSTT1*	deletion	-0.067	0.031	Paper II
	EPHX1	rs1051740	-0.077	0.013	Paper II
	SERPINE2	rs729631	0.094	0.003	Paper III
Bullae	*GSTT1*	deletion	-0.063	0.045	Paper II
	EPHX1	rs1051740	-0.093	0.003	Paper II

[1] Standardized coefficient

The associations between *EPHX1* rs1051740 SNP and emphysema did not appear in the stratified analysis. Instead, the *GSTT1* null genotype was found to pose a two-fold overall risk for emphysema (OR 2.01, 95% CI 1.33–3.03). When the emphysema subtypes were examined separately, the ORs were 2.05 for centrilobular (95% CI 1.31–3.20), 2.52 for paraseptal (95% CI 1.60–3.97), 1.73 for panlobular (95% CI 1.10–2.74), and 2.43 for bullae-type changes (95% CI 1.51–3.91). Furthermore, the carriers of the *GSTT1* null genotype had an almost four-fold risk (OR 3.70, 95% CI 2.15–6.36) for developing pathological changes compared to the subjects who possessed the gene. The risks for subnormal or pathological changes did not greatly differ from the risk of overall changes in the different emphysema subgroups (Table 18).

Similarly to *EPHX1* rs1051740 SNP, no association between *TIMP2* rs2277698 SNP and overall emphysema emerged in the stratified analysis. Instead, the carriage of at least one *TIMP2* rs2277698 variant A-allele was found to pose a two-fold risk for pathological paraseptal emphysema (OR 1.94, 95% CI 1.14–3.30). In addition, the carriage of at least one *TNF* rs1800629 variant A-allele was found to pose a two-fold risk for overall (OR 2.03, 95% CI 1.38–2.98), subnormal (OR 2.12, 95% CI 1.30–3.48), and pathological (OR 2.10, 95% CI 1.24–3.56) paraseptal emphysema. In contrast, the carriage of at least one *TGFB1* rs2241712 variant A-allele was found to halve the risk for pathological centrilobular emphysema (OR 0.53, 95% CI 0.30–0.90), as did the carriage of at least one *MMP9* rs3918242 variant T-allele (OR 0.51, 95% CI 0.30–0.86) (Table 18). In combination, the variant allele genotypes of *TGFB1* rs2241718 and *MMP9* rs3918242 loci were found to reduce the risk of centrilobular emphysema to one fifth compared to the wild type genotypes (OR 0.22, CI 95% 0.08–0.61) (Table 19). No other gene-gene interactions were observed.

The homozygous variant of *SERPINE2* rs729631 SNP was found to pose an over two-fold risk for developing panlobular emphysema (OR 2.22, 95% CI 1.05–4.72). The risk was slightly increased also for the heterozygotes (OR 1.66, 95% CI 1.15–2.38), and it appeared to be mainly attributable to the pathological emphysematous changes; the risk was over two-fold for heterozygotes (OR 2.19, 95% CI 1.23–3.91) and over four-fold for homozygotes (OR 4.37, 95% CI 1.61–11.86) as compared to subjects with the wild type genotype (Table 18).

In the haplotype analysis, the *SERPINE2* rs729631–rs840088 haplotype was found to be associated with panlobular emphysema ($p = 0.014$) (data not shown). In stratified analysis, the CC-haplotype (variant allele for rs729631, wild type allele for rs840088) showed almost a 1.5-fold risk for overall panlobular emphysema (OR 1.41, 95% CI 1.04–1.92) and an over two-fold risk for pathological panlobular changes (OR 2.23, 95% CI 1.41–3.54) in comparison to the most common haplotype with the wild type allele in both SNPs (GC). The haplotype with a variant allele for both SNPs (CT) showed an almost four-fold risk for overall panlobular changes (OR 3.72, 95% CI 1.56–8.90), and subnormal panlobular changes (OR 3.98, 95% CI 1.55–10.20) in comparison to the most common haplotype (GC) (Table 20).

In addition, the *TGFB1* haplotype (rs1800469–rs1800470) was found to be associated with centrilobular emphysema ($p = 0.018$) (data not shown). In the stratified analysis, the AT-haplotype was found to almost halve the risk for pathological centrilobular emphysema (OR 0.55, 95% CI 0.33–0.93) in comparison to the most common haplotype (GT) (Table 21).

Table 18. Distribution of *GSTT1, TGFB1, MMP9, TNF, TIMP2,* and *SERPINE2* genotypes according to the existence and severity of emphysematous changes.

Phenotype	Genotype	No changes[1]	Any changes	OR (95% CI)	Subnormal changes	OR (95% CI)	Pathological changes	OR (95% CI)
Emphysema, all	*GSTT1* deletion							
	null	562 (89.9)	301 (82.9)	1.0	207 (87.3)	1.0	94 (74.6)	1.0
	present	63 (10.1)	62 (17.1)	2.01 (1.33–3.03)	30 (12.7)	1.34 (0.82–2.19)	32 (25.4)	3.70 (2.15–6.36)
Centrilobular	*GSTT1* deletion							
	null	670 (89.1)	193 (81.8)	1.0	87 (81.3)	1.0	106 (82.2)	1.0
	present	82 (10.9)	43 (18.2)	2.05 (1.31–3.20)	20 (18.7)	1.92 (1.10–3.34)	23 (17.8)	1.93 (1.10–3.37)
	TGFB1 rs2241718							
	G/G	518 (72.8)	173 (77.6)	1.0	74 (71.2)	1.0	99 (83.2)	1.0
	G/A or A/A	194 (27.2)	50 (22.4)	0.76 (0.52–1.11)	30 (28.8)	1.08 (0.68–1.73)	20 (16.8)	0.53 (0.30–0.90)
	MMP9 rs3918242							
	C/C	519 (72.5)	171 (75.7)	1.0	74 (70.5)	1.0	97 (80.2)	1.0
	C/T or T/T	197 (27.5)	55 (24.3)	0.77 (0.53–1.12)	31 (29.5)	1.08 (0.68–1.72)	24 (19.8)	0.51 (0.30–0.86)
Paraseptal	*GSTT1* deletion							
	null	724 (89.3)	139 (78.5)	1.0	75 (78.1)	1.0	64 (79.0)	1.0
	present	87 (10.7)	38 (21.5)	2.52 (1.60–3.97)	21 (21.9)	2.69 (1.52–4.76)	17 (21.0)	2.33 (1.27–4.28)
	TNF rs1800629							
	G/G	605 (78.3)	111 (66.1)	1.0	61 (66.3)	1.0	50 (65.8)	1.0
	G/A or A/A	168 (21.7)	57 (33.9)	2.03 (1.38–2.98)	31 (33.7)	2.12 (1.30–3.48)	26 (34.2)	2.10 (1.24–3.56)

