GENETIC RISK FACTORS FOR CHRONIC OBSTRUCTIVE PULMONARY DISEASE

by

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ABSTRACT

Chronic obstructive pulmonary disease (COPD) is a major cause of morbidity and mortality. The worldwide increase in cigarette smoking means that COPD is becoming an even greater health problem and will have an astounding impact on health care costs and services. COPD is often thought of as a self-inflicted disease, however, not all people who smoke develop COPD, and not all patients with COPD are smokers. The pathogenesis of COPD is complex and not fully understood. There is an urgent need to improve present drug therapy. The identification of genetic risk factors for COPD will help elucidate the cellular and molecular mechanisms involved in the disease process and aid in the development of therapeutic regimens that can be tailored on an individual basis. I conducted population-based association studies to see whether alpha- and beta-defensins, plasminogen activator inhibitor 1, and tissue-type plasminogen activator polymorphisms influenced smokers' susceptibility to lung function decline in a relatively large population of outbred Caucasian individuals. I failed to find associations between polymorphisms in these four genes with rate of decline in lung function in smokers. In addition, I studied patients undergoing surgical resection for lung carcinoma in order to determine whether promoter polymorphisms in five proteinases produced by alveolar macrophages were related to alveolar macrophage mRNA expression and the development of emphysema. Furthermore, I wanted to determine whether mRNA expression of these five proteinases was related to emphysema. I found that the matrix metalloproteinase 9 C-to-T single nucleotide polymorphism (-1562C→T) significantly influenced matrix metalloproteinase 9 mRNA expression in alveolar macrophages. Matrix metalloproteinase 1 and cathepsin L expression levels in uncultured alveolar macrophages were significantly related to emphysema, and a matrix metalloproteinase 9 CA repeat was significantly related to a qualitative measure of emphysema. Matrix metalloproteinase 1, matrix metalloproteinase 9, and cathepsin L represent interesting biomarkers of COPD and their study may help in the development of therapeutic interventions that are individually tailored to patients needs.

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LIST OF ABBREVIATIONS AND ACRONYMS

bp	Base pair(s)
g	Gram(s)
h	Hour(s)
kb	Kilo base
kDa	Kilo Dalton
min	Minute(s)
mM	Millimolar
mL	Milliliter(s)
ng	Nanogram(s)
s	Second(s)
U	Unit(s)
µg	Microgram(s)
µL	Microliter(s)
µM	Micromolar
5'UTR	5'-untranslated region
3'UTR	3'-untranslated region
A	Adenine
ACTB	Beta actin
ASPOLG	Polymerase gamma accessory subunit
BAL	Bronchoalveolar lavage
C	Cytosine
CD	Cluster of differentiation designation
COPD	Chronic obstructive pulmonary disease
CT	Computed tomography
CTSL	Cathepsin L gene
CTSS	Cathepsin S gene
ECM	Extracellular matrix
FEV1	Forced expiratory volume in one second
FVC	Forced vital capacity

G	Guanine
GM-CSF	Granulocyte monocyte colony stimulating factor
HBD	Human beta-defensin
HD	Human defensin
HNP	Human neutrophil peptide
IL	Interleukin
LPS	Lipopolysaccharide
MMP	Matrix metalloproteinase
PA	Plasminogen activator
PAI	Plasminogen activator inhibitor
PCR	Polymerase chain reaction
PLAT	Tissue-type plasminogen activator gene
SNP	Single nucleotide polymorphism
T	Thymine
TDT	Transmission test of linkage disequilibrium
TGF	Transforming growth factor
TIMP	Tissue inhibitor of metalloproteinases
TNF	Tumor necrosis factor
tPA	Tissue-type plasminogen activator
VNTR	Variable numbers of tandem repeats
uPA	Urinary-type plasminogen activator

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DEDICATION

This book is affectionately dedicated with boundless love and gratitude to those nearest and dearest to me, in order of their appearance: father Brian, mother Carolynne, sister Michelle, my grandparents, Taku, and especially, Todd.

To my parents, for being lifelong learners and providing a stimulating and enriching environment to grow up in. I could not have done it without your love, encouragement, and support. To my sister, for the motivation and being my big sister. To my late grandparents, for their inspiration. To Taku, for his unconditional love and enthusiasm. I wish you were here now... And to Todd, for being there everyday and understanding.

CHAPTER 1. CHRONIC OBSTRUCTIVE PULMONARY DISEASE

1.1 DEFINITION

Until recently, chronic obstructive pulmonary disease (COPD) was poorly defined. Based on current knowledge, the Global Initiative for Chronic Obstructive Lung Disease defined COPD as "a disease state characterized by airflow limitation that is not fully reversible. The airflow limitation is usually progressive and associated with an abnormal inflammatory response of the lungs to noxious particles and gases."[1] For the first time the definition highlights the importance of airflow limitation in defining the clinical condition and emphasizes the persistent inflammation.

The term COPD includes chronic bronchitis (persistent productive cough for at least three months in at least two consecutive years), bronchiolitis (small airway disease; inflammatory thickening of airway walls and narrowing of the small airways), and emphysema (permanent enlargement of the airspaces distal to the terminal bronchioles accompanied by destruction of lung parenchyma). Most patients with COPD have all three conditions, to varying degrees. Symptoms associated with COPD include cough, sputum production, and dyspnea. Spirometry is used to diagnose COPD. The presence of a postbronchodilator forced expiratory volume in one second (FEV1) < 80% of the predicted value in combination with a ratio of FEV1 to forced vital capacity (FEV1/FVC) < 70% confirms the presence of airflow limitation that is not fully reversible.[1]

1.2 EPIDEMIOLOGY

COPD is a chronic disabling disease that is a major and increasing cause of global morbidity and mortality. During the past two decades, COPD steadily increased. In 1990, COPD was the sixth commonest cause of death worldwide.[2-4] By 2000, COPD had become the fourth leading cause of mortality worldwide.[5] The World Health Organization predicts that COPD will become the third commonest cause of death and the fifth most pressing public health problem worldwide by 2020.[6] A marked increase in cigarette smoking and environmental pollution in developing countries contribute to the dramatic increase in COPD as well as reduced mortality from cardiovascular diseases in industrialized countries and infectious diseases in developing countries.

COPD is the most underfunded disease in relation to the global burden of disease.[7] Despite the enormous global burden and the escalating healthcare costs, there has been relatively little research into the cellular and molecular mechanisms involved in the disease process. Currently, there are no treatments available to prevent the progression of COPD.

1.3 PATHOLOGIC CHANGES

Pathologic changes characteristic of COPD are found in the large airways, small airways (less than 2 mm), and alveoli. Chronic inflammation leads to fixed narrowing of small airways and alveolar wall destruction, which contribute to airflow limitation. The changes vary between patients and at different stages of the disease. Figure 1.1 summarizes the pathogenic mechanisms in COPD.

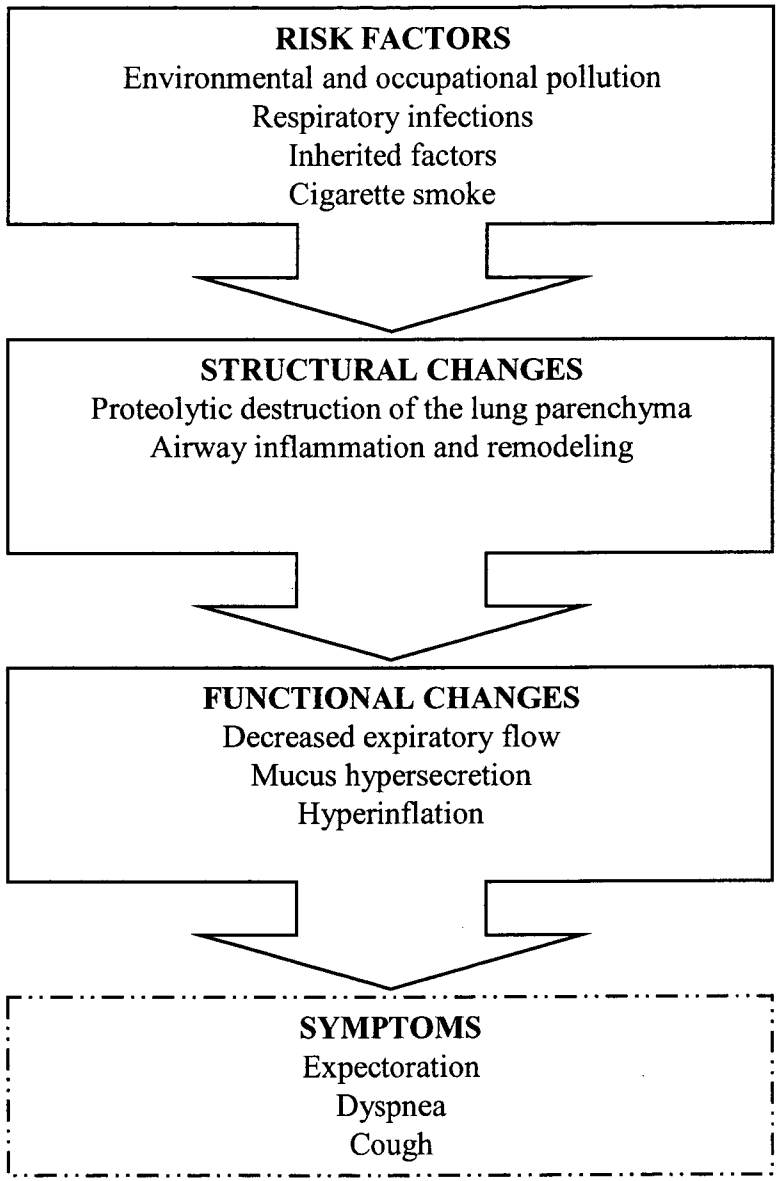


Figure 1.1 Summary of the pathogenic mechanisms in chronic obstructive pulmonary disease.
Exposure to cigarette smoke is the most important risk factor in the pathogenesis of COPD; however, it is not the only risk factor. Smoke exposure interacts with other risk factors to produce structural changes in the lung that result in functional changes and symptoms.

1.3.1 Large airways

The large airways include the trachea, bronchi, and bronchioles greater than 2 mm in internal diameter. Changes noted in the large airways due to chronic inflammation include mucus gland enlargement, goblet cell hyperplasia, smooth muscle hyperplasia, cartilage atrophy, and bronchial wall thickening and encroachment on the lumen.[8] These changes are responsible for the symptoms of chronic cough and mucus production that define chronic bronchitis, however, it is unlikely that these changes directly cause airflow limitation. Another change observed is dysfunctional and damaged cilia, which may lead to impaired mucociliary clearance and an increased risk of airway infections. Proteinases released from macrophages and CD8+ T lymphocytes, the predominant cell types in the inflammatory exudate as well as bacterial proteinases, may promote airway remodeling, which contributes to airflow obstruction.[9, 10]

1.3.2 Small airways (less than 2 mm)

More than thirty years ago, Hogg and associates[11] proposed the concept that COPD is a small airways disease based on the observation that the peripheral airways are the major site of airflow limitation in COPD. Changes that contribute to the narrowing of the small airways include loss of alveolar and bronchiolar attachments, goblet-cell metaplasia, edema, inflammatory cellular infiltration, reduced surfactant, smooth muscle hyperplasia, and fibrosis.[8] Loss of alveolar and bronchiolar attachments due to proteolytic lung destruction is most likely the result of mononuclear inflammatory cells collecting in the peripheral airways and the release of proteolytic enzymes. The resulting distortion and narrowing contributes to airflow obstruction. Goblet cells increase in number and extend into the airway lumen, which also contributes to airway narrowing. A reduction in surfactant may increase surface tension leading to airway narrowing and eventually collapse. Fibrosis in the respiratory bronchioles may be the most critical contributor to small airway narrowing.[12] Fibrosis is characterized by the accumulation of myofibroblasts and fibroblasts as well as the buildup of extracellular connective tissue matrix. The mechanisms of fibrosis around the airway are unclear, however, it likely involves an attempt to repair chronic inflammation. Fibrosis may be attributable, at least in part, to increased expression of transforming growth factor (TGF) -beta in the

peripheral airways[13, 14] given that TGF-beta is the most potent inducer of connective tissue growth factor thus far identified. TGF-beta may induce fibrosis via the release of connective tissue growth factor, which stimulates collagen deposition in the airways.[15, 16] Other cells and mediators involved in the process have yet to be defined.

1.3.3 Alveoli

The reduction in FEV1 that confirms COPD diagnosis is primarily due to an increase in resistance in the peripheral airways as discussed above, however, loss of elastic recoil pressure due to pulmonary emphysema is also a contributing factor.[17, 18] Emphysema is defined as "the abnormal permanent enlargement of airspaces distal to the terminal bronchiole, accompanied by destruction of their walls, without obvious fibrosis."[19] Both centrilobular and panlobular emphysema occur in smokers,[20] the former being the most common type in COPD patients. Centrilobular emphysema is the result of dilation and destruction of the respiratory bronchioles. These lesions are larger and more numerous in the upper lung regions, however, in advanced disease the lesion may appear diffusely throughout the entire lung and may involve the destruction of the pulmonary capillary bed. Panlobular or panacinar emphysema results from a uniform destruction of the acini within the secondary lobule, involving dilation and destruction of the alveolar ducts and sacs as well as the respiratory bronchioles. In contrast to centrilobular emphysema, panacinar emphysema tends to effect the lower lobes. Panacinar emphysema is the characteristic lesion associated with alpha-1 antitrypsin deficiency but it is also found in cases when there is no genetic abnormality of alpha-1 antitrypsin. The distinctions between cigarette smoking-related emphysema and alpha-1 antitrypsin-related emphysema are imprecise. A proteinase:antiproteinase imbalance and oxidative stress, both consequences of chronic lung inflammation and/or environmental and genetic factors, may contribute to parenchymal destruction and the subsequent loss of elastic recoil pressure.

1.4 PATHOGENESIS

Important processes in the pathogenesis of COPD include inflammation, an imbalance of proteinases and antiproteinases, oxidative stress, and apoptosis.

1.4.1 Inflammatory cells and mediators

COPD involves several inflammatory cells types and numerous inflammatory mediators (Figure 1.2). Even after cigarette smoking stops, there are markedly increased numbers of inflammatory cells present in the lung.[21] The precise role and importance of each cell type in the pathogenesis of COPD still remains to be established and another challenge is to determine how various cell types and mediators interact and contribute to the disease process. Churg et al.[22] demonstrate the importance of studying the interaction between cell types with the finding that, in an acute model of emphysema, elastin and collagen degradation is dependent on an interaction between both macrophages and neutrophils and that this interaction occurs in a stepwise fashion.

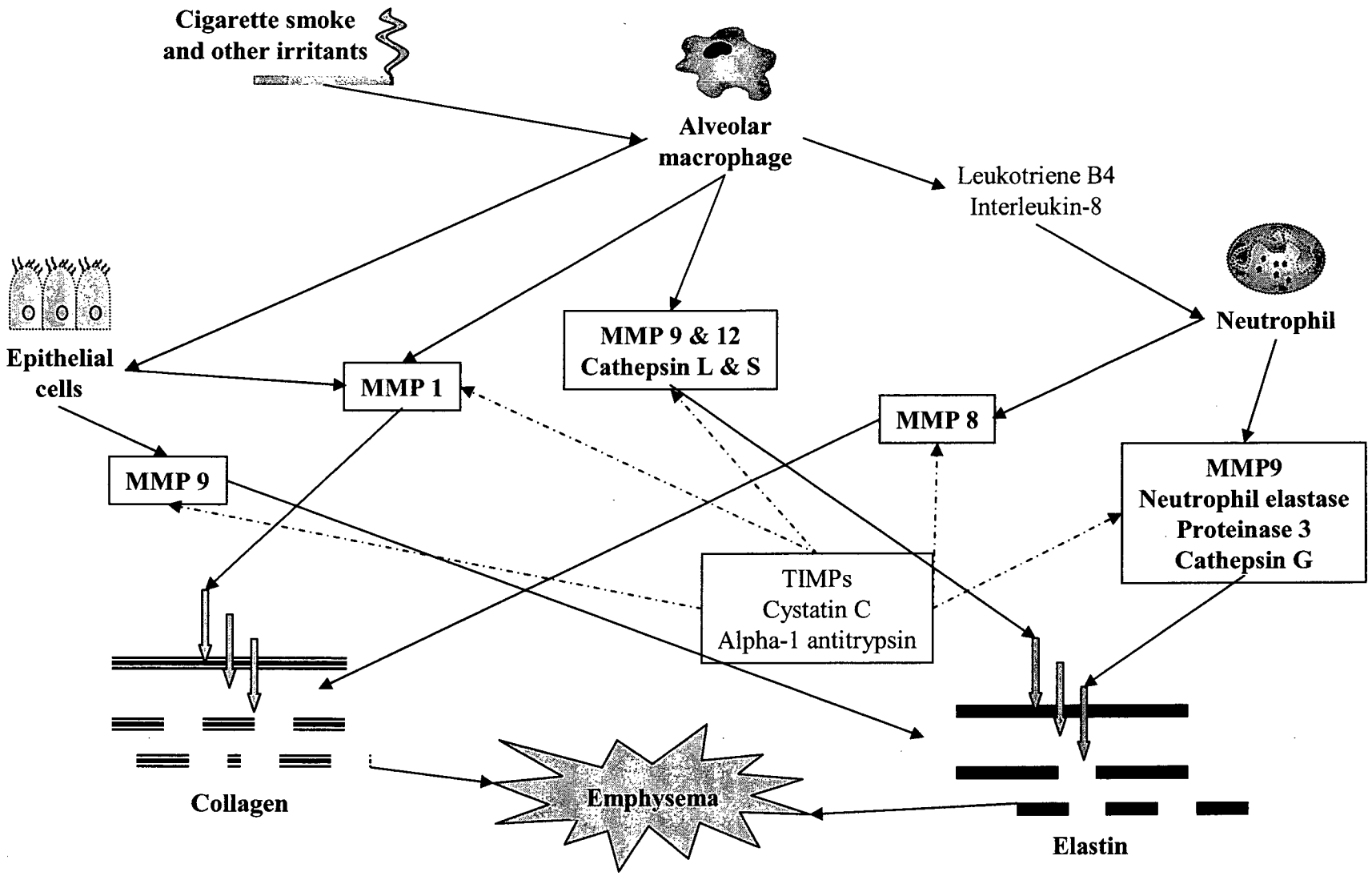


Figure 1.2 Inflammatory mechanisms in chronic obstructive pulmonary disease.

Cigarette smoke activates alveolar macrophages, which activate epithelial cells and release neutrophil chemotactic agents (e.g. leukotriene B4 and interleukin-8). Activated macrophages, epithelial cells, and neutrophils then release proteinases. Under normal conditions, proteinases are inhibited by proteinases inhibitors, however, when proteinases are released in excess of inhibitors they destroy the lung parenchyma, resulting in emphysema. MMP denotes matrix metalloproteinase; TIMP, tissue inhibitor of metalloproteinases.

1.4.1.1 Neutrophils

Alpha-1 antitrypsin, the only proven genetic risk factor for COPD, is the primary inhibitor of neutrophil elastase. Because neutrophil elastase is expressed almost exclusively by neutrophils, these cells were historically considered to play a major role in the pathogenesis of emphysema. Increased numbers of activated neutrophils are found in sputum and bronchoalveolar lavage (BAL) fluid of patients with COPD[23] as well as smokers without COPD.[24] In contrast, increased numbers of neutrophils are not seen in the airways and parenchymal tissue.[25] A possible explanation for the discrepancy between the increased number of neutrophils in sputum and BAL versus low numbers in lung tissue is that neutrophils migrate rapidly across the tissue and into the lumen; therefore, these cells may not be detectable by tissue analysis.

Neutrophils secrete a number of proteinases stored in their granules that may contribute to alveolar destruction including serine proteinases (neutrophil elastase, cathepsin G, and proteinase 3) as well as members of the matrix metalloproteinase (MMP) family (MMP8 and MMP9). Upon neutrophil activation, there is a rapid release of the granule contents. Furthermore, upon stimulation with cytokines and bacterial endotoxin, neutrophils generate a web of extracellular fibers, known as neutrophil extracellular traps, that kill extracellular bacteria.[26] Neutrophil extracellular traps may help minimize the proteinase damage to host cells by focusing proteinase activity and preventing their diffusion.[27] In addition to their contribution to alveolar destruction, the neutrophil serine proteinases mentioned above are potent stimulants of mucus secretion from submucosal glands and goblet cells in the epithelium.[28, 29] Although the contribution of mucus hypersecretion to airflow obstruction in COPD is unclear, mucus hypersecretion is a potential risk factor for an increased rate of decline in lung function.[30] In severe COPD, chronic mucus hypersecretion is associated with mortality and this may reflect an increased risk of terminal infection.[28, 29, 31]

Several studies suggest that neutrophils play a role in the pathogenesis of COPD. Experimental animals receiving intratracheal instillation of neutrophil elastase into their lungs develop emphysema.[32] In humans, cigarette smoking increases neutrophil retention in the lung.[33] Increased concentrations of myeloperoxidase and human neutrophil lipocalin, indicators of neutrophil activation, are detected in the sputum supernatant.[34-36]

An association between the number of circulating neutrophils and decline in FEV1 is documented.[37] However, a relationship between presence of neutrophils in the lung parenchyma and its destruction has not been reported. Finkelstein et al.[38] show that the number of neutrophils decrease in the lung parenchyma with increasing emphysema suggesting that neutrophils do not play an essential role in the lung destruction characteristic of emphysema. This was not the first time a negative correlation was found between the number of tissue neutrophils and the extent of lung destruction.[39] In addition, neutrophils are not a prominent feature of chronic parenchymal inflammation suggesting that they are a minor player in the generation of emphysema. Animal models of emphysema also support these findings.[40-42] Neutrophil accumulation may be an important initial event in the development of emphysema. A delay in transit time and increased retention of neutrophils in the lung after smoke exposure is demonstrated in humans and animals.[43, 44] However, two studies fail to find support for the notion that emphysema is associated with an increased sequestration of neutrophils.[45, 46] In support of both previous statements, Ofulue et al.[47] report that cigarette smoke-exposed rats have an increased number of interstitial and lavage fluid neutrophils at one month and that by two months the number of neutrophils decline to and remain at control levels for the duration of the study. In addition, evidence of ECM degradation is not evident until two months. In a very acute model of smoke-induced injury, neutrophils are found to be the initial effector cells involved in connective tissue breakdown.[48] The authors suggest that these findings should be interpreted with caution, as the model used does not provide information about long-term changes that may lead to emphysema.[48] In summary, during the acute phase of the disease, there is a predominance of neutrophils and there is no ECM breakdown. In the chronic phase, there is a decrease in neutrophils and a shift towards increased alveolar macrophages and T cells, and lung destruction occurs.

1.4.1.2 Macrophages

Macrophages play an important role in the development of COPD. Both smokers and COPD patients have elevated macrophage numbers. Macrophages accumulate in the alveoli and small airways. In addition to increases in number, alveolar macrophages from smokers are larger in size, often multinucleated, and contain characteristic dark

basophilic smokers' inclusion bodies within the cytoplasm as a result of ingestion of inhaled smoke particulates.[49-53] Alveolar macrophages life for approximately eighty days.[54] Studies have shown that it may take two to three years after smoking cessation before the percentage of alveolar macrophages with inclusions reaches similar levels as nonsmokers, suggesting the smokers' alveolar macrophages may persist for a considerably longer time than the estimated life span of these cells.[55, 56]

In healthy nonsmokers, macrophages are the major host defense cell in the lower airspace; the BAL fluid cell content consists of 95% macrophages. In response to cigarette smoking, the total number of cells recovered by BAL is elevated many fold; 95-98% of these cells are macrophages.[57] The amount of alveolar macrophages recovered by BAL is related to the development of emphysema in smokers.[58] There is a correlation between macrophage numbers in the airways and the severity of COPD.[59] Furthermore, macrophages accumulate in the respiratory bronchioles of cigarette smokers where emphysematous changes are first evident and there is a positive association between macrophage numbers and the degree of small airways disease in COPD patients.[38, 60]

The role of macrophages in the lung is not limited to the phagocytosis of deposited debris; they are also involved in antigen presentation and produce numerous mediators of inflammation such as cytokines, growth factors, and nitric oxide. Two potent neutrophil chemotactic factors produced by macrophages are leukotriene B4 and interleukin (IL) -8. In addition, macrophages produce a number of proteinases that have been implicated in the pathogenesis of emphysema. Unlike neutrophils, which rapidly release their proteinases from storage granules upon activation, the synthesis and secretion of macrophage proteolytic enzymes is strictly regulated and acts in response to the cellular environment. Cytokines, endotoxin, phagocytosis, and growth factors modify their expression. Human alveolar macrophage derived elastases include cathepsin L and S (two of the most potent elastases), MMP9, and MMP12. Animal and human studies show that emphysema is characterized by the breakdown of elastin as well as the breakdown and resynthesis of collagen. In addition to the elastinolytic enzymes mentioned, macrophages also synthesize and secrete MMP1, a potent collagenase.

1.4.1.3 T Lymphocytes

The role of T cells in the pathophysiology of COPD is uncertain, however, it was suggested that T cells influence the recruitment of inflammatory cells and the release of proteolytic enzymes.[61] T cells, particularly CD8+ cells, accumulate in the lungs of patients with COPD.[9, 38, 62, 63] Furthermore, Finkelstein et al.[38] provide evidence suggesting that the amount of lung destruction is correlated with the number of T cells. One way CD8+ cells may contribute to the pathogenesis of COPD is through the release of perforin, granzyme-B, and tumour necrosis factor (TNF) –alpha, which leads to cytolysis and apoptosis of alveolar epithelial cells.[64] An association between CD8+ cells and apoptosis of alveolar cells in emphysema is observed.[65] There is a predominance of T cells, which are directly related to the severity of emphysema, in a mouse model of cigarette-induced emphysema.[66] In another mouse model, interferon-gamma released from CD8+ cells causes emphysema and contributes to the proteinase:antiproteinase imbalance in adult mice lungs.[67] The altered proteinases include cathepsins L and S, MMP9, and MMP12.

1.4.1.4 Eosinophils

The role of eosinophils in COPD is unclear. Reports concerning eosinophil levels are conflicting and may be influenced by coexisting asthma in some COPD patients.[9, 68] A few studies, by the same group, agree that during acute exacerbations of COPD there is an increase in airway eosinophils.[69, 70] High levels of neutrophil elastase cause eosinophil degranulation.[71] This suggests that eosinophils may participate directly in the degradation of alveolar extracellular matrix (ECM) by releasing proteinases such as MMP9. Eosinophils express MMP9, which is increased upon stimulation with TNF-alpha;[72, 73] TNF-alpha is increased in the sputum of patients with COPD.[23]

1.4.1.5 Epithelial cells

Epithelial cells lining the airways and alveoli may be important sources of proteinases and inflammatory mediators in COPD. Epithelial cells produce the proteolytic enzymes urokinase-type plasminogen activator and MMP9. Cigarette smoke-induced activation of epithelial cells leads to the production of TNF-alpha, IL-1beta, GM-CSF, and IL-8.[74-76] In

addition, defensins produced by conducting and respiratory airway epithelial cells are involved in inflammation and repair in the lung.

1.4.1.6 Inflammatory mediators

Activated inflammatory cells are the source of a variety of mediators that themselves are capable of recruiting inflammatory cell and causing lung damage. There is an increased concentration of leukotriene B4, which is chemotactic for neutrophils, in the sputum of patients with COPD.[77] Another neutrophil-chemotactic cytokine, IL-8, and a cytokine upstream from IL-8, TNF-alpha, are also increased in the sputum of patients with COPD.[23] Alveolar macrophages are probably the major sources of leukotriene B4, IL-8, and TNF-alpha. IL-8 is also secreted by neutrophils and epithelial cells. TNF-alpha controls the IL-8 gene via the transcription factor nuclear factor-kappaB in epithelial cells and macrophages.

1.4.2 Proteinase:antiproteinase imbalance

The proteinase:antiproteinase hypothesis of lung injury originated in the 1960s. The starting point was two findings, one by Laurell and Eriksson and another by Gross and colleagues. Laurell and Eriksson[78] observed that individuals with severe alpha-1 antitrypsin deficiency developed emphysema at an early age. Gross and colleagues[79] developed the first reproducible model of emphysema by injecting rat lungs with the plant proteinase papain. Based on these two observations it was believed that emphysema was induced by proteolytic injury to the lung ECM. It is generally accepted that cigarette smoke leads to the recruitment of inflammatory cells into the terminal airspaces of the lung. These inflammatory cells release proteinases, in excess of inhibitors, leading to lung ECM degradation and resulting in airspace enlargement that defines pulmonary emphysema. A proteinase:antiproteinase imbalance may be caused by a number of different factors such as cigarette smoke, oxidative stress, and genetic polymorphisms that alter the expression or function of these enzymes. The proteinase:antiproteinase hypothesis continues to dominate current thinking, however, many questions are still unanswered. It is unclear which cell types and proteinases are responsible for the ECM

degradation that characterizes emphysema. In addition, the ECM components targeted by proteinases are uncertain.

Early studies focused on neutrophil-derived proteinases, particularly neutrophil elastase, because of the finding that individuals with alpha-1 antitrypsin deficiency develop emphysema. The pathogenic roles of neutrophil elastase, proteinase 3, and cathepsin G have been confirmed in animal models of COPD. Neutrophil elastase almost undoubtedly accounts for emphysema related to alpha-1 antitrypsin deficiency, however, it may not be a key player in smoking-related emphysema without an alpha-1 antitrypsin deficiency.

There is now increasing evidence that macrophage derived proteinases are involved in the generation of emphysema. The importance of macrophage-derived proteinases is underscored by the study showing that MMP12 knock out mice do not develop emphysema when exposed to cigarette smoke.[80] MMP12 is the most highly expressed MMP in mice (expressed at low levels in humans), which has raised some doubt about its importance in human emphysema, however, this study still demonstrates that MMPs are capable of destroying lung tissue. Another important study shows that IL-13 is a potent stimulator of a number of cathepsins and MMPs, including the macrophage-derived proteinases cathepsin L, cathepsin S, MMP9, and MMP12 and its overexpression leads to the formation of emphysema in mice.[81] In the same study, when the mice are treated with cathepsin and MMP inhibitors, the amount of emphysema and inflammation is significantly decreased.

Proteolytic enzymes are regulated by a number of inhibitors. Alpha-1 antitrypsin and secretory leukocyte protease inhibitor are the major inhibitors of neutrophil elastase and proteinase 3, respectively. Cystatin 3 inhibits cathepsin activity. MMPs are inhibited by the endogenous and specific tissue inhibitor of metalloproteinases (TIMPs). To date, four TIMPs have been identified. Recently, a new inhibitor of MMPs, reversion-inducing cysteine-rich protein with Kazal motifs, has been identified although it may be limited to MMP2 inactivation.[82, 83]

The proteinase:antiproteinase imbalance may result from altered production or activity of both proteinases and antiproteinases. In smokers who develop COPD the cigarette smoke-induced production of proteinases may be too great for the

antiproteinases to neutralize. Moreover, genetic polymorphisms that effect the expression and the function of these enzymes are probably important determinants of which smokers develop COPD.

1.4.3 Oxidative stress

There is now considerable evidence that oxidative stress is an important pathogenic mechanism in COPD.[84] Increased oxidant exposure and/or antioxidant depletion or deficiency contributes to oxidative stress. Sources of oxidants in COPD patients include cigarette smoke and inflammatory cells. Markers of increased oxidative stress have been found in the airspaces, breath, blood, and urine of patients who have COPD.[23, 85]

The increased presence of oxidants contributes to COPD through several mechanisms. Oxidative stress influences the proteinase:antiproteinase imbalance through the oxidative inactivation of alpha-1 antitrypsin.[86] Oxidants activate the transcription factor nuclear factor-kappaB, which induces the transcription of several proinflammatory genes including TNF-alpha and IL-8, thus enhancing airspace inflammation.[23] Increased oxidative stress may also result in airspace epithelial injury, mucus hypersecretion, the activation of MMPs, and degradation of TIMPs.[87]

1.4.4 Apoptosis

Recently, the concept that vascular changes are important in the pathogenesis of pulmonary emphysema proposed by Liebow[88] in the 1950s has been revisited. Liebow examined histological sections of emphysema lungs and noted that the alveolar septa in centrilobular emphysema appeared to be thin and somewhat avascular. He proposed that the disappearance of alveolar septa might be accounted for by a reduction in the blood supply to the small precapillary vessels. Studies by Tuder and colleagues[89, 90] in rats and humans have highlighted the importance of endothelial and epithelial cell apoptosis in the pathogenesis of emphysema. Apoptosis is a form of programmed cell death, which occurs naturally as part of the normal development, maintenance, and renewal of tissues within an organism. Tuder and colleagues revived Liebow's vascular atrophy model and there are now a number of studies suggesting that alveolar cell apoptosis triggered by cigarette smoke may contribute to the development of pulmonary emphysema.[89-93] These findings

have led to an alternative to the widely accepted proteinase:antiproteinase hypothesis. The newly proposed hypothesis states that apoptosis of endothelial and epithelial cells initiates the process of ECM degradation. Whereas, traditionally it is thought that airspace enlargement occurs through the destruction of the ECM by proteinases and that the loss of cell attachment to the basement membrane leads to the subsequent death of surrounding endothelial and epithelial cells. Furthermore, studies have shown that oxidative stress and apoptosis interact and cause emphysema.[91, 92] The findings that apoptosis interacts with other pathological mechanisms leading to the lung destruction characteristic of emphysema underscores the importance of investigating the interaction between inflammation, proteinase:antiproteinase imbalance, oxidative stress, and apoptosis when investigating pathogenic mechanisms in pulmonary emphysema.[94] In contrast to the above findings, a study shows that cigarette smoke-induced necrosis (the death of cells due to stimulation by a toxic substance), not apoptosis, is responsible for the destruction of alveolar walls in emphysema.[95] In summary, evidence indicates that some form of cell death, be it apoptosis or necrosis, causes alveolar apoptosis and contributes to the development of pulmonary emphysema.