	TIMP2 rs2277698							
	G/G	609 (78.9)	123 (73.2)	1.0	72 (78.3)	1.0	51 (67.1)	1.0
	G/A or A/A	163 (21.1)	45 (26.8)	1.41 (0.94–2.13)	20 (21.7)	1.04 (0.59–1.81)	25 (32.9)	1.94 (1.14–3.30)
Panlobular	*GSTT1* deletion							
	null	723 (88.5)	140 (81.9)	1.0	90 (83.3)	1.0	50 (79.4)	1.0
	present	94 (11.5)	31 (18.1)	1.73 (1.10–2.74)	18 (16.7)	1.54 (0.88–2.66)	13 (20.6)	2.00 (1.02–3.91)
	SERPINE2 rs729631							
	G/G	500 (64.6)	85 (51.2)	1.0	60 (56.1)	1.0	25 (42.4)	1.0
	G/C	245 (31.7)	70 (42.2)	1.66 (1.15–2.38)	42 (39.3)	1.43 (0.93–2.20)	28 (47.4)	2.19 (1.23–3.91)
	C/C	29 (3.7)	11 (6.6)	2.22 (1.05–4.72)	5 (4.7)	1.43 (0.52–3.92)	6 (10.2)	4.37 (1.61–11.86)
Bullae	*GSTT1* deletion							
	null	766 (88.9)	97 (77.0)	1.0	70 (76.9)	1.0	27 (77.1)	1.0
	present	96 (11.1)	29 (23.0)	2.43 (1.51–3.91)	21 (23.1)	2.46 (1.44–4.22)	8 (22.9)	2.35 (1.03–5.39)

Data are presented as n (%), unless otherwise stated

[1] Reference category

Table 19. Distribution of *MMP9* rs3918242/*TGFB1* rs2241718 combined genotypes according to the existence and severity of centrilobular emphysema.

Genotype combination	No changes[1]	Any changes	OR (95% CI)	Subnormal changes	OR (95% CI)	Pathological changes	OR (95% CI)
Wild type–wild type[2]	375 (52.8)	130 (58.3)	1.0	52 (50.0)	1.0	78 (65.5)	1.0
Heterozygote/ homozygote[3]	281 (39.6)	81 (36.3)	0.83 (0.60–1.15)	43 (41.3)	1.07 (0.69–1.68)	38 (31.9)	0.66 (0.43–1.02)
Heterozygotes or homozygotes[4]	54 (7.6)	12 (5.4)	0.63 (0.36–1.09)	9 (8.7)	1.29 (0.68–2.46)	3 (2.5)	0.22 (0.08–0.61)

Data are presented as n (%), unless otherwise stated
[1] Reference category
[2] *MMP9* CC/*TGFB1* GG
[3] *MMP9* CT/*TGFB1* GG or *MMP9* CC/*TGFB1* GA
[4] *MMP9* CT/*TGFB1* GA or *MMP9* TT or *TGFB1* AA

Table 20. Distribution of *SERPINE2* haplotypes according to the existence and severity of panlobular emphysema.

SERPINE2 haplotype	No changes[1]	Any changes	OR (95% CI)	Subnormal changes	OR (95% CI)	Pathological changes	OR (95% CI)
rs729631–rs840088							
GC	834 (53.3)	165 (49.1)	1.0	117 (53.7)	1.0	48 (40.7)	1.0
GT	429 (27.4)	79 (23.5)	0.92 (0.68–1.24)	49 (22.5)	0.80 (0.56–1.14)	30 (25.4)	1.20 (0.74–1.94)
CC	289 (18.5)	83 (24.7)	1.41 (1.04–1.92)	45 (20.6)	1.09 (0.75–1.59)	38 (32.2)	2.23 (1.41–3.54)
CT	14 (0.9)	9 (2.7)	3.72 (1.56–8.90)	7 (3.2)	3.98 (1.55–10.20)	2 (1.7)	2.71 (0.58–12.59)

Data are presented as number of chromosomes n (%), unless otherwise stated
[1] Reference category

Table 21. Distribution of *TGFB1* haplotypes according to the existence and severity of centrilobular emphysema.

TGFB1 haplotype	No changes[1]	Any changes	OR (95% CI)	Subnormal changes	OR (95% CI)	Pathological changes	OR (95% CI)
rs1800469–rs1800470							
GT	834 (57.7)	283 (62.1)	1.0	125 (59.5)	1.0	158 (64.2)	1.0
GC	398 (27.5)	119 (26.1)	0.88 (0.68–1.14)	52 (24.8)	0.91 (0.64–1.30)	67 (27.2)	0.90 (0.65–1.26)
AT	187 (12.9)	50 (11.0)	0.79 (0.55–1.13)	30 (14.3)	1.10 (0.71–1.72)	20 (8.1)	0.55 (0.33–0.93)
AC	27 (1.9)	4 (0.9)	0.44 (0.15–1.32)	3 (1.4)	0.72 (0.21–2.44)	1 (0.4)	0.22 (0.03–1.70)

Data are presented as number of chromosomes n (%), unless otherwise stated
[1] Reference category

5.6 Genetic association to lung function

Significant associations between studied polymorphisms and lung function among all construction workers are shown in Table 22. The *CARD8* rs2043211 SNP was found to be associated with FEV_1, FVC, and MEF50 ($p = 0.022$, $p = 0.047$, and $p = 0.032$, respectively). The *GSTT1* deletion polymorphism was associated with DL_{CO} and DL_{CO}/VA ($p = 0.021$ and $p = 0.002$, respectively) as was the *NAT2* rs1041983 SNP ($p = 0.007$ and $p = 0.006$, respectively). The *GSTM3* rs1332018 SNP was associated with FEV_1/FVC and MEF50 ($p = 0.010$ and $p = 0.032$, respectively), and *EPHX1* rs1051740 and *TIMP2* rs2277698 SNPs with MEF50 ($p = 0.008$ and $p = 0.013$, respectively) (Table 22). No associations were found between the studied polymorphisms and TLC.

Table 22. Significant associations between gene polymorphisms and lung function.

Phenotype	Gene	Polymorphism	β[1]	p-value	Reference
FEV_1	*CARD8*	rs2043211	0.073	0.022	Unpublished[2]
FVC	*CARD8*	rs2043211	0.065	0.047	Unpublished[2]
FEV_1/FVC	*GSTM3*	rs1332018	-0.077	0.010	Paper II
	TIMP2	rs2277698	-0.066	0.035	Paper IV
DL_{CO}	*GSTT1*	deletion	0.062	0.021	Paper I
	NAT2	rs1041983	-0.075	0.007	Paper I
DL_{CO}/VA	*GSTT1*	deletion	0.095	0.002	Paper I
	NAT2	rs1041983	-0.086	0.006	Paper I
MEF50	*TIMP2*	rs2277698	-0.084	0.008	Paper IV
	GSTM3	rs1332018	-0.067	0.032	Unpublished[2]
	EPHX1	rs1051740	0.078	0.013	Unpublished[2]
	CARD8	rs2043211	0.067	0.032	Unpublished[2]

[1] Standardized coefficient
[2] Adjusted with pack-years and years of asbestos exposure

The associations between *CARD8* rs2043211 SNP and FEV_1, FVC, and MEF50 did not emerge in the stratified analysis. Instead, the *GSTT1* null genotype was found to pose a 1.8-fold risk (OR 1.77, 95% CI 1.06–2.95, $p = 0.029$) for decreased DL_{CO} and 2.4-fold risk (OR 2.37, 95% CI 1.33–4.23, $p = 0.003$) for decreased DL_{CO}/VA (Table 23); the average values were 87.2±21.5 (DL_{CO}) and 93.2±20.7 (DL_{CO}/VA) among subjects with *GSTT1* null genotype, and 91.6±20.0 and 99.0±18.0 among subjects with the wild type genotype ($p = 0.030$ and $p = 0.002$, respectively) (unpublished data). When only subjects with fibrotic changes were considered, the risk for decreased pulmonary diffusing capacity was a somewhat higher; the ORs were 1.88 (95% CI 1.10–3.21) for DL_{CO} and 2.81 (95% CI 1.55–5.12) for DL_{CO}/VA (Paper I). In addition, the DL_{CO} and DL_{CO}/VA values were significantly lower ($p = 0.004$ and $p = 0.003$, respectively) among subjects homozygous with *NAT2* rs1041983 variant T-allele (83.8±18.2 and 92.6±17.1) compared to subjects with the wild type genotype (92.6±20.6 and 99.9±18.0, respectively) (unpublished data).

Table 23. Distribution of *GSTT1* genotypes according to pulmonary diffusing capacity.

***GSTT1* genotype**	**DL_{CO}**		
	< 74% pred	**≥ 74% pred**	**OR (95% CI)**
null	145 (81.9)	686 (87.8)	1.0
present	32 (18.1)	95 (12.2)	1.77 (1.06–2.95)
	DL_{CO}/VA		
	< 74% pred	**≥ 74% pred**	**OR (95% CI)**
null	72 (77.4)	761 (87.8)	1.0
present	21 (22.6)	106 (12.2)	2.37 (1.33–4.23)

Data are presented as n (%), unless otherwise stated

No associations between *EPHX1* rs1051740 SNP and MEF50 were detected in the stratified analysis. Instead, subjects with at least one *TIMP2* rs2277698 variant A-allele had a significantly ($p = 0.011$) lower

MEF50 (61.0 ±27.0) than subjects with the wild type G/G genotype (67.0±29.9). Similarly, the FEV_1/FVC-ratio tended to be lower (p = 0.136) in those subjects who were homozygous for the *TIMP2* rs2277698 variant A-allele (89.1±9.3) when they were compared to subjects with the wild type G/G genotype (93.8±12.7) (Paper IV). In addition, subjects homozygous for the *GSTM3* rs1332018 variant C-allele had significantly (p = 0.049) lower MEF50 (62.0±26.7) and somewhat (p = 0.107) lower FEV_1/FVC-ratio (92.2±11.8) than subjects with wild type A/A genotype (68.6±30.8 and 94.8±12.5, respectively) (unpublished data).

In Paper III, lung function was analysed also among subjects with panlobular emphysema. The FEV_1 appeared to decrease in parallel to the number of the variant C alleles in *SERPINE2* rs729631 locus (GG 77.8±22.5, GC 73.7±23.0 and CC 70.2±19.0). The same trend was seen for FVC, which was significantly (p = 0.023) lower with homozygous CC variants in comparison to the carriers of GG genotype (GG 94.1.8±17.2, GC 87.5±20.1, CC 81.3±16.6).

6 DISCUSSION

Tobacco smoking is a major global health burden being responsible for several millions of deaths yearly all over the world. It has been shown to increase the risk for different malignancies, and to promote the development of cardiovascular and respiratory disorders [3].

Since there are currently national bans and restrictions in place, the widespread use of asbestos has passed into history. However, the previous exposures still represent a significant health concern due to the huge amounts of asbestos mined and used in different insulation and construction purposes since the early 1900s and the long latency period of most asbestos associated diseases [2].

Both tobacco smoke and asbestos fibers enter the body mainly by inhalation, which makes the respiratory tract especially vulnerable to their toxic effects. In the lungs, foreign compounds may induce oxidative stress, alter the protease-antiprotease balance, induce innate and adaptive immune responses, and create the conditions of persistent inflammation leading eventually to lung injury. However, the type and severity of lung injury vary greatly between individuals, even with similar exposure histories. These differences are believed to originate from the complex interplay between genetic, epigenetic, environmental, and life course factors [13].

6.1 State of the association studies investigating asbestos and smoking related non-malignant lung diseases

The genetic background of COPD has been extensively studied with hundreds of publications including also large meta-analysis and GWA-

studies. These investigations have identified more than a hundred genes with positive associations to COPD, COPD related traits, and lung function [265], probably due to the high prevalence of the disorder and relatively simple diagnostic procedures. The genetics of pulmonary emphysema has also attracted considerable interest, not least because of the influence of the hereditary AAT-deficiency. Nonetheless, the high cost and inconvenience of the imaging methods have limited the availability of the study material, and only a handful of association studies have been published examining CT-assessed emphysema, emphysema severity, or different emphysema subtypes.

The factors that determine individual susceptibility to asbestos associated non-malignant lung diseases have been rather poorly studied. There are, however, a few association studies on asbestosis; these have mainly concentrated on the genes participating in the metabolism of xenobiotics and products of oxidative stress.

6.2 Xenobiotic metabolizing enzyme genes

6.2.1 *GSTM1*

The gene deletion of the most widely studied member of the GST family, *GSTM1*, has been associated with an increased risk of several malignant and non-malignant conditions, including COPD [118, 119]. Although several reports have failed to confirm this finding [140, 145, 266, 267], the results from recent meta-analyses have indicated that the *GSTM1* null genotype could pose a slightly elevated risk for COPD in both Asian and Caucasian populations [106, 108, 109]. The lack of *GSTM1* gene has also been proposed to increase the risk for pulmonary emphysema [120].

In the current study, no significant associations were found between the *GSTM1* polymorphism and neither emphysema nor a lung function decline. This is in contrast to a previous report linking *GSTM1* genotype to the development of emphysema [120]. However, that finding was based on a very small sample size (43 smoking cases and 179 controls without known smoking status), and it is unclear whether any potential confounders were taken into account in the statistical analysis. Moreover, two subsequent studies have failed to associate the *GSTM1* genotypes

to CT-defined emphysema and emphysema distribution [141, 142]. It therefore seems that even though *GSTM1* deficiency may increase the risk of COPD, this is not attributable to the emphysema component of the disease.