1.5 RISK FACTORS

Susceptibility to develop COPD results from a combination of environmental and genetic factors. Cigarette smoking is undoubtedly the main environmental risk factor for COPD in the developed world. Although the majority of cases of COPD occur in individuals who have smoked (>95%), one of the most important questions is why only 15 to 20% of smokers develop this condition.[96] Other risk factors identified include indoor and outdoor air pollution (particularly indoor air pollution from biomass fuel in less developed countries), exposure to occupational dusts and chemicals, airway hyperresponsiveness, severe childhood respiratory infection, low birth weight, and poor nutrition.[1] In addition, genetic factors are influential in the development of COPD. There are good reasons to assume that multiple genes, each with only a modest effect, contribute to the development of COPD. It can also be speculated that multiple predisposing gene variants are interacting with each other and with environmental risk factors.[97]

1.6 ALPHA-1 ANTITRYPSIN

The only proven genetic risk factor for the development of COPD is a severe alpha-1 antitrypsin deficiency. Alpha-1 antitrypsin is a member of the serine proteinase inhibitor superfamily. It is the most abundant circulating proteinase inhibitor and has a broad spectrum of activity. Hepatocytes and monocytes are the principal producers of the protein. Protein from these sources reaches the lung via the blood. In addition, alveolar macrophages and epithelial cells produce alpha-1 antitrypsin within the lung. The role of alpha-1 antitrypsin in the lung is to protect the alveolar matrix from proteolytic attack by neutrophil elastase. A single gene located on the long arm of chromosome 14 encodes the highly polymorphic 52 kDa protein. The normal gene is designated PiM. Over 75 allelic variants have been reported.[98] PiS and PiZ are the two most common defective alleles for alpha-1 antitrypsin deficiency. The S and Z forms, which arise from single nucleotide polymorphisms (SNPs) at positions 264 and 342, respectively, are associated with reduced plasma levels of alpha-1 antitrypsin. A sixty percent decrease in normal alpha-1 antitrypsin plasma levels is associated with the S allele (Glu264Val). The Z variant (Glu342Lys) results in a severe deficiency of protein plasma levels, which are 10-15% of that produced by the normal M allele.[99] In North America, the mean gene frequencies of PiM, PiS, and PiZ are 0.95, 0.03, and 0.01, respectively.[100] The six major genotypic classes include PiMM (normal), PiMS, PiMZ, PiSS, PiSZ, and PiZZ. Individuals who are carriers of the deficiency alleles (PiMZ) and individuals with deficiency allele combinations (PiSS, PiSZ, and PiZZ) are at risk of a wide variety of adverse health effects, including lung and liver diseases. Individuals homozygous for the Z allele (PiZZ) are predisposed to the development of emphysema; smoking greatly increases the risk.[101, 102] Only 1 to 2% of all cases of COPD are the result of a severe PiZ alpha-1 antitrypsin deficiency, and current and ex-smokers with the same PiZ genotype have considerable FEV1 variability.[103] This suggests that other coexisting genetic factors, environmental exposures, and gene-environment interactions may influence the predisposition of PiZ individuals to COPD. The genetic aspects of alpha-1 antitrypsin deficiency in relation to emphysema risk have been thoroughly reviewed by DeMeo and Silverman.[104]

CHAPTER 2. FINDING GENES THAT UNDERLIE COMPLEX TRAITS

Although both genetic and non-genetic factors contribute to COPD susceptibility, it is clear from a host of family and twin studies that genetic factors play a major role in the etiology of this disease.[105, 106] The identification of the genetic factors will hopefully provide insight into the biochemical pathways and the cellular and molecular mechanisms of this disease as well as provide a rational basis for the design of effective therapies.

2.1 VARIATION IN THE HUMAN GENOME

Since before the dawn of civilization humans have been interested in hereditary phenomena. Ancient peoples improved plant crops and livestock by selecting desirable specimens for future generations. It was not until the 1860s when experiments performed by an Augustan monk named Gregor Mendel first pointed to the existence of biological elements called genes. In addition, he started to elucidate the mechanisms by which heredity affects phenotype. These early experiments paved the way for modern genetic principles and procedures. It is widely accepted that many human diseases have a genetic basis. Until recently, researchers were limited to studying monogenic (Mendelian) disorders. During the last twenty years, molecular-genetic techniques have become applicable to finding and isolating multiple interacting genes underlying complex genetic disorders.

Polymorphisms are positions in the genome sequence for which two or more alleles are present at appreciable frequency (traditionally, at least 1%) in the human population.[107] Studies in human genetics have benefited enormously by the identification of polymorphic loci that serve as genetic markers, such as variable numbers of tandem repeats (VNTRs)[108] and microsatellites. A VNTR is a chromosomal locus at which a particular repetitive sequence is present in different numbers in different individuals. A microsatellite is another type of repetitive DNA sequence based on very short repeat sequences, usually dinucleotide and trinucleotide sequences.

SNPs, the most common form of non-repetitive sequence variants and the most frequent type of genetic variation throughout the human genome, are a valuable resource for medical research. After the completion of a draft sequence of the human genome, SNP discovery became a subject of considerable interest. More than 1.42 million SNPs have been identified in the human genome;[109] on average they occur every several hundred base pairs.[110] Most SNPs do not effect gene function, but a proportion may alter expression levels and/or biological function of gene products and contribute to disease susceptibility and resistance. Using SNPs, it is often possible to test for association between a phenotype and a functional variant directly.

SNPs became popular amongst molecular geneticists when research groups shifted from studying monogenic disorders toward the analysis of complex multifactorial diseases, such as diabetes, schizophrenia, and asthma. While generally not as informative as VNTRs and microsatellites, SNPs are utilized by molecular geneticists for five main reasons: first, SNPs are more prevalent than microsatellite repeats; second, SNP inheritance is more stable because they are less prone to germline mutations; third, SNPs may alter gene expression and/or function which means that they can be directly responsible for the trait studied; fourth, population frequency estimations are made easier due to the fact that most SNPs are biallelic; and fifth, they can be used in high-throughput genotyping methods.

The assessment of individual SNPs is not the only approach to identify disease susceptibility genes. Individual genes sometimes contain over 100 SNPs and to investigate all of them in a given study group is often impractical. Fortunately, this is often not necessary since alleles at one polymorphic locus are frequently found to be associated with alleles at other sites. This association between alleles is termed "linkage disequilibrium" and it can reduce the amount of genotyping that is necessary to assess each candidate gene. If the presence of an allele at one site is always accompanied by the presence of specific alleles at several other sites, then genotyping of all the polymorphisms at each site is redundant. The arrangement of alleles along an individual's chromosome is called a haplotype. SNPs that are used to determine the presence of alleles at other sites in the haplotype are called haplotype-tagging SNPs.[111]

2.2 IDENTIFYING GENETIC DETERMINANTS

The two main approaches that are used to search for susceptibility genes are linkage analysis and association studies.

2.2.1 Linkage analysis

Linkage analysis uses family data to compare the inheritance of the disease with the inheritance of genetic markers in families with multiple affected members. Linkage analysis determines whether any of the markers are inherited with the disease more often than predicted by chance. If a genetic marker is inherited more often than predicted by chance, it is inferred that the marker is located on a chromosome near to the disease causing gene(s). Once a marker is identified, the next step is to identify candidate genes near the marker.

An advantage of linkage analysis is that novel genetic determinants can be discovered. A disadvantage is that a lot of time and resources are required for linkage analysis to be successful. In addition, linkage analysis is difficult to perform in most families with COPD because symptoms do not usually manifest until the fourth and fifth decades; therefore, in most cases, the parents of cases have died and the next generation is too young to detect significant airflow obstruction. Genome-wide linkage analysis has been very successful for multiple complex disorders (e.g. diabetes and asthma), however, until recently linkage analysis failed to identify regions of the genome likely to contain susceptibility genes for COPD. In the last few years, genome-wide linkage analysis has suggested a number of regions linked to lung function and a few regions have been identified in more than one study. Loci on chromosomes 2q and 8p were significantly linked to airflow obstruction susceptibility.[112] In another study, chromosome 2q was also significantly linked to FEV1/FVC suggesting that gene(s) at this locus may influence the development of airflow obstruction.[113] This study also found FEV1/FVC linkages to chromosomes 1 and 17, and chromosome 12p was linked to FEV1. Furthermore, another study provided evidence to suggest that there is a locus on chromosome 12p, which contributes to susceptibility to early-onset COPD.[114] A genome-wide scan in the Framingham Study suggested that loci on chromosomes 4, 6, and 21 influence FEV1 and FVC.[115]

2.2.2 Association studies

In population-based association studies, such as longitudinal cohort studies and case-control studies, polymorphisms in genes that have been implicated in the disease process, based on their known or presumed biological functions, are identified. In a longitudinal study, the incidence of disease is compared in groups of individuals with different genotypes at the putative susceptibility gene. In a case-control study, the frequency of the polymorphism is compared between a set of patients and control subjects. The findings are potentially very important but should be viewed with caution. A positive association for a tested candidate gene could occur if the gene is linked to the disease-causing gene.

In addition, population-based association studies are limited by a number of factors and careful consideration of each factor needs to be taken into account when designing these studies. Weiss discussed the problems with genetic association studies and listed ten issues that need to be addressed in order to perform a high-quality genetic association study.[116] Briefly, small sample sizes and lack of statistical power, ethnically mixed study populations, laboratory error in genotype detection, or study recruitment bias may explain why some studies are not reproducible. Ethnically mixed study populations (also known as stratified populations) can lead to false positive results if the prevalence of COPD is different in the ethnic groups. This may lead to a preponderance of one ethnic group in the cases compared with the controls. In this case, any polymorphism that varies in frequency between the ethnic groups will appear to be associated with COPD.

One way to avoid potential confounding factors due to ethnic differences between cases and controls in population-based association studies is to use a "family-based association study". Family-based association tests are carried out on families that have at least one affected offspring and a transmission linkage disequilibrium test (TDT) is applied. A TDT compares the number of times heterozygous parents transmit a specific allele to affected offspring. If the transmission frequency differs from that expected by chance, then linkage disequilibrium exists, meaning, the gene is associated with the disease. An advantage of the TDT is that it tests for both an association and linkage. As the transmitted and nontransmitted alleles are from the same parent, there is perfect ethnic matching and the possibility of spurious results due to ethnic differences can be

avoided. However, as mentioned previously, it is hard to perform family-based studies when investigating genetic risk factors for COPD.

Numerous association studies have been performed to assess candidate genes that may predispose individuals to COPD (Table 2.1). Although researchers are limited to the assessment of genes that have already been described when performing association studies, associations between variants of a number of genes and COPD have been reported. A few reviews have been written summarizing COPD genetics and associations studies.[117-119] Early enthusiasm about COPD association studies has been substituted with pessimistic perspectives because replication of these studies has proven to be difficult. Given the limitations in sample sizes and power in each study it may not be possible to replicate the results of many of the published studies. Moreover, allele frequencies of the genes may be highly variable between different ethnic groups and populations or the original reports may represent false-positive associations or linkages.

2.3 FUTURE DIRECTIONS

Until recently, genome-wide association studies were not feasible. Recent progress in genotyping techniques and the availability of closely spaced SNP markers on the genome facilitate new opportunities in exploring the impact of functional candidate genes. The increasing efficiency of high throughput techniques makes genome-wide association studies feasible. In addition, the SNP-marker system with 2-3 million of biallelic polymorphisms will facilitate this strategy which requires at least 500 000 equidistant polymorphic markers for a genome-wide coverage. Furthermore, the method of genomic controls will help to exclude stratification artifacts.[120] One obstacle is that, in order to ensure sufficient statistical power, very large case-control populations are necessary. With these parameters in place, it is expected that in the near future the detection of multiple disposition genes with modest or small effects can be identified by this strategy.

Table 2.1 Candidate genes that have been tested for an association with COPD.

Genes
ABH secretor, Lewis, and ABO blood groups[121-126]
Alpha1-antichymotrypsin[127-130]
Alpha-1 antitrypsin[97, 101, 102, 127, 131-151]
Beta-2-adrenergic receptor[152]
Calcium-dependent chloride channel-1[153]
Cystic fibrosis transmembrane regulator[154-156]
Cytochrome P-450[157, 158]
Glutathione S-transferase M1[159-164]
Glutathione S-transferase T1[159, 160, 163, 164]
Glutathione S-transferase P1[159, 163-167]
Heat shock protein 70[168]
Heme oxygenase-1[164, 169]
Human leukocyte antigens[125, 170, 171]
Human beta-defensin 1[172]
Immunoglobulin deficiency[173-176]
Interleukin-1β and interleukin-1 receptor antagonist[177, 178]
Interleukin-4 receptor antagonist[178]
Interleukin-13 and interleukin-13 receptor antagonist 1[178, 179]
Mannose-binding lectin[180]
Matrix metalloproteinase 1[181]
Matrix metalloproteinase 2[182]
Matrix metalloproteinase 9[181, 183]
Matrix metalloproteinase 12[181]
Microsomal epoxide hydrolase[159, 160, 166, 184-186]
Surfactant protein gene A, B, and D[187]
Toll-like receptor 4[188]
Transforming growth factor-beta 1[189, 190]
Tumour necrosis factor[177, 186, 191-200]
Vascular endothelial growth factor[201]
Vitamin D binding protein[202-206]

CHAPTER 3. COPD SUSCEPTIBILITY GENES

Genes directly implicated in the pathogenesis of COPD can be divided into four main categories based on function: 1) proteinases and antiproteinases; 2) xenobiotic metabolizers; 3) those involved in regulating the inflammatory response to cigarette smoke; and 4) those involved in the efficiency of mucociliary clearance in the lung.[106] Genes that do not directly influence COPD pathogenesis but may modify the disease phenotype are also important to consider when looking for susceptibility genes. Other genes of interest include substance abuse genes, and genes that influence susceptibility to allergies and infections.

3.1 DEFENSINS

Defensins play an important role in infection and inflammation in the lung and have the ability to directly and indirectly cause lung tissue injury (Figure 3.1). Increased defensin expression may increase individuals' susceptibility to COPD. A number of genetic variants have been identified in the human defensin genes.