The present study did not reveal any significant associations between the *GSTM1* deletion polymorphism and asbestos induced fibrotic changes or pleural plaques. This contrasts both the previous finding from our research group that the *GSTM1* deficiency in combination with *NAT2* slow acetylator genotype increases the risk for asbestos associated non-malignant lung diseases [102], and the observation that the *GSTM1* deletion alone elevates the risk for asbestos related parenchymal changes [101]. The findings of the current study are, however, in agreement with three other reports which also failed to detect any associations between *GSTM1* and asbestos-related abnormalities [100, 104, 105].

The discrepancy between the different studies may partly be due to the different inclusion criteria for cases; in some of the previous studies all non-malignant disorders were grouped together [102, 105] while in the current study, fibrotic changes and pleural plaques were studied separately. The duration and intensity of the asbestos exposure, which are very hard to assess, have also been inconsistently reported in the published reports. In addition, most of the previous studies were rather small, with less than 100 cases included [100–102, 104].

6.2.2 *GSTT1*

Similarly to the *GSTM1* polymorphism, the *GSTT1* deletion polymorphism has been widely studied in relation to lung diseases, and a deficiency of the gene has been associated to increased risk of COPD and lung function decline [121, 266–269]. The recent meta-analyses, however, have indicated that the *GSTT1* deficiency does not significantly affect the risk of COPD [106, 108, 109, 113], and it has also been failed to be associated to emphysema [141]. On the other hand, *GSTT1* deficiency has been postulated to protect against the development of asbestosis [105].

In the current study, significant associations were found between the *GSTT1* genotype and pulmonary emphysema, interstitial lung fibrosis, and diffusing capacity for carbon monoxide (DL_{CO} and DL_{CO}/VA); these

associations were significant in both of the study cohorts. In the stratified analysis, deletion of the *GSTT1* gene was found to increase the risk for severe fibrotic changes, severe emphysematous changes of any type, and decreased gas transfer capacity.

Although the present findings do not agree with the results from previous studies, they are consistent with the functional consequences of the associated polymorphism; deletion of an XME gene would be anticipated to lead to a reduced detoxification capacity and the accumulation of oxidative agents, which, in turn, could evoke a lung tissue injury and promote the development of pulmonary disorders and functional impairment.

The main difference between the present and previous studies is the sample size; our study population comprised of over one thousand construction workers, 351 of whom had emphysematous changes of different types and 750 of whom exhibited various degrees of interstitial fibrosis. In earlier studies the amount of emphysema and fibrosis patients has been much smaller, varying from a few tens to a couple of hundred [100–105, 120]. Consequently, the elevated risk which was detected only for severe changes in the present study could well have gone unrecognized in the previous studies with much smaller sample sizes, where stratification was not appropriate.

Since a reduced diffusing capacity is a characteristic of both emphysema and asbestosis, the associations found in the present study clearly support each other suggesting that the absence of GSTT1 activity might lead to an increase in many kinds of structural changes in the lungs, simultaneously or separately, depending probably on the type and duration of the exposures and their interaction with other inherited factors. The exact mechanism of this process and the genes involved, however, remain to be clarified.

6.2.3 *GSTP1*

The *GSTP1* gene has also been fairly extensively studied in relation to lung diseases; the functional Ile105Val polymorphism in *GSTP1* has previously been associated with COPD, emphysema distribution, asbestosis, and impairment of lung function [104, 121, 127, 128, 142, 268, 269]. Results from recent meta-analyses are, however, rather inconsistent;

in one study, the Val-allele was found to be a risk factor for COPD in Caucasians but protective among Asian populations [108]. A subsequent study supported the disadvantageous effect of Val-allele among Caucasians, but disputed its protective influence among Asians [111], and in a third study the Val-allele was found to be protective among a mixed Caucasian/Asian population [110]. Moreover, yet another meta-analysis failed to detect any effects for *GSTP1* Ile105Val polymorphism on the development of COPD [109].

In the current study, no significant associations were found between *GSTP1* Ile105Val polymorphism and non-malignant pulmonary or pleural changes. This contrasts with the results of some of the earlier studies, most of which were carried out in smaller research frames than the current study. For instance, only 61 asbestos exposed persons were included in one of these studies [104]. In addition, the contradictory results from meta-analyses indicate that the relationship between *GSTP1* Ile105Val polymorphism and COPD is far from being straightforward; although racial differences may partly explain the discrepancies, there most probably are other, yet unidentified genetic and/or epigenetic factors contributing to the pathogenesis of COPD along with *GSTP1*. It is clear that better characterized study populations would be valuable if one wishes to reveal the exact phenotypes or COPD related traits that could be attributed to changes in the function of GSTP1.

6.2.4 *GSTM3*

The *GSTM3* gene has been much less studied in terms of pulmonary disorders than the other *GST* genes, and its polymorphisms have not been associated to COPD or asbestos related lung diseases. However, in the current study, a significant association was found between the *GSTM3* promoter polymorphism (rs1332018), FEV_1/FVC-ratio, and MEF50; the MEF50 was significantly reduced and FEV_1/FVC-ratio somewhat, but not statistically significantly, reduced among individuals with homozygous rs1332018 variant allele genotype. Both reduced MEF50 and FEV_1/FVC-ratio indicate peripheral obstruction typical of smoking associated COPD and emphysema [270].

Interestingly, one previous report has indicated a role for *GSTM3* expression in emphysema severity; the gene was found to be over-expressed

in the lungs of former heavy-smokers with a low FEV_1/FVC-ratio [123]. In another large cohort of children, a certain *GSTM3* haplotype and a three base-pair deletion (rs1799735) were found to be associated with a decrease in the growth for maximum midexpiratory flow rate (MMEF) [271]. In the same cohort, the deletion polymorphism rs1799735 was found to exist in tight linkage with the promoter polymorphism rs1332018, which was associated with altered MEF50 in the current study.

The rs1332018 SNP has been postulated to affect the *GSTM3* expression [122], and by using the eQTL Browser [264], we found a microarray study supporting this view [272]. Hence, based on the results from the current and previous works, it seems plausible that the variant genotype of *GSTM3* promoter polymorphism, associated with decreased MEF50 and FEV_1/FVC-ratio in the present study, increases the expression of the gene in the lungs, leading to increased metabolism of certain tobacco smoke components. In this way it could contribute to the development of pulmonary obstruction and possibly to emphysema.

6.2.5 *EPHX1*

The two functional *EPHX1* polymorphisms, Tyr113His (rs1051740) and His139Arg (rs2234922), predicting slow or fast EPHX1 activity, have been widely studied in relation to malignant and non-malignant lung diseases [273–275]. The slow activity related His-alleles and a putative slow EPHX1 activity phenotype have previously been associated to the development of COPD, emphysema, and impairment of lung function [118, 128, 137–143]. Other *EPHX1* polymorphisms have also been connected to several COPD-related traits including emphysema distribution and airway wall phenotype [129, 142, 144]. However, there are several reports which have not found any association between *EPHX1* genotypes and COPD, emphysema and/or lung function [145, 146, 148, 150, 151]. In addition, the results from previous meta-analyses are rather conflicting; two studies [108, 109] could not confirm the associations between *EPHX1* polymorphisms and COPD, while two other studies [107, 112] suggested that the risk for COPD might be marginally increased by the His-alleles.

In the current study, significant associations were observed between *EPHX1* rs1051740 SNP (Tyr113His), MEF50, and emphysema. This is essentially in agreement with previous positive association studies concerning emphysema [129, 141, 142]. However, in the stratified analysis none of the initial associations emerged and therefore the role of *EPHX1* Tyr113His polymorphism in the development of pulmonary emphysema and obstruction remains tentative until replicated in an independent study cohort.

The EPHX1 slow activity phenotype has also been associated with the development of pleural plaques among asbestos associated workers [104]. Since almost all of our study subjects had pleural plaques, we were only able to examine the extent, greatest thickness, and calcification of pleural plaques instead of their existence. However, none of these measures were found to be associated with *EPHX1* genotypes or phenotypes.

6.2.6 *NAT2*

Although genetic variation of *NAT2* has been extensively studied in relation to lung cancer [276], much less data exists on *NAT2* polymorphisms and chronic pulmonary disorders. Our research group has previously found an association between the *NAT2* slow acetylator genotype and non-malignant pulmonary disorders among asbestos exposed workers; the risk of having non-malignant changes was over four-fold for patients with a combination of *NAT2* slow acetylator genotype and *GSTM1* null genotype [102].

Unfortunately, it was not possible to confirm the previous findings in the current study. As discussed in the context of *GSTM1*, this discrepancy could be due to the different inclusion criteria for cases; in the previous study all non-malignant disorders were grouped together, while in the current study fibrotic changes and pleural plaques were studied separately. In addition, the incoherence between the previous and current findings could be partly explained by differences in the exposure level and/or the asbestos type to which the subjects were exposed; in the earlier report, the workers were all heavily exposed to asbestos, and the exposure was confirmed with a lung fiber burden measurement, whereas in the current study, the workers were exposed to various levels of asbestos and no fiber measurements were performed. The sample size was also remarkably smaller in the earlier study.

The slow *NAT2* acetylator genotype has also been previously associated with the development of COPD [133]. In the current study, DL_{CO} and DL_{CO}/VA values were found to be significantly lower among subjects homozygous with *NAT2* rs1041983 variant allele compared to subjects with the wild type genotype. Although the rs1041983 SNP defines the NAT2 acetylator phenotype together with rs45532639, it was not possible to detect any associations between the acetylator phenotype and emphysema or lung function. Hence, there is inadequate evidence to support the involvement of *NAT2* in the development of asbestos and tobacco smoke related non-malignant pulmonary disorders until this association can be confirmed in an independent study population.

6.3 Genes involved in protease-antiprotease balance

6.3.1 *SERPINE2*

SERPINE2 was identified as a COPD candidate gene in 2006 by gene expression analysis [156]. Subsequently, several *SERPINE2* SNPs have been associated with COPD in two large family and case-control -based Caucasian populations [156, 162] and in two Asian populations [163, 164]. There are, however, one Caucasian [165] and two Asian [166, 167] studies that have failed to replicate these findings. Interestingly, certain *SERPINE2* polymorphisms were recently associated to pulmonary emphysema [129, 168].

In the present study, the *SERPINE2* rs729631 SNP and the rs729631–rs840088 haplotype were found to be associated with panlobular emphysema; these associations were significant in both of the study cohorts. Stratified analysis revealed a two-fold risk for panlobular emphysema for the homozygous variant genotypes of *SERPINE2* rs729631 SNP, and when only pathological changes were considered, the risk increased to as high as four-fold.

These findings are in agreement with the two previous reports concerning *SERPINE2* genotypes and emphysema [129, 168]. Interestingly, also the study subjects of the first association study [156] were subsequently declared as suffering from emphysema [277].

The converging results from the three previous reports [129, 156, 168] and the current study indicate that the disease phenotype behind the observed association between *SERPINE2* and COPD might in fact be structural emphysema. This would also explain the discrepant results from some of the earlier studies that were not able to find associations between *SERPINE2* genotypes and COPD among patients with unknown emphysema status [165–167]. In this regard, the HRCT evaluation becomes superior over lung function measurements; emphysema patients with normal lung function may be missed unless diffusing capacity measurements or HRCT are performed.

The role of *SERPINE2* polymorphisms in the development of different emphysema subtypes has not been assessed before. Hence, the present study was the first to demonstrate an association between *SERPINE2* genotypes and panlobular emphysema. Intriguingly, also a deficiency of AAT, encoded by the *SERPINA1* gene, predisposes to early onset emphysema of panlobular type [154]. The role of *SERPINA1* in the development of emphysema is much more evident than that of *SERPINE2*; AAT has a major role in inactivating neutrophil elastase and in that way protecting lung tissue from damage [155], while PN1, the protein product of *SERPINE2*, is known to be mainly involved in coagulation and fibrinolysis with trypsin, thrombin, plasmin, and urokinase as the major substrates [157, 158]. Recent proteolytic analysis has, however, revealed a link between metalloproteinases and PN1; MMP9 has been shown to regulate the degradation of PN1 by proteolytic cleavage [278]. In the same study, the down-regulation of MMP9 was shown to lead to a dramatic accumulation of PN1 in prostate carcinoma cells, and mutations in the *SERPINE2* gene near MMP9 cleavage sites were shown to make PN1 more resistant to MMP9 dependent degradation [278]. Based on the observations in the current study and the outcomes of the proteolytic analysis [278], it seems very likely that PN1 has a role in inhibiting as yet unidentified protease(s) involved in the development of emphysema, especially the panlobular type.

Since tobacco smoke, which is an essential factor in our study, increases the number of MMP9 secreting inflammatory cells in the airways and lung parenchyma [152], smoking could result in enhanced degradation of PN1 leading to accumulation of proteases that break down the extracellular matrix. In the case of mutations that increase

the expression of *SERPINE2* gene or make the protein product more resistant to degradation, the actions of these proteases would be more efficiently inhibited.

In an attempt to further unravel the ambiguous link between genetic variation of *SERPINE2* and destruction of lung parenchyma, a microarray database search was undertaken to locate genes that are co-expressed with *SERPINE2* by using the MADCOW-tool [262]. The genes in the resulting list were then classified by the DAVID 6.7 functional annotation tool [263]. The most enriched clusters included several genes related to glycosylation, extracellular matrix, extracellular region, and cell adhesion (data not shown). This supports the working hypothesis that SERPINE2 may, directly or via interactions with other proteins, be involved in the extracellular matrix organization.