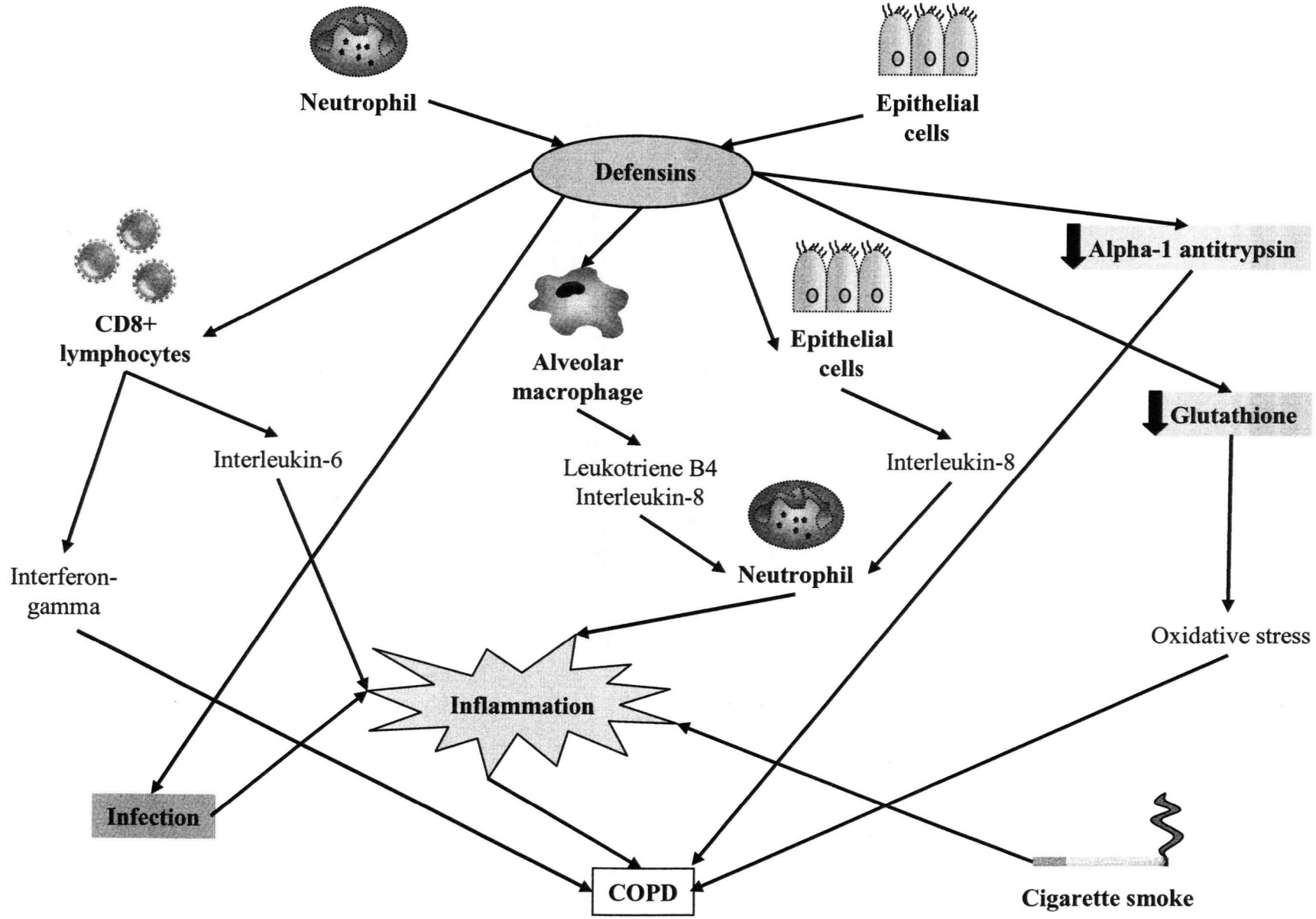


Figure 3.1 Role of defensins in chronic obstructive pulmonary disease (COPD).

3.1.1 Overview

Defensins are small (29-47 amino acids) cationic microbicidal peptides that are believed to be part of both the innate and adaptive immune responses. Defensins show activities against Gram-positive and Gram-negative bacteria, fungi, yeast, and enveloped viruses.[207] Defensins, or similar peptides, are found in mammals, birds, insects, and plants[208] and are known primarily for their antimicrobial activities. However, evidence indicates that the scope of their activities extends beyond immune responses (Figure 3.2).

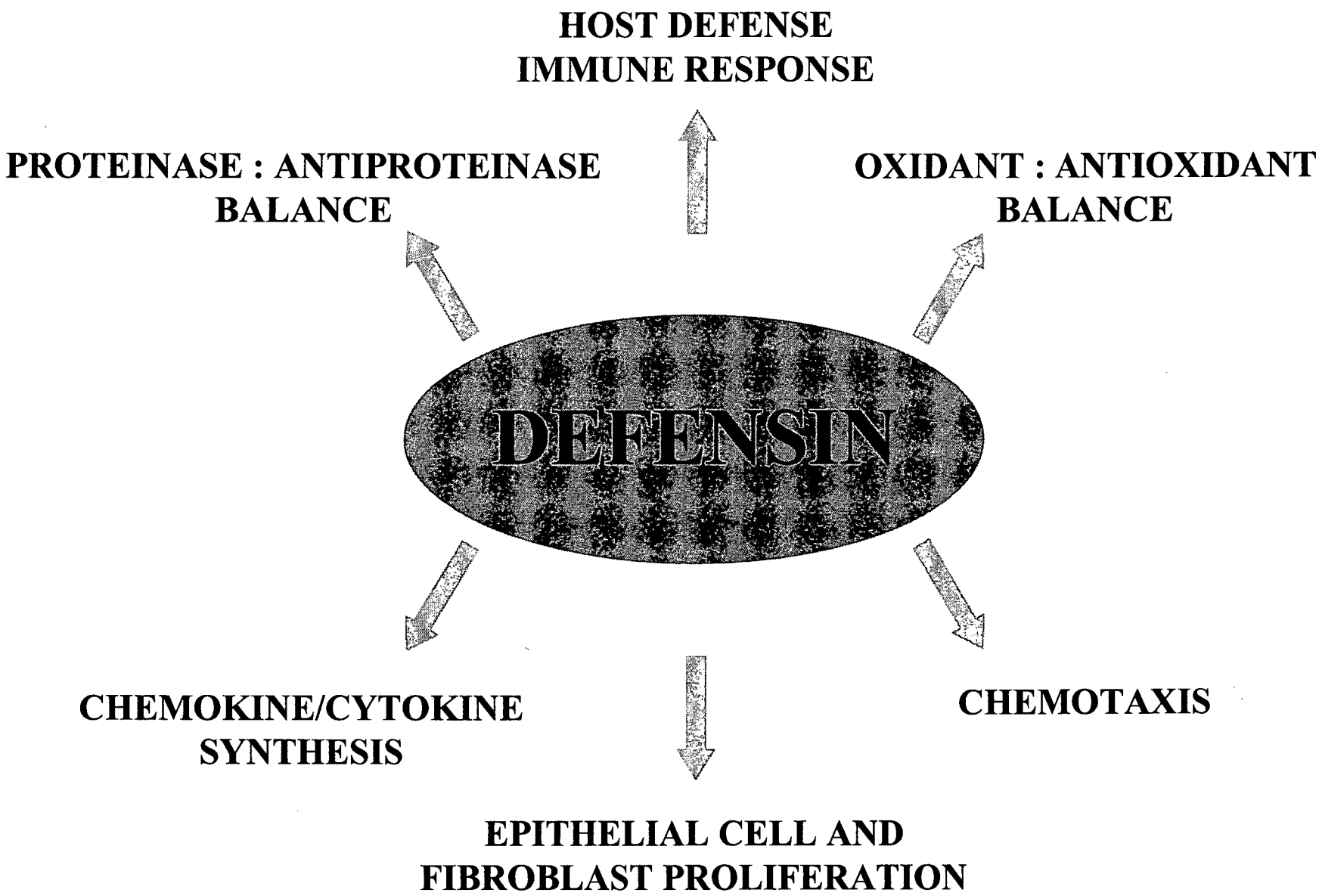


Figure 3.2 Role of defensins.

Defensins are salt sensitive peptides; high salt concentrations inhibit their antimicrobial activity (e.g., in the lungs of cystic fibrosis patients and in vitro > 75 mM NaCl). In vitro, the degree of antimicrobial and/or viricidal properties exhibited also varies depending on defensin concentration. The details of defensins' antimicrobial mechanism still need to be explored, however, it is known that defensins exert their antimicrobial activity by disrupting bacterial membranes and it is clear that membrane interaction is essential for antimicrobial defensin function.[209] Defensins act on microbes by permeabilizing the membrane of the target, either through an electrostatic charge-based mechanism[210] or the formation of multimeric transmembrane pores.[211] Defensin dimers interact to form pores in lipid bilayers. It has been suggested that a minimum number of defensin dimers must interact, forming multimers, for a pore to form.[212] During membrane permeabilization, defensins become internalized. Cell death may be achieved through one or a combination of the following methods: 1) defensin-induced DNA damage; 2) interference with protein synthesis; and/or 3) defensin-induced leakage of the cellular contents.[213] One proposed killing mechanism is depicted in Figure 3.3.

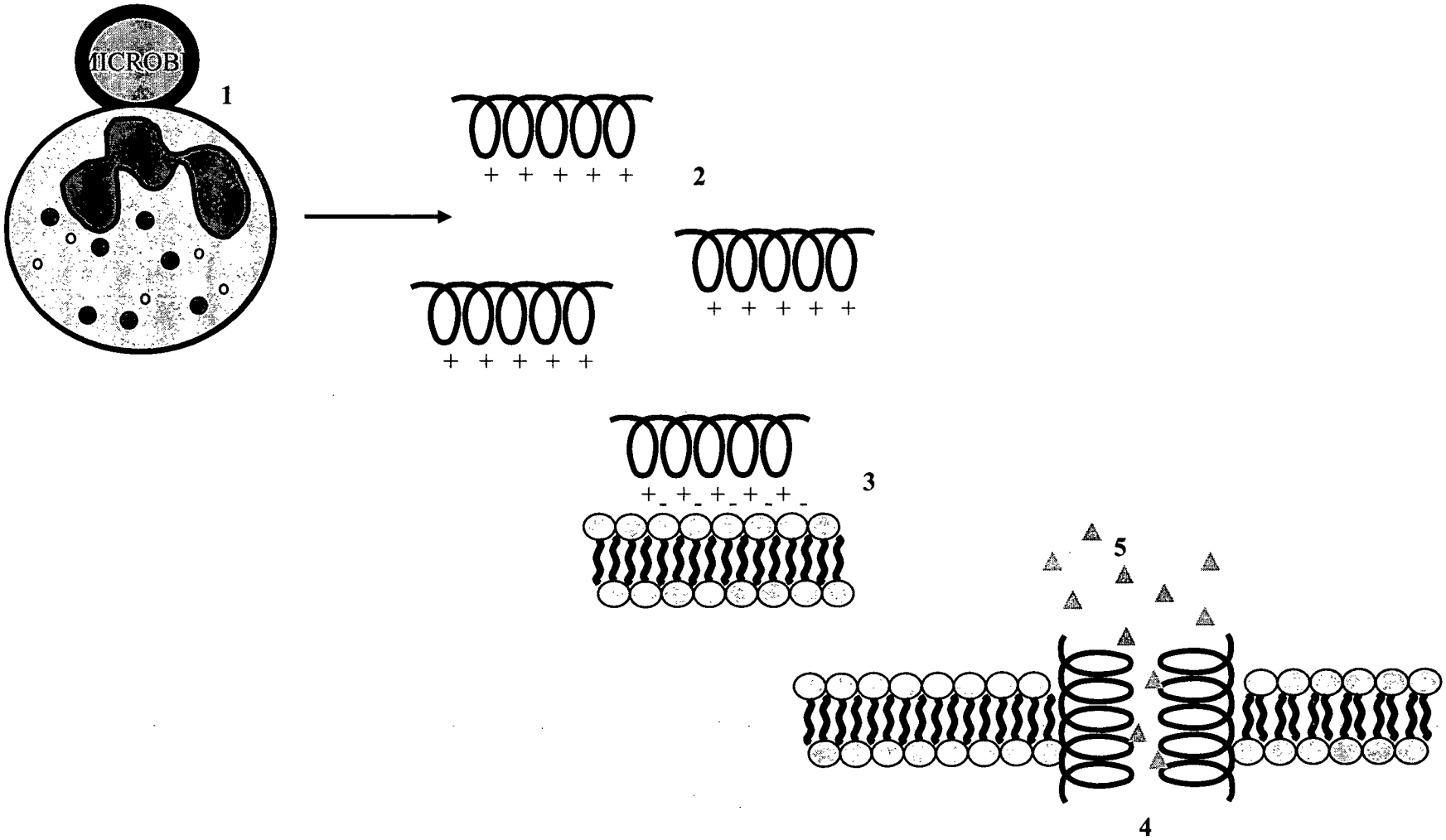


Figure 3.3 Proposed defensin killing mechanism.

1. Microbes activate the cell. 2. Positively charged defensins are released into the extracellular space. 3. Positively charged defensins bind to the negatively charged external surface of the microbes lipid bilayer which leads to localized thinning of bilayer. 4. Defensin dimers interact and insert into the microbe's membrane forming multimeric transmembrane pores. 5. Cytoplasmic molecules leak out of the cell and cell death occurs.

Based on a characteristic three-dimensional fold and a 6-cysteine/3-disulfide pattern, human defensins are divided into two groups, alpha- and beta- defensins. The alpha-defensin 6-cysteine/3-disulfide pattern is 1-6, 2-4, and 3-5; beta-defensins are connected 1-5, 2-4, and 3-6. Although alpha- and beta-defensins adopt very similar molecular conformations, they differ at the level of gene, cDNA, and prepropeptide sequences.[214] Until recently, the human alpha- and beta-defensin genes were thought to be limited to 450 kb region on chromosome 8p23.[215] In humans, five alpha-defensins and at least four beta-defensins cluster in this region (Figure 3.4). This organization supports the hypothesis that members of the defensin family evolved by gene duplication and divergence from a common ancestor. Genome wide search strategies have been employed to identify new members of the defensin family that reside at different chromosomal locations. In the past few years, new human beta-defensin genes have been identified.

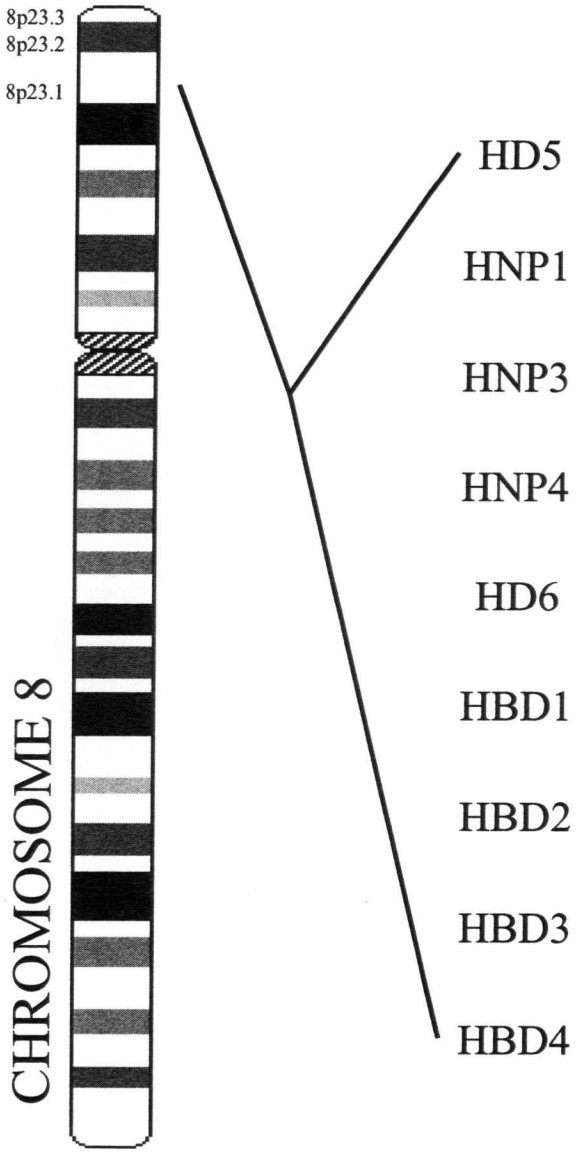


Figure 3.4 Organization of human defensin genes on chromosome 8 in a 450 kb region.
HD denotes human defensin; HNP, human neutrophil peptide; HBD, human beta-defensin; and kb, kilo base.