The *SERPINE2* SNPs examined in the current study are not located in the MMP9 cleavage site or in any other functional regions of the protein product. Instead, they are intronic variants which have no known functional consequences, and which have been selected based on previous association studies. The F-SNP program [260], connected to the main databases, predicts a role for the rs729631 SNP in transcriptional regulation, but finds no regulatory role for the rs840088 SNP. However, by using the eQTL Browser [264], it was possible to identify a microarray study associating the rs840088 SNP with the expression level of *SERPINE2* gene [279]. Although it is still possible that the associated SNPs are in linkage with other, yet unidentified functional variants in *SERPINE2* or in completely different genes, the rs729631 and rs840088 SNPs showed tight linkage ($r^2 > 0.8$) only to other *SERPINE2* SNPs (1000 Genomes Population; 1KGP). Hence, these intronic SNPs could be considered as potential causal variants modifying the risk for pulmonary emphysema.

6.3.2 *MMP1, MMP9,* and *MMP12*

MMPs, that are capable of degrading elastin and other matrix macromolecules and thereby participating in the protease-antiprotease balance, have been abundantly examined concerning COPD and emphysema [153, 170]. Animal experiments have shown that overexpression of *MMP1* and *MMP9* predispose to emphysematous changes, and that mice lacking the

MMP12 gene were protected from emphysema despite of a long term exposure to tobacco smoke [172–174].

In the current study, a significant association between the *MMP9* rs3918242 variant T-allele and a lowered proneness to pathological centrilobular emphysema was detected. This is in agreement with a recent Korean study which reported that the T-allele was protective against COPD [187], but contrasts to some of the earlier findings suggesting the T-allele as a risk factor for COPD and emphysema [178, 181, 183]. There are also reports failing to find any associations between *MMP9* polymorphisms and COPD and/or lung function [180, 188].

In addition to the discrepant observations from the original studies, the results from recent meta-analyses concerning *MMP9* rs3918242 SNP appear quite inconsistent; the first meta-analysis identified an increased risk for the variant T-allele, whereas the second analysis found a decreased risk of COPD for this allele in ethnically mixed populations, but neither of these associations were statistically significant [108, 109]. The most recent meta-analysis, in which separate analysis were performed for Caucasian and Asian populations, found the T-allele to be a significant risk factor for COPD only in Asian populations by using dominant model [194]. When all the sub-studies with only healthy smokers as controls were included, the risk was even more obvious.

As for the *MMP1* and *MMP12*, no significant associations were detected between their polymorphisms and emphysema or lung function. This contrasts with the previous works reporting associations between *MMP1* and *MMP12* polymorphisms and COPD, emphysema, lung function, and gas transfer capacity [180, 185, 186, 188, 189]. Similarly to the results of the present study, however, some earlier reports have failed to associate *MMP1* [184, 187, 188] or *MMP12* [184, 185, 187] to COPD and/or lung function.

The somewhat contradictory results from the *MMP* association studies may partly originate from ethnic differences, lack of power due to small sample sizes, or phenotypic heterogeneity of COPD. Other unidentified genetic and/or epigenetic factors may also confound the association, which makes it hard to clarify the role of *MMP9* rs3918242 SNP and other *MMP* polymorphisms in the development of pulmonary emphysema from the currently existing data.

In the present study, no associations were observed between the studied *MMP* genes and fibrotic changes. Although differential expression of MMP1, MMP9, and MMP12 have been implicated in the development of fibrotic lung diseases [175, 176], the genetic association studies concerning *MMP*s and fibrosis are scarce; one report has described an association between *MMP1* promoter polymorphism and IPF [280], but no previous association study has examined the *MMP* polymorphisms in relation to asbestos associated non-malignant diseases. Hence, the differential expression of MMPs detected in asbestos associated fibrosis [171] is probably mainly attributed to other factors than genetic variation of *MMP*s.

6.3.3 *TIMP2*

TIMPs are involved in tissue repair and remodeling and protease-antiprotease balance by binding MMPs and inhibiting their actions [169]. TIMP2, released by alveolar macrophages, can inhibit most of the MMPs including collagenases (MMP1) and gelatinases (MMP9). The TIMPs have been proposed to play a role in the development of pulmonary fibrosis and emphysema [153, 171], and certain *TIMP2* polymorphisms have been connected to COPD [179, 182].

In the present study, the *TIMP2* rs2277698 SNP was found to be associated with overall and paraseptal emphysema, FEV_1/FVC ratio, and MEF50. Stratified analysis revealed a two-fold risk for pathological paraseptal changes for individuals with at least one rs2277698 variant A-allele. In addition, FEV_1/FVC ratio tended to be lower among carriers of homozygous variant A-allele genotype, and MEF50 was significantly reduced among individuals with at least one variant A-allele.

The rs2277698 SNP is a synonymous base-substitution with unconfirmed functional consequences. Although it has previously been speculated to associate with a down-regulation of TIMP2 activity leading to matrix degradation and COPD [179], this has remained unconfirmed. Interestingly, the F-SNP program predicts that rs2277698 SNP is highly likely to be involved in splicing regulation [260]. Hypothetically, if the A-allele were to create an unstable or incomplete *TIMP2* message RNA, it would lead to deficient protein product, decreased MMP inhibition, and increased matrix degradation. This, again, could explain the pre-

sent findings indicating that the A-allele was a risk factor for paraseptal emphysema and peripheral obstruction (*i.e.,* decreased FEV_1/FVC ratio and MEF50) typical of smoking related emphysema.

An association was also found between *TIMP2* rs2277698 SNP and pleural thickenings; the carriage of rs2277698 variant A-allele was shown to predispose to high calcification degree of the thickenings. The relationship between TIMP2 and pleural plaques has not been explored before. Instead, results from animal experiments have indicated that the over-expression of TIMP2 could inhibit the development of atherosclerotic plaques and prevent plaque destabilization [281]. However, the mechanisms involved in the development of atherosclerotic plaques are probably sufficiently different from the development of pleural plaques to allow firm conclusions to be drawn.

There is, however, also the possibility that the causal variant behind paraseptal emphysema and peripheral obstruction is not the phenomenon studied here; the *TIMP2* rs2277698 SNP is in strong linkage (r^2 > 0.8 in 1KGP) with other *TIMP2* SNPs, some of which (rs9889410 and rs11654470) reside in an area predicted to alter the transcriptional regulation [260]. However, in 1KGP, SNPs from other genes are not strongly linked to the rs2277698 SNP. Based on these observations and the TIMP2 function, it is very likely that it is involved in the pathogenesis of emphysema.

6.4 Inflammation and innate immunity related genes

6.4.1 *TNF*

Both asbestos and tobacco smoke are known to induce TNF secretion from alveolar macrophages and other inflammatory cells [8, 11], which, in turn, enhances the expression of TGFB1 [196]. Together TNF and TGFB1 may provoke immune suppression and lung fibrogenesis, and participate in the down-regulation of collagen degradation through MMPs and TIMPs [197, 198]. *TNF* gene polymorphisms have previously been associated with the development of emphysema and asbestosis [199, 282], and these have been studied widely also in relation to COPD [108, 109, 190, 192, 193].