Four alpha-defensins have been characterized, human neutrophil peptides (HNP) - HNP1, HNP2, HNP3, and HNP4. They are major constituents (30-50% of the total protein content) of neutrophil azurophilic granules.[216] HNP 1-4 contain six conserved cysteine residues that participate in three characteristic intramolecular disulfide bridges that define them as alpha-defensins. The intramolecular disulfide bridges are considered essential for their antimicrobial activity. During neutrophil development, inactive 93 to 100 amino acid prepropeptides must undergo proteolytic processing to form the mature defensins (29 to 35 amino acids in length) (Figure 3.5).[217, 218] Early processing steps involve the formation of intermediate prodefensin forms.[219] Some alpha-defensin propeptides are activated by MMP7.[220] The formation of propeptides is unlikely to be of importance for the targeting of defensins to azurophilic granules. Rather, propeptides may prevent autotoxicity, misfolding, or retention of the mature defensins during their passage through the biosynthesis machinery of the cell.[221-223] HNP1, HNP2, and HNP3 are closely related; they differ by only one amino acid. HNP1 and HNP3 are 30 amino acids long and differ only at the N-terminal amino acid, which is Ala in HNP1 and Asp in HNP3.[224] It is believed that 29 amino acid long HNP2 is the proteolytic product of HNP1 and/or HNP3 as it begins with Cys, the second amino acid of both HNP1 and HNP3; HNP1 and HNP3 are encoded by separate genes.[224] The sequence similarities of HNP1, HNP2, and HNP3 are shown in Figure 3.5. HNP4 shares only 72% identity with HNP1 and HNP3 at the cDNA level[224] and the other three HNPs are approximately 100 times more abundant than HNP4.[225] Two other alpha defensins, human defensins (HD5 and HD6), are expressed in the Paneth cells of the small intestine.[226, 227]

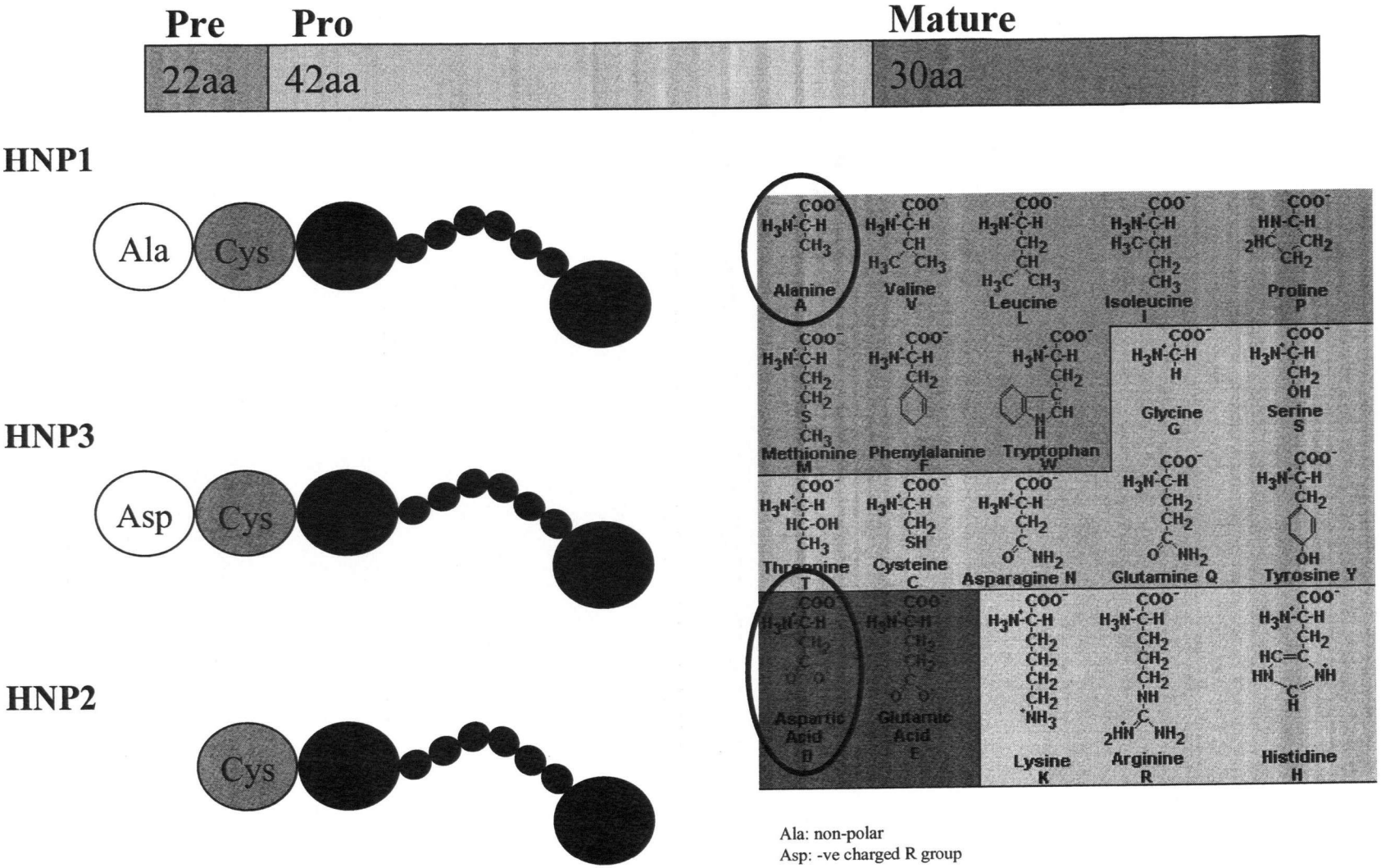


Figure 3.5 Human neutrophil peptides 1-3.
During neutrophil development, inactive 94 amino acid prepropeptides must undergo proteolytic processing to form the mature peptides. HNP1, HNP2, and HNP3 are closely related; they differ by only one amino acid. HNP1 and HNP3 are 30 amino acids long and differ only at the N-terminal amino acid, which is Ala in HNP1 and Asp in HNP3. It is believed that 29 amino acid long HNP2 is the proteolytic product of HNP1 and/or HNP3 as it begins with Cys, the second amino acid of both HNP1 and HNP3. AA denotes amino acid; HNP, human neutrophil peptide; Ala, alanine; Asp, aspartic acid; and Cys, cysteine.

Until recently, only four human beta-defensins (HBD1, HBD2, HBD3, and HBD4) were described.[228-234] The genes encoding these peptides have two exons and one 4 kb intron. HBDs are 36 to 42 amino acids in length. Similar to alpha-defensins, beta-defensins are synthesized as 64 to 68 amino acid long prepropeptides. The 26 to 32 amino acids at the N-terminus compromise putative signal peptide and propeptide segments. The intracellular processing, storage, and release pathways remain to be defined. HBDs are expressed in the epithelial cells of several organs and play a role in the antimicrobial defense of these epithelial surfaces. HBD1 is most abundant in the kidney and HBD2 and HBD3 are most abundant in inflamed skin. All three are expressed in conducting and respiratory airway epithelium as well.[231, 235-237] In vivo and in vitro evidence suggests that HBD2-4 are upregulated by inflammatory stimuli such as specific microorganisms, IL-1beta, TNF-alpha, and interferon-gamma,[230-234, 238-241] however, HBD1 expression is not inducible.[236] The mature HBD1 peptide is 36 amino acids long. Multiple isoforms of HBD1, ranging in length from 36 to 47 amino acids, have been identified that represent N-terminal extensions of the 36 amino acid peptide and the isoforms differ in their antimicrobial activity.[229]

As defensins exist as large families in a number of species and there is a high frequency of gene duplications within the defensin gene cluster, it was suspected that more defensin genes existed. With the help of the human genome sequence and new computational methods, new genes have been recently identified. Genome wide search strategies identified twenty-eight new HBD genes.[242] New regions identified containing clusters of HBD genes are chromosomes 6p, 20p, and 20q.[242] There is no experimental evidence to verify whether these genes are expressed or associated with microbicidal activity. This may be also be a gross overestimate as some of these putative twenty-eight genes may not belong to the HBD family. Kao et al.[243] found that the amino acid spacing between the highly conserved cysteine residues of the six-cysteine motif is very different in some of the putative HBD genes from the known spacing in well-characterized HBD genes. In 2003, six novel HBD genes were identified and the expression pattern of these genes was confirmed.[243] Three of the genes are clustered on chromosome 8p23.1. Two others are localized to chromosome 20q11.21 and 20p13; one newly identified gene has an ambiguous chromosomal location. Expression analysis revealed that two of the novel

HBDs are expressed in the lung. Proper translation start sites were identified for five of the newly found HBDs suggesting that these genes are translated into proteins. Again, the clustering of genes and sequence similarities supports the idea that defensin genes evolve by gene duplication and divergence from a common ancestor.

Patients with COPD have recurrent lower respiratory tract infections. Cigarette smoke may impair the innate and adaptive immune responses of the airway epithelium leading to an increased susceptibility to infection. Evidence suggests that respiratory infection may account for 5-70% of exacerbations in COPD.[244] Thirty percent of infectious exacerbations may be due to viruses (influenza/parainfluenza, adenoviruses [non-enveloped], and rhinovirus [non-enveloped]) and atypical microorganisms, the remaining 70% are attributed to pathogenic bacteria. Defensins may not play an important role in fighting viral infections in the lung as they only inactivate enveloped viruses. Furthermore, Daher and coworkers[209] tested the antiviral activity of HNP1, HNP2, and HNP3 against influenza virus and observed that influenza virus A/WSN was inactivated by incubation with HNP1; incubation with HNP2 and HNP3 did not result in inactivation. On the other hand, defensins are effective at fighting bacterial infections. The importance of bacterial infection in exacerbations of COPD is highlighted by trials of antibiotic therapy. Patients with exacerbations recover faster with antibiotic therapy than without, especially if the exacerbations are accompanied by purulent sputum.[245, 246]

Haemophilus influenzae is the most frequent bacterium isolated from patients with COPD, followed by *Moraxella catarrhalis*, *Streptococcus pneumonia*, and *Pseudomonas aeruginosa*.[244, 247-249] Sethi et al.[250] studied sputum specimens from patients with COPD during stable periods and exacerbations. A significant association between the appearance of *Streptococcus pneumoniae* or *Moraxella catarrhalis* in sputum cultures and exacerbations was found. In addition, exacerbations were associated with changes in bacterial strain as opposed to the simple presence or absence of a bacterial strain in sputum cultures. Studies have shown that as the bacterial load increases, the local inflammatory response of the host increases,[251] which may potentially cause damage to the lungs. Defensins' antimicrobial properties contribute to the generation of local inflammatory signals and lead to an amplified inflammatory response by recruiting additional leukocytes, specifically CD4+ and CD8+ T lymphocytes,[252-254] to the site of

infection. The ability to recruit CD4+ and CD8+ T lymphocytes amplifies the inflammatory response and provides a bridge between innate and adaptive immune responses.

A few studies have failed to demonstrate the role of respiratory illness in a change in FEV1.[255-257] In contrast, in some studies a more rapid decline in FEV1 was associated with more frequent episodes of lower respiratory infection.[258] Furthermore, Kanner and coworkers[259] investigated interactions between lower respiratory illness and smoking status on the annual change in lung function and found that active smokers with lower respiratory illness had a significant increase in rate of decline in FEV1 over a five year period suggesting that respiratory infections may accelerate disease progression in smokers.

Defensins may also contribute to bacterial colonization in COPD patients. In vitro, defensins were found to increase the adherence of *H. influenzae* to cultured epithelial cells.[260] However, it is important to note that this defensin-stimulated adherence may actually aid in the removal of the bacteria. When adhered to the epithelium the bacteria will be exposed to high levels of epithelia-derived antimicrobial peptides such as HBDs and secretory leukocyte proteinase inhibitor.[261] If genetic variation impairs the function of these antimicrobial peptides then this would have a negative effect.

Studies have shown that defensins are capable of inhibiting the antiproteinase activity alpha-1 antitrypsin.[262] The defensin-mediated inactivation of alpha-1 antitrypsin may leave the lung susceptible to the injurious actions of neutrophil elastase. However, defensins increase the release of secretory leukocyte proteinase inhibitor, another elastase inhibitor, by airway epithelial cells, which may help protect the lung when alpha-1 antitrypsin levels are low.[263] Elevated levels of both alpha-1 antitrypsin and secretory leukocyte proteinase inhibitor can be detected in the BAL fluid of patients with COPD and emphysema.[264, 265]

Defensins modulate the proliferation of epithelial cells and fibroblasts.[266] They also stimulate epithelial cells to produce chemokines, such as IL-8, promoting neutrophil infiltration.[267] In addition, alveolar macrophages are stimulated by defensins to produce leukotriene B4 and IL-8.[268] Other studies have shown that neutrophil defensins display chemotactic activity for monocytes[269] and T-lymphocytes.[254] In addition to their

chemotactic activity for T cells, evidence suggests that defensins induce interferon-gamma, IL-6, and IL-10 release by T cells.[270] In mice, interferon-gamma released from CD8+ cells caused emphysema and contributed to the proteinase:antiproteinase imbalance.[67] IL-6 is an inflammatory cytokine; increased levels have been detected in patients with COPD.[271] On the other hand, IL-10 is a cytokine with a wide spectrum of anti-inflammatory actions, which also regulates the release of proteinases and antiproteinases by airway macrophages; therefore, it is of interest as a therapeutic agent for COPD.[272]

An oxidant-antioxidant imbalance may contribute to the pathogenesis of COPD. There are signs of increased oxidative stress and a decreased antioxidant capacity in the lungs of COPD patients.[273, 274] Defensins may disrupt this balance by reducing glutathione levels in airway epithelial cells.[275]

I am interested in the role of polymorphisms in HNP1-3 and HBD1 in promoting FEV1 decline in subjects with COPD (Table 3.1). Mars and coworkers[224] have shown that individuals can inherit variable numbers of copies of HNP1 and HNP3, and that HNP3 is often absent. Several polymorphisms of HBD1 have been reported.[276-278] The frequency of coding region SNPs is low compared to SNPs in the promoter, 5'-untranslated region (5'UTR), and 3'-untranslated region (3'UTR). Of the reported polymorphisms, three SNPs in the 5'UTR region of the HBD1 gene have minor allele frequencies greater than twenty percent in a Caucasian population.[277] The SNP at position -20 (-20G→A) in the 5'UTR results in the formation of an nuclear factor-kappaB transcription factor-binding sequence, however, as HBD1 is constitutively expressed and this site is in the 5'UTR, the functional impact is unclear.[278] The only association reported with the three common 5'UTR polymorphisms thus far is an association with the SNP at position -44 (-44C→G) with *Candida* carriage in type I diabetics.[279] A newly detected SNP in exon 2 (Val38Ile) of HBD1 was found to be significantly associated with COPD in a Japanese population.[172] I genotyped 306 Caucasian subjects for this SNP and found only one heterozygous subject (unpublished data) suggesting that this polymorphism is very rare (<1%) in the Caucasian population. As demonstrated by Jurevic et al.,[278] HBD1 SNPs show frequency differences between ethnic groups,

underscoring the fact that it is important to control for ethnicity when performing genetic association studies.

Table 3.1 Human neutrophil peptide 1-3 and human beta-defensin 1 polymorphisms.

Gene	Polymorphism	Effect
HNP1-3	Varying numbers of HNP1-3 genes	Unknown; possibly increased defensin production
HBD1	-20G→A (5'UTR)	Formation of transcription factor-binding sequence[278]
	-44C→G (5'UTR)	Unknown
	-52G→A (5'UTR)	Unknown
	Val38Ile (exon 2)	Unknown; associated with COPD in Japanese population[172]

The abbreviation HNP denotes human neutrophil peptide; and HBD, human beta-defensin.

3.1.2 Genetic susceptibility to chronic obstructive pulmonary disease: contribution of human defensins to decline in lung function and infection in the Lung Health Study participants

3.1.2.1 Introduction

Alpha-defensins (major constituents of neutrophil azurophilic granules) and beta-defensins (expressed in conducting and respiratory airway epithelium) contribute to the pathogenesis of COPD by amplifying the cigarette smoke-induced and the infection-induced inflammatory reactions leading to tissue injury.

3.1.2.2 Hypothesis

I hypothesized that specific polymorphisms of the alpha- and beta-defensin genes increase smokers' susceptibility to a rapid decline in lung function and infection.

3.1.2.3 Specific aims

Due to a copy number polymorphism, individuals inherit variable numbers of HNP1 and HNP3.[224] It has also been shown that although some individuals inherit at least one copy of both the HNP1 and the HNP3 genes, other individuals only inherit copies of HNP1.[224] In this study I determined whether individuals inherited both HNP1 and HNP3 or HNP1 only and looked at the frequency of the copy number polymorphism in two groups of participants in the Lung Health Study (fast decliners and non-decliners). I further investigated whether having more copies of HNP1 and/or HNP3 was correlated with an increase in defensins in neutrophils. I also determined the frequency of three HBD1 5'UTR polymorphisms in fast decliners and non-decliners from the Lung Health Study. In addition, I investigated the relationship between the three HBD1 SNPs and individuals' susceptibility to lower respiratory infection.

3.1.2.4 Methods

PART I: ASSOCIATION STUDIES

Study subjects

Subjects were selected from participants in Phase I of the NHLBI Lung Health Study. Details of the study have been previously published.[280] Study participants were current smokers, 35-60 years of age, with mild to moderate airflow obstruction (FEV1 55–90% predicted and FEV1/FVC $\leq$ 0.70). Exclusion criteria included serious illnesses such as cancer, heart attack or stroke, or other important conditions that required medical treatment. The primary outcome variable was rate of decline in FEV1 over a follow-up period of five years. Annually, participants were asked to report on episodes of bronchitis, pneumonia, influenza, or chest colds resulting in physician visits during the previous year. Of the 3216 continuing smokers in this cohort, 275 were chosen with a fast decline in lung function (decline in percent predicted FEV1>3.0% per year), and 304 were selected with no decline in lung function over the same period (increase in percent predicted FEV1>0.4% per year); all 579 selected participants were Caucasian (white, non-Hispanic). In addition, 27 African American Lung Health Study participants were genotyped; the data were used to determine the prevalence of genotypes across racial groups.

DNA extraction

Genomic DNA was isolated from frozen whole blood with a standard phenol/chloroform protocol.[281]

Polymerase chain reaction (PCR) to distinguish HNP1 and HNP3 genes

Restriction fragment length polymorphism analysis was performed in order to distinguish the HNP1 and HNP3 genes. The analysis was performed as previously described.[215] An amplicon of 950 bp was generated by 35 cycles of PCR using the sense primer 5'-CAGCGGACATCCCAGAAGTGG and the antisense primer 5'-GCGTTTTGGTACGTGTATCC. PCR were performed in a total reaction volume of 20 µL with 100 ng of genomic DNA, 0.5 U *Taq* polymerase (Invitrogen), 10X PCR buffer (Invitrogen), 3 mM Mg^{2+}, 0.4 µM forward and reverse primers, and 200 µM dNTPs, with

an annealing temperature of 57°C. After PCR, 20 μL of the reaction mixture was digested with 1.25 U *Hae* III restriction endonuclease (New England BioLabs Inc., Beverly, MA). The digest mixture was resolved on a 2% agarose gel stained with ethidium bromide. DNA from individuals with HNP1 only produced two bands, one at 300 bp and one at 650 bp; individuals with HNP3 only produced one band at 950 bp; and individuals with both HNP1 and HNP3 produced all three bands (Figure 3.6).

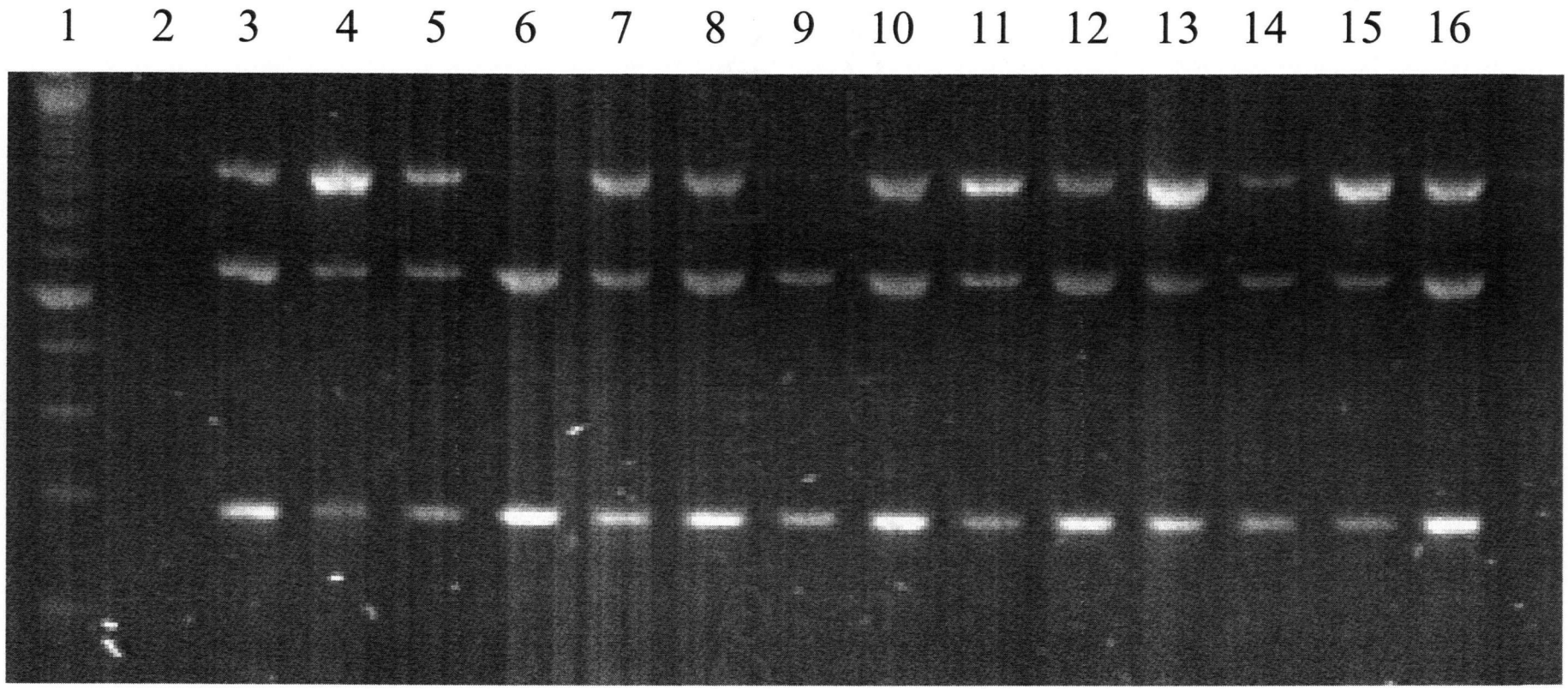


Figure 3.6 The PCR-RFLP method was used to distinguish the HNP1 and HNP3 genes.

The PCR product was digested with the *Hae* III restriction endonuclease. After digestion, the digest mixture was resolved on a 2% agarose gel stained with ethidium bromide. Lane 1, size marker (100 bp DNA Ladder from New England BioLabs); lane 2, negative control, no bands produced; lanes 3, 4, 5, 7, 8, and 10-16, individuals with copies of both HNP1 and HNP3 genes; and lanes 6 and 9, individuals with copies of the HNP1 gene only. HNP denotes human neutrophil peptide.

HBD1 genotyping

Genotyping of the polymorphisms at positions -20, -44, and -52 in the 5'UTR were performed by restriction fragment length polymorphism analysis. An amplicon of 260 bp was generated by 35 cycles of PCR using the sense primer 5'-GTGGCACCTCCCTTCAGTTCCG and the antisense primer 5'-CAGCCCTGGGGATGGGAAACTC. PCRs were performed in a total reaction volume of 60 μL with 100 ng of genomic DNA, 0.5 U *Taq* polymerase (Invitrogen), 10X PCR buffer (Invitrogen), 1.5 mM Mg^{2+}, 0.75 μM forward and reverse primers, and 200 μM dNTPs, with an annealing temperature of 67°C. After PCR, 20 μL of the reaction mixture was digested with 2 U *Scr* FI restriction endonuclease (New England BioLabs Inc.) to detect variation at position -20. The digest mixture was resolved on a 3% agarose gel stained with ethidium bromide. DNA from individuals with the homozygous G genotype (GG) produced three bands at 136 bp, 118 bp, and 6 bp; the homozygous A genotype (AA) produced two bands, one at 254 bp and one at 6 bp; and the heterozygous genotype (GA) produced all four bands (Figure 3.7). 20 μL of the reaction mixture was digested with 4 U *Hga* I restriction endonuclease (New England BioLabs Inc.) to detect variation at position -44. The digest mixture was resolved on a 3% agarose gel stained with ethidium bromide. DNA from individuals with the homozygous C genotype (CC) produced two bands, one at 71 bp and one at 189 bp; the homozygous G genotype (GG) produced three bands at 71 bp, 30 bp, and 159 bp; and the heterozygous genotype (CG) produced all three bands (Figure 3.8). 20 μL of the reaction mixture was digested with 2 U *Nla* IV restriction endonuclease (New England BioLabs Inc.) to detect variation at position -52. The digest mixture was resolved on a 3% agarose gel stained with ethidium bromide. DNA from individuals with the homozygous G genotype (GG) produced three bands at 108 bp, 122 bp, and 30 bp; the homozygous A genotype (AA) produced two bands, one at 230 bp and one at 30 bp; and the heterozygous genotype (GA) produced all four bands (Figure 3.9).

Template-free controls and known genotype controls were included in each experiment. Twenty samples were selected at random and sequenced to confirm the genotyping protocols. Genotypes were assigned by two independent investigators who were unaware

of the patients' identities and phenotypes. Inconsistencies were resolved by two additional genotyping reactions.

Statistical analysis

An association with rate of decline in lung function was assessed by the chi-square test. The associations were also analyzed by multiple logistic regression to adjust for potential confounding factors. Age, sex, smoking history (pack year), methacholine response, and initial level of lung function (pre-bronchodilator FEV1 percent predicted) were included as covariates in the multivariate model. Haplotypes were estimated for each subject using PHASE version 2.0.[282, 283] Arlequin version 2.0[284] (http://anthro.unige.ch/arlequin) was used to test for Hardy-Weinberg equilibrium and to test for linkage disequilibrium. Lower respiratory infection rate was quantified using the number of self-reported visits at each follow-up in secondary outcomes analysis. All tests were performed using the JMP Statistics software package (SAS Institute Inc., Cary, NC). Statistical significance was defined at the standard 5% level.

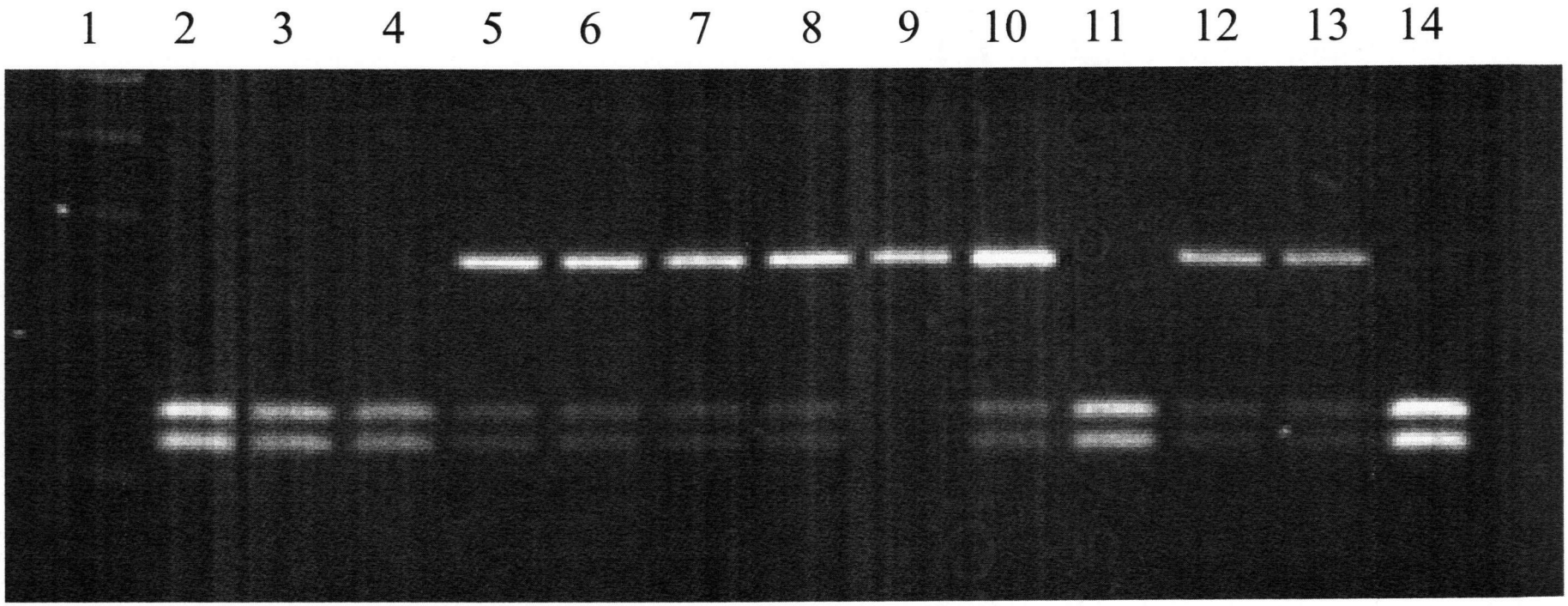


Figure 3.7 Genotyping of the HBD1 -20G→A SNP.

The PCR product was digested with the *Scr* FI restriction endonuclease. After digestion, the digest mixture was resolved on a 3% agarose gel stained with ethidium bromide. Lane 1, size marker (100 bp DNA Ladder from New England BioLabs); lanes 2, 3, 4, 11, and 14, homozygotes for the G allele; lanes 5-8, 10, 12, and 13, heterozygotes for the G and A alleles; and lane 9, homozygote for the A allele. HBD1 denotes human beta-defensin 1.

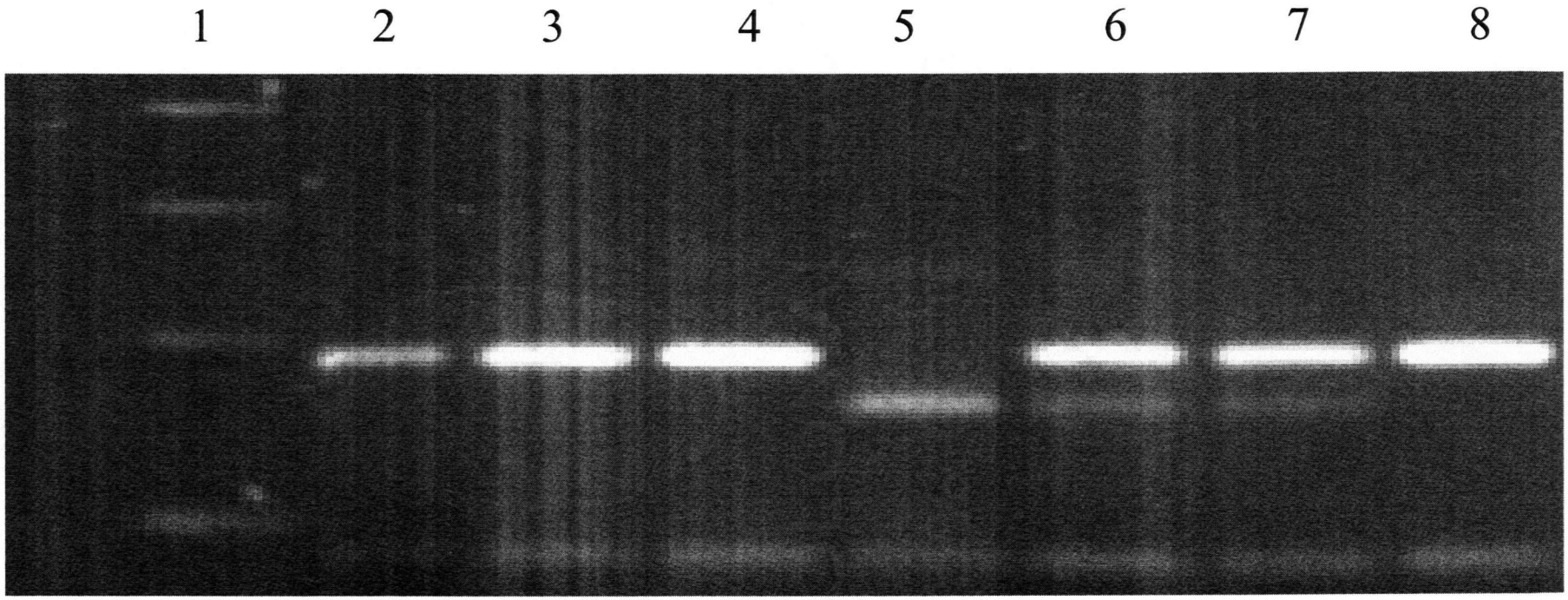


Figure 3.8 Genotyping of the HBD1 -44C→G SNP.

The PCR product was digested with the *Hga* I restriction endonuclease. After digestion, the digest mixture was resolved on a 3% agarose gel stained with ethidium bromide. Lane 1, size marker (100 bp DNA Ladder from New England BioLabs); lanes 2-4, and 8, homozygotes for the C allele; lane 5, homozygote for the G allele; and lanes 6 and 7, heterozygotes for the C and G alleles. HBD1 denotes human beta-defensin 1.