In the current study, no associations were found between *TNF* polymorphisms and asbestos related fibrosis. This could be due to phenotypic heterogeneity; in the previous study, a strict diagnosis of asbestosis was used, whereas in the current study fibrotic changes were used as such. In addition, since the control subjects of the previous study were not exposed to asbestos, the analyses were not adjusted for asbestos exposure level [199].

On the contrary, significant association was observed between the *TNF* rs1800629 SNP and paraseptal emphysema. Further analysis revealed a twofold risk for pathological paraseptal changes for individuals with at least one variant A-allele. This finding is in agreement with a recent meta-analysis with over 5500 COPD patients and controls [109]. However, meta-analyses stratifying the study population based on ethnicity seem to suggest that the risk for COPD is statistically significant only among Asian subjects [108, 192, 193].

Another insight supporting our finding is that the rs1800629 variant A-allele has been associated with enhanced TNF expression [283], which, in turn has been shown to induce emphysematous changes in mouse models [203, 284]. The possible mechanism explaining the association between TNF and paraseptal type of emphysema, however, remains to be clarified.

6.4.2 *TGFB1*

The *TGFB1* polymorphisms have previously been associated with asbestosis and emphysema [200, 205, 206], and several meta-analyses investigating the relationship between *TGFB1* and COPD have been performed [108, 109, 191].

In the present study, *TGFB1* rs2241718 SNP and a haplotype consisting of the rs1800469 and rs1800470 SNPs were found to be associated to centrilobular emphysema. Stratified analysis showed that the variant A-allele in rs2241718 locus and a haplotype consisting of rs1800469 variant A-allele and rs1800470 wild-type T-allele were protective against pathological centrilobular changes. Together with the *MMP9* rs3918242 variant T-allele the *TGFB1* rs2241718 variant A-allele reduced the risk of pathological centrilobular emphysema into fifth compared to the wild-type genotype.

The results from recent meta-analyses concerning the rs1800469 and rs1800470 SNPs and COPD are somewhat divergent; in two meta-analyses [108, 109] the variant allele of rs1800470 SNP was found to protect from COPD in ethnically mixed populations, while a third meta-analysis [191] failed to detect any effects for this allele. Conversely, in the third meta-analysis, the variant allele of rs1800469 was shown to increase the risk of COPD among Asian populations, while the two other meta-analyses failed to see any effect for it in mixed populations.

We also found the rs2241718 SNP to be associated with asbestos induced visceral pleural fibrosis, and two *TGFB1* haplotypes consisting of rs1800469 and rs1800470 SNPs to be associated with pleural plaque calcification. In stratified analysis, the variant allele of *TGFB1* rs2241718 SNP was found to confer protection against visceral pleural fibrosis.

The *TGFB1* rs2241718 SNP is located in the non-coding area near to the gene and there are no known functional consequences for this polymorphism. 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 serum levels of TGFB1 [285, 286]. Moreover, the F-SNP program predicts that the rs1800470 SNP might be involved in splicing regulation [260].

It is also possible that the causative variant resides outside the *TGFB1* gene; the studied *TGFB1* SNPs are in tight linkage with polymorphisms in *e.g., TMEM91*, *HNRNPUL1*, and *B9D2* genes ($r^2 > 0.9$ in 1KGP). However, since TGFB1 is a down-stream effector of NLRP3 mediated innate immunity response, and also involved in the regulation of protease-antiprotease balance, it could be speculated to play a role in the pathogenesis of lung diseases related to external exposure and inflammation.

Both TGFB1 and MMP9, genetic polymorphisms of which were found protective against centrilobular emphysema in the present study, can be secreted from AMs. AMs, in turn, are activated by cigarette smoke and found in increased numbers in airways and lung parenchyma of COPD patients [152]. Since centrilobular emphysema is the most common type of pulmonary emphysema and closely associated to cigarette smoke [86, 287], the effects of *TGFB1* and *MMP9* polymorphisms in the development of centrilobular disease could be mediated through macrophages via an interaction with cigarette smoke.

6.4.3 *NLRP3* and *CARD8*

There is growing evidence that inflammasomes are involved in the pathogenesis of chronic respiratory disorders, and there is also increasing interest in examining how the genes of innate immunity are related to these diseases [288]. The NLRP3 inflammasome complex, consisting of several components including NLRP3, PYCARD, and CARD8, can be activated by asbestos leading to IL-1β secretion [28]. This complex has been proposed to play a role in the development of fibrotic diseases [289], but so far, very few data exists on the association of inflammasome complex genes and pulmonary fibrosis.

The current study revealed a significant association between *NLRP3* rs35829419 SNP and asbestos induced interstitial lung fibrosis; the carriage of at least one variant A-allele was found to pose almost a 2.5-fold risk for pathological fibrotic changes compared to individuals with the wild type genotype. Moreover, a truncating polymorphism (C10X; rs2043211) in another member of the complex, *CARD8*, was associated with the greatest thickness of pleural plaques.

Both rs35829419 and rs2043211 SNPs have previously been linked to increased IL-1β production and severe inflammation [207]. The rs35829419 SNP changes amino acid (Q705K) in the NLRP3 protein, and there is recent evidence to suggest that it is a gain-of-function mutation leading to constantly active NLRP3 inflammasome and increased IL-1β levels [290]. This, again, may induce fibrosis and is therefore in agreement with our findings.

Interestingly, there is a recent study pointing to an association between the variant T-allele of another *NLRP3* SNP (rs1539019) and coal workers´ pneumoconiosis (CWP) [291]. CWP is a lethal fibrotic lung disease induced by inhalation of airborne coal mining dust, including crystalline silica [32]. Although the functional consequences of the rs1539019 SNP are not known, this finding is in agreement with the present results and strengthens the hypothesis of the involvement of the NLRP3 inflammasome complex in the development of fibrotic lung diseases.

The role of NLRP3 inflammasome complex has also been implicated in the development of COPD [292], but genetic association studies supporting this view are lacking to date. The current study did not discover any new evidence concerning the NLRP3 inflammasome genes and pulmonary emphysema.

6.4.4 *GC*

GC is a multifunctional serum protein, which is known to participate in several immunologically important functions such as macrophage activation [208]. GC has been proposed to be involved in the chronic inflammation process of the lungs, and its gene polymorphisms have been extensively studied in several pulmonary disorders [209]. There are three common variants (haplotypes) in the *GC* gene (GC1F, GC1S, and GC2), due to the presence of two SNPs (rs4588 and rs7041), that affect the function of the protein [208]. These haplotypes and SNPs have been repeatedly associated with COPD and impairment of lung function [210–219].