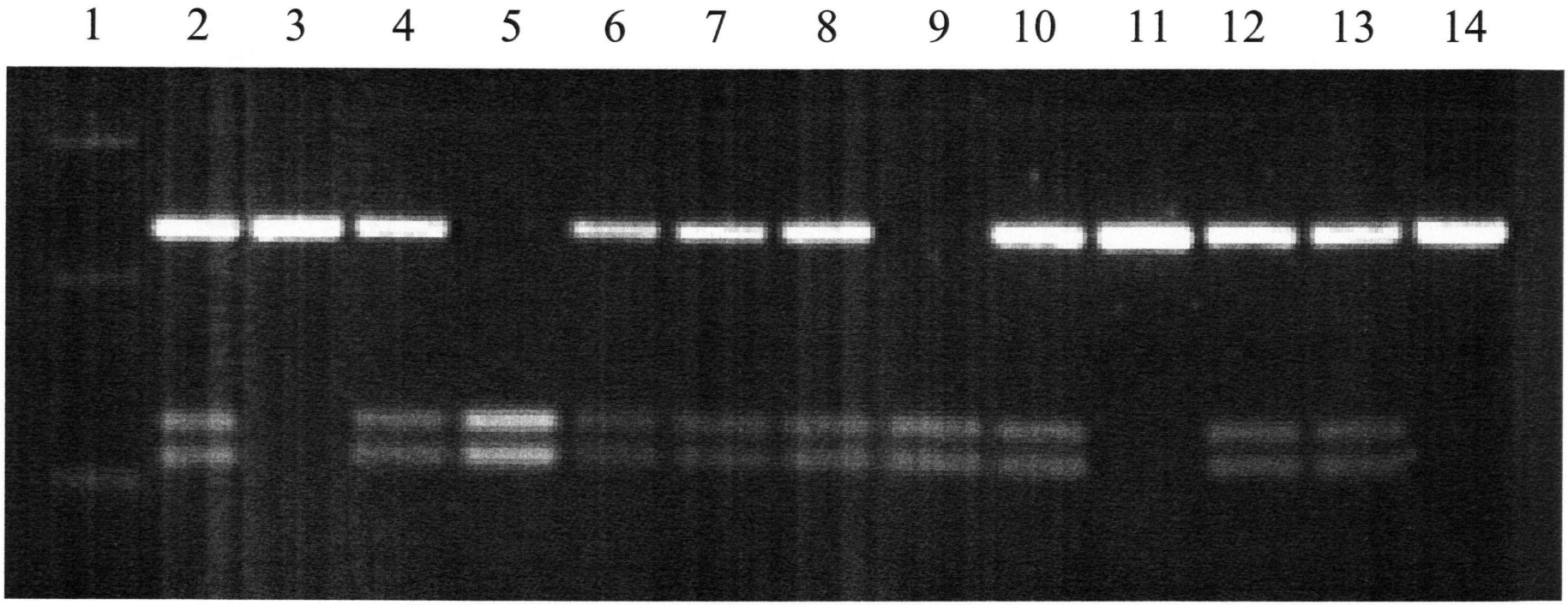


Figure 3.9 Genotyping of the HBD1 -52G→A SNP.

The PCR product was digested with the *Nla* IV restriction endonuclease. After digestion, the digest mixture was resolved on a 3% agarose gel stained with ethidium bromide. Lane 1, size marker (100 bp DNA Ladder from New England BioLabs); lanes 2, 4, 6-8, 10, 12, and 13, heterozygotes for the G and A alleles; lanes 3, 11, and 14, homozygotes for the A allele; and lanes 5 and 9, homozygotes for the G allele. HBD1 denotes human beta-defensin 1.

PART II: HNP1-3 GENE COPY NUMBER AND LEVELS IN PERIPHERAL BLOOD NEUTROPHILS

Volunteers

Eighteen healthy Caucasian subjects were recruited for the study from University of British Columbia employees (7 women and 11 men; mean age 29; range 18-52 years). Four were current smokers with a cumulative smoking history of 5 to 10 pack years; fourteen were never smokers. Three of the subjects had asthma. All subjects were free of any other significant disease. One subject was receiving treatment with an anti-inflammatory agent. This study was approved by the University of British Columbia/Providence Health Care Research Ethics Boards. All subjects provided written informed consent.

DNA extraction

Genomic DNA was isolated from frozen whole blood using QIamp DNA Blood Midi Kit according to the manufacturer's protocol (Qiagen, Mississauga, ON).

HNP1-3 gene copy number determination

Quantitative real-time PCR, carried out on a LightCycler (Roche Molecular Biochemicals), was used to determine the gene copy numbers of HNP1-3 and the nuclear gene for the human polymerase gamma accessory subunit (ASPOLG). HNP1-3 and ASPOLG were quantified separately.

For HNP1-3, the PCR were performed using the sense primer 5'-CAAGAGCTGATGAGGTTGCTG and the antisense primer 5'-TAGACTGCTGAGACCCAACTC. The donor probe was labeled at the 3' end with fluorescein and contained the sequence 5'-GCATCCAGGTGAGAGAGGCAGGCATGC. The acceptor probe was labeled at the 5' end with LightCycler Red 640, was modified at the 3' end by phosphorylation to block extension, and contained the sequence 5'-AGCTGCTAAGTCTAGAGGGAAGGAC. Both fluorophore-labeled probes were synthesized and purified by reverse-phase HPLC by TIB Molbiol LLC (Adelphia, NJ). HNP1-3 real-time PCR were performed in a total reaction volume of 20 μL with 100 ng of genomic DNA, 0.3 μM forward and reverse

primers, 0.1 μM donor probe and 0.2 μM acceptor probe, 3 mM Mg^{2+}, and 2 μL LightCycler FastStart DNA Master Hybridization Probes Mix (Roche Molecular Biochemicals). The samples were loaded in the LightCycler glass capillaries, closed, centrifuged, and placed in the LightCycler rotor. The cycling program consisted of 10 minutes of initial denaturation at 95°C and 45 cycles at 95°C for 0 s, 60°C for 10 s, and 72°C for 5 s (ramp rates 20°C/s). The analytical melting program was 95°C for 0 s and 55°C for 10 s, increasing to 95°C at a ramp rate of 0.2°C/s, with continuous fluorescence acquisition.

For the nuclear DNA ASPOLG gene, the PCR were performed using the sense primer 5'-GAGCTGTTGACGGAAAGGAG and the antisense primer 5'-CAGAAGAGAATCCCGGCTAAG. The donor probe was labeled at the 3' end with fluorescein and contained the sequence 5'-GAGGCGCTGTTAGAGATCTGTCAGAGA. The acceptor probe was labeled at the 5' end with LightCycler Red 640, was modified at the 3' end by phosphorylation to block extension, and contained the sequence 5'-GGCATTTCCTAAGTGGAAGCAAGCA. Both fluorophore-labeled probes were synthesized and purified by reverse-phase HPLC by TIB Molbiol LLC (Adelphia, NJ). ASPOLG real-time PCR were performed in a total reaction volume of 20 μL with 100 ng of genomic DNA, 0.25 μM forward and reverse primers, 0.1 μM donor probe and 0.2 μM acceptor probe, 5 mM Mg^{2+}, and 2 μL LightCycler FastStart DNA Master Hybridization Probes Mix (Roche Molecular Biochemicals). The samples were loaded in the LightCycler glass capillaries, closed, centrifuged, and placed in the LightCycler rotor. The cycling program consisted of 10 minutes of initial denaturation at 95°C and 45 cycles at 95°C for 0 s, 60°C for 10 s, and 72°C for 5 s (ramp rates 20°C/s). The analytical melting program was 95°C for 0 s and 55°C for 10 s, increasing to 95°C at a ramp rate of 0.2°C/s, with continuous fluorescence acquisition.

A standard curve was included in each run. To generate the standard curves, PCR amplification was performed with serially diluted template. The data were analyzed by using the second-derivative maximum of each amplification reaction and relating it to its respective standard curve. The results from the quantitative PCR were expressed as the ratio of the mean HNP1-3 measurements to the mean ASPOLG value for a given sample (HNP1-3:ASPOLG). Each sample was run in triplicate.

Flow cytometry

Peripheral blood was drawn into tubes containing EDTA. HNPs are stored within neutrophil azurophilic granules; intracellular staining was performed with the use of FIX & PERM according to the manufacturer's instructions (Caltag Laboratories, Burlingame, CA). Cells were labeled with 1 μg/mL of anti-HNP 1-3 (BACHEM, Torrance, CA) and as control, 0.6 μg/mL non-immune mouse IgG1 (Sigma, Saint Louis, MO). Labeled cells were incubated for 15 minutes at room temperature with 1.3 μg/mL of fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse secondary antibody (Sigma) and fixed with 1% paraformaldehyde. The fluorescence intensity per cell was measured in a Beckman Coulter EPICS XL flow cytometer (Miami, FL). A total of 5000 events were analyzed. Each sample was run in duplicate. Forward light scatter versus side light scatter was used to identify granulocytes and exclude subcellular debris, lymphocytes, and monocytes (Figure 3.10). Data were recorded as frequency distribution histograms and mean fluorescence intensity units.

Statistical analysis

Relative copy number and protein level was compared by linear regression analysis. P values less than 0.05 were considered significant.

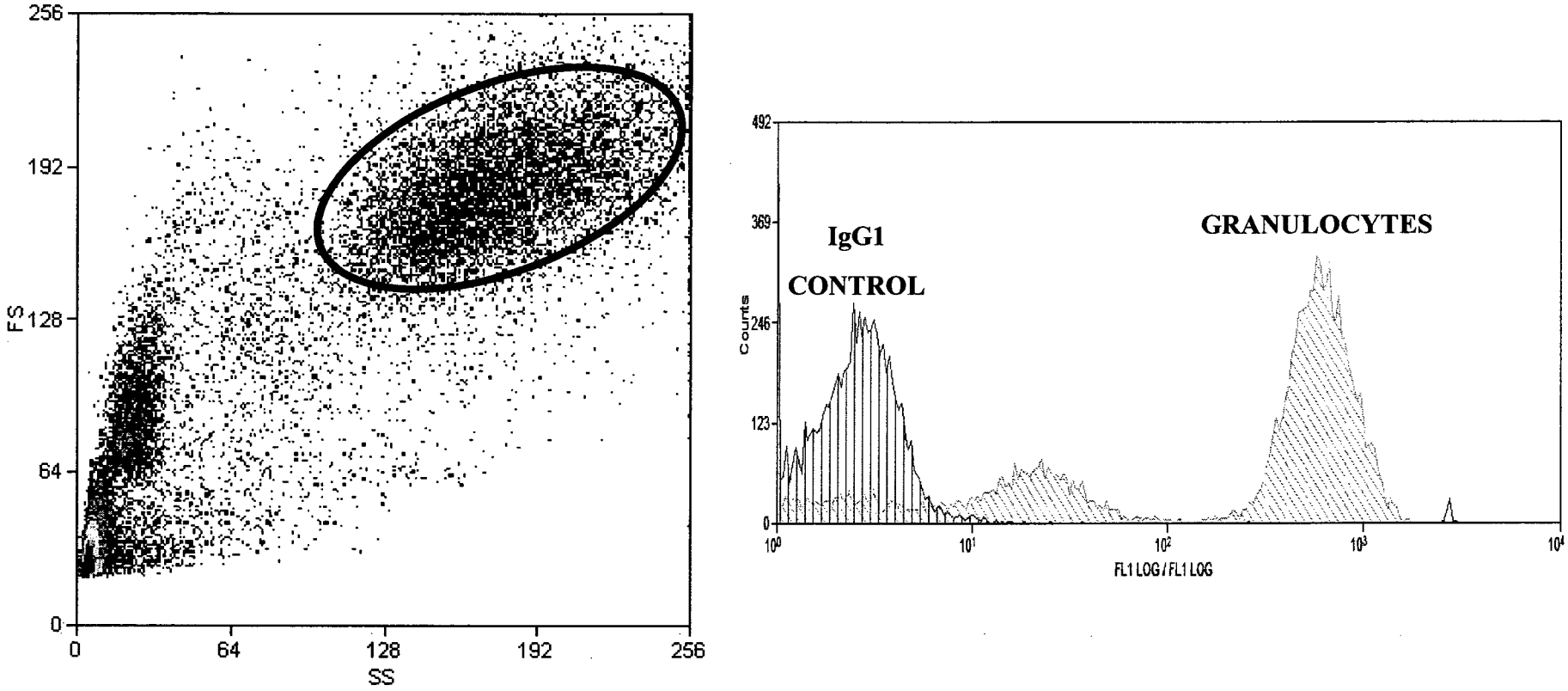


Figure 3.10 Flow cytometric analysis.

Forward light scatter versus side light scatter was used to gate on the granulocyte population (circle). FS denotes forward light scatter; and SS, side light scatter.

3.1.2.5 Results

PART I: ASSOCIATION STUDIES

General characteristics

The study population consisted of 579 Caucasian (white, non-Hispanic) smokers; 275 smokers with a fast decline in lung function and 304 smokers with no decline in lung function over the five year study period. The characteristics of the study group are described in Table 3.2. Age, sex, smoking history (pack year), baseline FEV1, and methacholine response differed significantly between the two groups.

Table 3.2 Description of the study population.

Variable	Fast decline (n=275)	No decline (n=304)	p Value*
Age, yr†	49.5 (6.4)	47.6 (6.9)	0.0007
Sex, n (%)	163 men (59)	203 men (67)	0.06
Smoking history, pk yrs†‡	43.3 (19.1)	38.3 (18.1)	0.0005
Baseline FEV1, % predicted†~	72.7 (8.9)	75.7 (8.1)	<0.0001
Methacholine response†∞	-23.4 (32.7)	-7.5 (14.0)	<0.0001

* p Values derived from Wilcoxon test or chi-square analysis.

† Mean (SD).

‡ Number of packs of cigarettes smoked per day x number of years of smoking.

~ Lung function at the start of the study.

∞ Measurement of the responsiveness of the airways to methacholine expressed as percent decline in FEV1 per final cumulative dose of methacholine administered.[368] Methacholine response is strongly associated with rate of decline in lung function.[369, 370]

HNP1 and HNP3 genes

The frequency of individuals with only HNP1 and both HNP1 and HNP3 genes are shown in Table 3.3. Univariate analysis did not suggest any significant association between the groups and rate of decline in lung function. Logistic regression modeling to adjust for confounding variables confirmed the lack of association (data not shown).

Table 3.3 HNP1 only and HNP1/3 frequencies by fast decline in lung function or no decline in lung function status.

	HNP1 only	HNP1 and HNP3
Fast decline, n (%)	31 (11.8)	244 (88.7)
No decline, n (%)	33 (10.9)	271 (89.1)
Univariate analysis	χ^2=0.03, p=0.87 OR=1.0 (CI=0.6-1.8)*	

* OR for possession of HNP1 and HNP3 genes versus HNP1 only.

The abbreviation HNP denotes human neutrophil peptide; OR, odds ratio; CI, 95% confidence interval.

HBD1 5'UTR polymorphisms

Genotype association analysis. Allelic and genotypic frequencies of the three HBD1 SNPs (-20G→A, -44C→G, and -52G→A) analyzed are given in Tables 3.4, 3.5, and 3.6, respectively. The observed distribution of genotypes was consistent with Hardy-Weinberg equilibrium (Table 3.7). Univariate analysis did not suggest any significant associations between the genotypes and rate of decline in lung function. Logistic regression modeling to adjust for confounding variables confirmed the lack of association (data not shown).

Table 3.4 Human beta-defensin 1 -20G→A allelic and genotypic frequencies by fast decline in lung function or no decline in lung function status.

	Allele		Genotype		
	G	A	GG	GA	AA
Fast decline, n (%)	297 (54.0)	253 (46.0)	83 (30.2)	131 (47.6)	61 (22.2)
No decline, n (%)	332 (54.6)	276 (45.4)	91 (29.9)	150 (49.3)	63 (20.7)
Univariate analysis	χ^2=0.04, p=0.84, OR=1.0 (CI=0.8-1.3)*			p=0.89†	

*OR for possession of A allele versus G allele.

†p value derived from chi-square test.

The abbreviation OR denotes odds ratio; CI, 95% confidence interval.

Table 3.5 Human beta-defensin 1 -44C→G allelic and genotypic frequencies by fast decline in lung function or no decline in lung function status.

	Allele		Genotype		
	C	G	CC	CG	GG
Fast decline, n (%)	439 (79.8)	111 (20.2)	177 (64.4)	85 (30.9)	13 (4.7)
No decline, n (%)	487 (80.1)	121 (19.9)	198 (65.1)	91 (29.9)	15 (4.9)
Univariate analysis	χ^2=0.01, p=0.91, OR=1.0 (CI=0.8-1.4)*			p=0.97†	

*OR for possession of G allele versus C allele.

†p value derived from chi-square test.

The abbreviation OR denotes odds ratio; CI, 95% confidence interval.

Table 3.6 Human beta-defensin 1 -52G→A allelic and genotypic frequencies by fast decline in lung function or no decline in lung function status.