In the current study, no significant associations were observed between *GC* SNPs (rs4588 and rs7041) or haplotypes and emphysema, fibrosis, pleural plaques, or lung function parameters. There are many potential explanations for this discrepancy; first, most of the previous studies were rather small, with 100 COPD patients or less [210–216], and in two of the most recent larger studies utilizing replication cohorts, only a few associations could actually be replicated [218, 219]. Second, there may be some confounding factors related to vitamin D that should be accounted for when studying GC. Third, including the present study, there are no reports associating GC with pulmonary emphysema; hence, it is possible that GC does not contribute to the emphysema phenotype of COPD.

6.5 Strengths and weaknesses of the present study

In the current study, two cohorts of asbestos exposed construction workers were combined and used in a case-control setting to examine the role of selected candidate gene polymorphisms in the development of different pleural, pulmonary, and lung function changes related to asbestos and tobacco smoke exposure.

One of the main strengths of the present study is the carefully characterized population; because lung function and CT-findings (interstitial and visceral pleural fibrosis, pleural plaques, and emphysema subtypes) were recorded separately and classified according to their severity, there

was the possibility to examine the genetic background of very finely defined phenotypes.

Another advantage is that the present patient material was considerably large and a lot of ex- and current smokers were included. This is useful in demonstrating the genetic predisposition to emphysema, which probably would not have manifested to such a marked degree without smoking.

This study also has some 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 the image assessment. However, since the Finnish population is very homogenous and the three cities, where the patients were enrolled, are all located in southern Finland very close to each other, it seems most unlikely that the geographic origin would be responsible for any significant bias appearing in the data analysis. Moreover, any inconsistency in image reading or in the technical image quality could cause an inaccuracy, *i.e.,* random noise added to the results which would lead to a loss of power rather than to a systematic error. This increases the error variance in computations and the detected associations are therefore likely to be underestimated.

Second, in addition to asbestos and tobacco smoke, it is most likely that the study subjects have also been occupationally exposed to other particles, such as concrete, silica, and wood dusts, and these could well have contributed to the development of pulmonary diseases. Unfortunately information about exposure data of these dusts was not available from our study subjects.

Finally, the multiple comparisons performed increase the possibility of detecting false-positive associations. However, no corrections were used since most of the methods which attempt to correct for multiple testing are very conservative, and it is not even clear what could be considered as the correct number of comparisons for which one should adjust [293]. Nonetheless, the present results should be considered with caution until replicated in an independent study population.

7 CONCLUSIONS

In this thesis, the roles of several genes involved in xenobiotic metabolism, protease-antiprotease balance, innate immunity, and inflammation were studied in the development of different asbestos and tobacco smoke exposure related pleural and pulmonary changes, peripheral obstruction, and impairment of pulmonary diffusing capacity.

According to the present results, polymorphisms of certain XME genes are potential modifiers of the risk of developing pleural and pulmonary changes related to asbestos and tobacco smoke exposure. The most convincing evidence came from *GSTT1*; the deletion of this gene was significantly associated with several types of changes in the whole study population and in both of the study cohorts separately. Thus, one could speculate that *GSTT1* has a crucial role in the metabolism of different kind of environmental agents entering the lungs.

Another principal observation was the association between serine protease inhibitor *SERPINE2* and panlobular emphysema. Together with the findings concerning *MMP9*, *TIMP2*, *TNF,* and *TGFB1*, this implies that polymorphisms of genes involved in protease-antiprotease balance likely contribute to the development of pulmonary emphysema and bronchial obstruction, and different molecular mechanisms may explain the development of different emphysema subtypes.

The present results also indicate that genetic variation in innate immunity related genes may have an important role in coping with asbestos exposure. One very interesting finding supporting this view was the association between interstitial lung fibrosis and polymorphism of *NLRP3*, an essential component of the NLRP3 inflammasome complex.

8 FUTURE PROSPECTS

This study is a typical example of candidate gene approach, where prior knowledge of the function of the gene is coupled to the current understanding of the pathogenesis of the phenotype of interest [294]. Unfortunately, candidate gene studies have not produced very repeatable results, as discussed above in relation to the genes investigated here.

Candidate genes are usually examined in a case-control setting, where study size is critical in order to obtain reproducible results. Many of the earlier studies have been performed in rather small populations leading to a lack of statistical power, which may partly explain the conflicting results of the literature. The inconsistencies could also be due to genetic heterogeneity and complexity; most often the disease phenotype is the outcome of multiple genetic and/or epigenetic factors that interact with the environment.

Another important source of error is the phenotypic heterogeneity; in the case of COPD, for example, it has become evident that a more accurate patient characterization is needed in order to clarify the molecular mechanisms and genetic factors behind different phenotypes, and ultimately, to develop more effective therapies for this disorder [80, 81].

New genome-wide applications are increasingly used in identifying the genomic regions associated with complex diseases. Although GWA studies are hypothesis-free and considered as unbiased, similarly to the situation with other association studies, they are confronted with many statistical and methodological problems such as disease misclassification, population stratification, genotyping errors, and statistical significance due to multiple comparisons [295, 296].

The advent of next generation sequencing (NGS) technologies has, in turn, lowered the costs of direct sequencing; these are now attractive

options for investigating the genetic variation associated with human diseases. Compared to the other technologies, sequencing offers the benefit that it can identify all sequence variants in an individual genome, exome, or sub-genomic area of interest [294].

Despite the new technological advances, candidate gene and case-control strategies are still needed to confirm and define the results obtained from genome-wide applications. However, if one hopes to conduct a reliable replication, the quality of candidate gene studies needs to be considerably improved. Based on the current study and the previous literature, one of the key factors in this process is the rigorous characterization of patients and controls in order to create homogeneous study populations. It has also been proposed that more stringent significance thresholds and independent replication cohorts should be used [297].

Since biological systems are complex and cannot be predicted by their single constituents, more than genetic association studies are needed if one hopes to obtain a better understanding of the human diseasome. A call for systems biology approach integrating functional genomics, transcriptomics, proteomics, and metabolomics by the means of bioinformatics has been expressed also in the field of pulmonary research, and the first steps have been taken in identifying the functional and regulatory pathways that play central roles in respiratory pathophysiology [298, 299]. After the primary causes and mechanisms of a disease development start to emerge, it is possible not only to diagnose and treat patients more efficiently, but also to identify people at-risk or individuals who would be especially vulnerable to certain environmental exposures.

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Both tobacco smoke and asbestos fibers enter the body mainly by inhalation. In the lungs, they may evoke oxidative stress, alter the protease-antiprotease balance, induce innate and adaptive immune responses, and create persistent inflammation leading eventually to lung injury. The type and severity of lung injury induced by foreign compounds varies greatly between individuals, even with similar exposure history. These differences are believed to originate from the complex interplay between genetic, epigenetic, environmental, and life course factors.

In this study, the effects of genetic variation on the risk and severity of asbestos and smoking related non-malignant pleural and pulmonary changes and lung function impairment was examined among Finnish construction workers. The studied polymorphic genes encode proteins involved in xenobiotic metabolizing, proteolytic balance, inflammation, and innate immunity; pathways that are potentially linked to asbestos and tobacco smoke exposure.

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