	Allele		Genotype		
	G	A	GG	GA	AA
Fast decline, n (%)	342 (62.2)	208 (37.8)	107 (38.9)	128 (46.6)	40 (14.6)
No decline, n (%)	388 (63.8)	220 (36.2)	130 (42.8)	128 (42.1)	46 (15.1)
Univariate analysis	χ^2=0.33, p=0.57, OR=1.1 (CI=0.8-1.4)*			p=0.55†	

*OR for possession of A allele versus G allele.

†p value derived from chi-square test.

The abbreviation OR denotes odds ratio; CI, 95% confidence interval.

Table 3.7 Hardy-Weinberg equilibrium shown for all subjects, subjects with a fast decline, and subjects with a slow decline in lung function.

Polymorphism	p Value*
HBD1 -20G→A	
All	0.87
Fast decline	0.79
Slow decline	1.00
HBD1 -44C→G	
All	0.47
Fast decline	0.80
Slow decline	0.57
HBD1 -52G→A	
All	0.47
Fast decline	0.99
Slow decline	0.31

* p Value derived from chi-square test.

The abbreviation HBD denotes human beta-defensin.

Linkage disequilibrium. There was strong linkage disequilibrium between the three HBD1 5'UTR SNPs (Table 3.8).

Table 3.8 Linkage disequilibrium.

	D' (D/Dmax)	RSquare	p Value*
-20G→A and -52G→A	0.75	0.29	<0.00001
-20G→A and -44C→G	0.88	0.17	<0.00001
-44C→G and -52G→A	0.93	0.13	<0.00001

* p Value derived from chi-square test.

Haplotype association analysis. Analysis of the three HBD1 SNPs revealed seven haplotypes. The imputed haplotype frequencies in the two groups are given in Table 3.9. Univariate analysis did not suggest any significant association between haplotypes and rate of decline in lung function.

Table 3.9 Human beta-defensin 1 haplotypes and association with rate of decline in lung function.

Imputed haplotypes (-20/-44/-52)	Fast decline, n (%)	No decline, n (%)	p Value*
ACA	24 (4.4)	18 (3.0)	0.29
GCA	181 (32.9)	201 (33.1)	
GGA	3 (1.0)	1 (<1.0)	
ACG	223 (40.6)	257 (42.3)	
GCG	11 (2.0)	11 (1.8)	
AGG	6 (1.1)	1 (<1.0)	
GGG	102 (18.6)	119 (19.6)	

* p Value derived from chi-square test.

Secondary outcomes analysis. Statistical analysis did not suggest any significant associations between the -20G→A, -44C→G, and -52G→A genotypes (Table 3.10) and haplotypes (Table 3.11) and the mean number of visits to a doctor for lower respiratory infections per year. I also investigated the relationship between the three 5'UTR SNPs (Table 3.10) and haplotypes (Table 3.11) and the mean number of days kept in bed for lower respiratory infections per year; no significant associations were detected.

Table 3.10 Human beta-defensin 1 genotypes and lower respiratory infections each year.

Polymorphism	Genotype	Doctor visits†‡	p Value*	Days in bed†~	p Value*
-20G→A	GG	0.27 (0.04)	0.84	0.49 (0.10)	0.88
	GA	0.26 (0.03)		0.48 (0.08)	
	AA	0.30 (0.05)		0.54 (0.11)	
-44C→G	CC	0.28 (0.03)	0.90	0.53 (0.07)	0.85
	CG	0.27 (0.04)		0.44 (0.10)	
	GG	0.18 (0.10)		0.28 (0.24)	
-52G→A	GG	0.29 (0.04)	0.79	0.47 (0.08)	0.67
	GA	0.25 (0.03)		0.47 (0.08)	
	AA	0.30 (0.06)		0.62 (0.14)	

* p Value derived from chi-square test.

† Mean (SE).

‡ Mean number of visits to a doctor for lower respiratory infections per year.

~ Mean number of days kept in bed for lower respiratory infections per year.

Table 3.11 Human beta-defensin 1 haplotypes and lower respiratory infections each year.

Haplotype	Doctor visits†‡	p Value*	Days in bed†~	p Value*
ACA	0.26 (0.08)	0.84	0.60 (0.19)	0.14
GCA	0.27 (0.03)		0.50 (0.06)	
GGA	0.50 (0.27)		2.50 (0.63)	
ACG	0.28 (0.02)		0.50 (0.06)	
GCG	0.45 (0.11)		1.00 (0.27)	
AGG	0.23 (0.20)		0.60 (0.48)	
GGG	0.24 (0.04)		0.36 (0.08)	

* p Value derived from chi-square test.

† Mean (SE).

‡ Mean number of visits to a doctor for lower respiratory infections per year.

~ Mean number of days kept in bed for lower respiratory infections per year.

Polymorphisms in alpha- and beta-defensins in different racial groups

I determined the prevalence of genotypes across Caucasian and African American participants of the Lung Health Study and found statistically significant differences between racial groups (Table 3.12).

Table 3.12 Frequency of defensin polymorphisms by racial group.

	Caucasian (n=579)	African American (n=27)	p Value*
HNP1/3			
1	11.1	22.2	0.08
1 + 3	88.9	77.8	
HBD1 -20G→A			
GG	30.1	44.4	0.21
GA	48.5	44.5	
AA	21.4	11.1	
HBD1 -44C→G			
CC	64.8	100.0	0.0008
CG	30.4	0.0	
GG	4.8	0.0	
HBD1 -52G→A			
GG	40.9	22.2	0.0013
GA	44.2	37.1	
AA	14.9	40.7	

Overall observed SNP frequency (%).

* p Value derived from chi-square test.

The abbreviation HNP denotes human neutrophil peptide; and HBD, human beta-defensin.

PART II: HNP1-3 GENE COPY NUMBER AND LEVELS IN PERIPHERAL BLOOD NEUTROPHILS

The characteristics of the eighteen volunteers are given in Table 3.13. Despite considerable interindividual differences in relative HNP1-3 copy numbers (Figure 3.11), statistical analysis did not show any relationship between gene copy number and defensin protein levels in peripheral blood neutrophils (RSquare=0.04; p=0.45). All eighteen subjects had copies of both HNP1 and HNP3.

Table 3.13 Description of the study population.

Variable	
Age, yr†	29 (9.7)
Sex, n (%)	11 men (61)
Current smoking, n (%)	4 (22)
Asthma, n (%)	3 (16)

†Mean (SD).

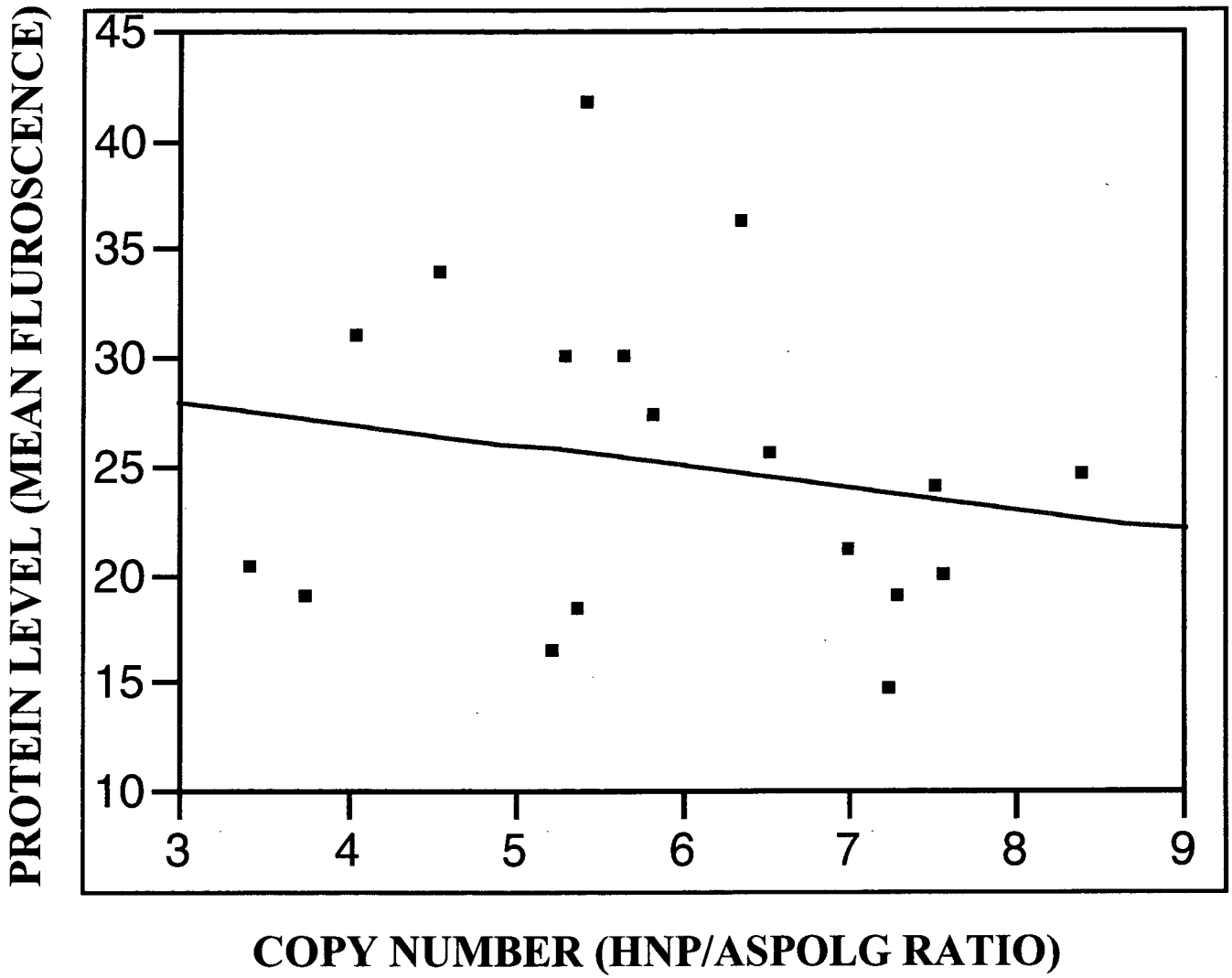


Figure 3.11 HNP1-3 gene copy number vs protein level in peripheral blood neutrophils.

RSquare=0.04; p=0.45. HNP denotes human neutrophil peptide; and ASPOLG, nuclear gene for the human polymerase gamma accessory subunit.

3.1.2.6 Discussion

Defensins may play a role in the pathogenesis of COPD and are excellent candidate genes for a COPD association studies. First, HNP1-3 are expressed in neutrophils and HBD1 is expressed by airway epithelial cells. Second, defensins may contribute to the pathogenesis of COPD by amplifying the cigarette smoke-induced and the infection-induced inflammatory reactions leading to lung tissue injury. Finally, members of the alpha- and beta-defensin families are located on chromosome 8p23, a genomic region linked to airflow obstruction susceptibility.[112] Matsushita et al. [172] reported a significant association between a SNP in exon 2 (Val38Ile) of the HBD1 gene and COPD in a Japanese population. I found that this polymorphism was very rare (<1%) in the Caucasian population (unpublished data).

I conducted a population-based association study to see whether alpha- and beta-defensin polymorphisms influenced smokers' susceptibility to lung function decline in a relatively large population of outbred Caucasian individuals. Analysis of individual SNPs and imputed haplotype frequencies failed to find associations between the polymorphisms and an increased rate of decline in lung function in smokers. To my knowledge, this is the first time the HNP1/3 copy number polymorphism and the three HBD1 5'UTR SNPs have been tested for an association with rate of decline in lung function in a Caucasian population.

I found seven haplotypes with imputed frequencies ranging from <1.0 to 42.3% in the subject group with no decline in lung function. The three most common haplotypes (GCA, ACG, and GGG) were reported by two other studies and were the only haplotypes reported by these studies.[172, 277] Some of the haplotypes I report are very rare. In the studies by Dork et al.[277] and Matsushita et al.[172] where there was a total of 103 random German blood donors and 60 Japanese patients with COPD, respectively, they may not have had large enough sample sizes to detect all of the haplotypes. In addition, racial/ethnic differences may account for the differences observed. In any case, the results suggest that the three haplotypes found in all three studies are common in most racial/ethnic populations.

It is known that the frequency of HBD1 SNPs differ between racial/ethnic groups.[278] I determined the prevalence of the genotypes across Caucasian and African

American subjects and found that the HBD1 -52G→A SNP differed significantly between Caucasian and African American populations. In addition, I was unable to detect the G allele of the HBD1 -44C→G SNP in twenty-seven African American subjects suggesting that this allele is very rare or non-existent in the African American population.

This genetic association study fulfills the major criteria for robust association studies[116] and has the following strengths: 1) defensins are excellent COPD candidate genes because defensins mediate inflammation and response to infection in the lung; 2) I included only Caucasian subjects in this study in order to potentially avoid the effects of population stratification; 3) this study is a large association study involving approximately 600 individuals; and 4) I have data on the frequency of lower respiratory infections in the study subjects. Again, the results indicate that the HNP1/3 copy number polymorphism and the three HBD1 5'UTR SNPs do not influence smokers' susceptibility to lung function decline.

Secondary analysis showed no association between individual HBD1 SNPs or imputed haplotype frequencies and the frequency of lower respiratory infection in the Lung Health Study participants. The functions of the three SNPs in the 5'UTR are unknown. As the SNPs were not important predictors of susceptibility to infection the possibility that they influence HBD1-mediated response to infection can be excluded.

In interpreting the data, a few limitations should be considered. Although I did not find an association between the HNP1/3 copy number polymorphism and genotype and haplotype analysis of the HBD1 SNPs and rate of decline in lung function, these polymorphisms may influence other COPD-related phenotypes. Moreover, the genetic basis of COPD may differ between racial/ethnic groups. I limited the association study to Caucasian subjects and I have shown that some of the genotype frequencies differed significantly between racial groups. Therefore, it may be of interest to investigate the role of these polymorphisms in other racial/ethnic populations.

I also investigated the relationship between HNP1-3 gene copy number and HNP1-3 protein levels in peripheral blood neutrophils and found no correlation. This finding needs to be interpreted with caution because the study was performed on healthy subjects. Neutrophils are terminally differentiated cells, incapable of cell division, and synthesize very low levels of RNA and protein. The production of alpha-defensins is first

initiated in late promyelocytes and continues throughout myeloid cell differentiation. The expression is mediated by combinations of transcription factors present at distinct stages of myeloid cell development. Patients with COPD may be equipped with a defensin armamentarium, different from that found in healthy subjects, as some of the 5,000 plus chemical compounds and in excess of 10 free radical molecules present in cigarette smoke may directly trigger the molecular pathways involved in defensins production. Another limitation to note is the fact that all of the healthy subjects had copies of both HNP1 and HNP3 (no subjects had copies of only HNP1). Therefore this study failed to answer the question of how an individual's protein level varies with respect to the inheritance of HNP1 only or both HNP1 and HNP3 genes.

Power analysis indicated that this study was not underpowered. It was estimated that for the HBD1 -20, -44, and -52 SNPs, the study design had eighty percent power to detect an association with a relative risk of 1.60, 1.72, and 1.61, respectively (Table 3.14). Post hoc power calculations are advocated because they are believed to provide insight into the interpretation of negative results. However, the number of papers opposing the use of post hoc power calculations is growing and some argue that post hoc power analysis is fundamentally flawed.[285-289] This analysis should be interpreted with caution as it may not accurately explain the negative results.

Table 3.14 Power calculations for a sample size of 275/6 cases and 304 controls with alpha=0.05 and beta=0.80 for a two sided test.

Prevalence of polymorphism	Odds ratio
5%	2.41
10%	1.98
15%	1.81
20%	1.72
25%	1.67
30%	1.64
35%	1.61
40%	1.60
45%	1.60
50%	1.60

In summary, I did not detect a difference in the frequency of the HNP1/3 copy number polymorphism in the fast decliners and non-decliners. In addition, there was no correlation between copies of HNP1-3 genes and defensin protein levels in peripheral blood neutrophils in healthy subjects. I also found no association between individual HBD1 SNPs or imputed haplotype frequencies and rate of decline in lung function or the frequency of lower respiratory infections.

3.2 PLASMINOGEN SYSTEM

3.2.1 Overview

The plasminogen system (Figure 3.12), also known as the fibrinolytic system, has an important role in controlling endogenous fibrosis and is essential for maintaining fluent blood flow. The major reaction of the plasminogen system involves the conversion of plasminogen into the active enzyme plasmin. Plasmin is the active serine proteinase formed from the inactive proenzyme plasminogen by the enzymatic activity of the serine proteinases, tissue-type (tPA) and urinary-type (uPA) plasminogen activators (PA). Plasmin can degrade fibrin, which forms the matrix structure of a blood clot.

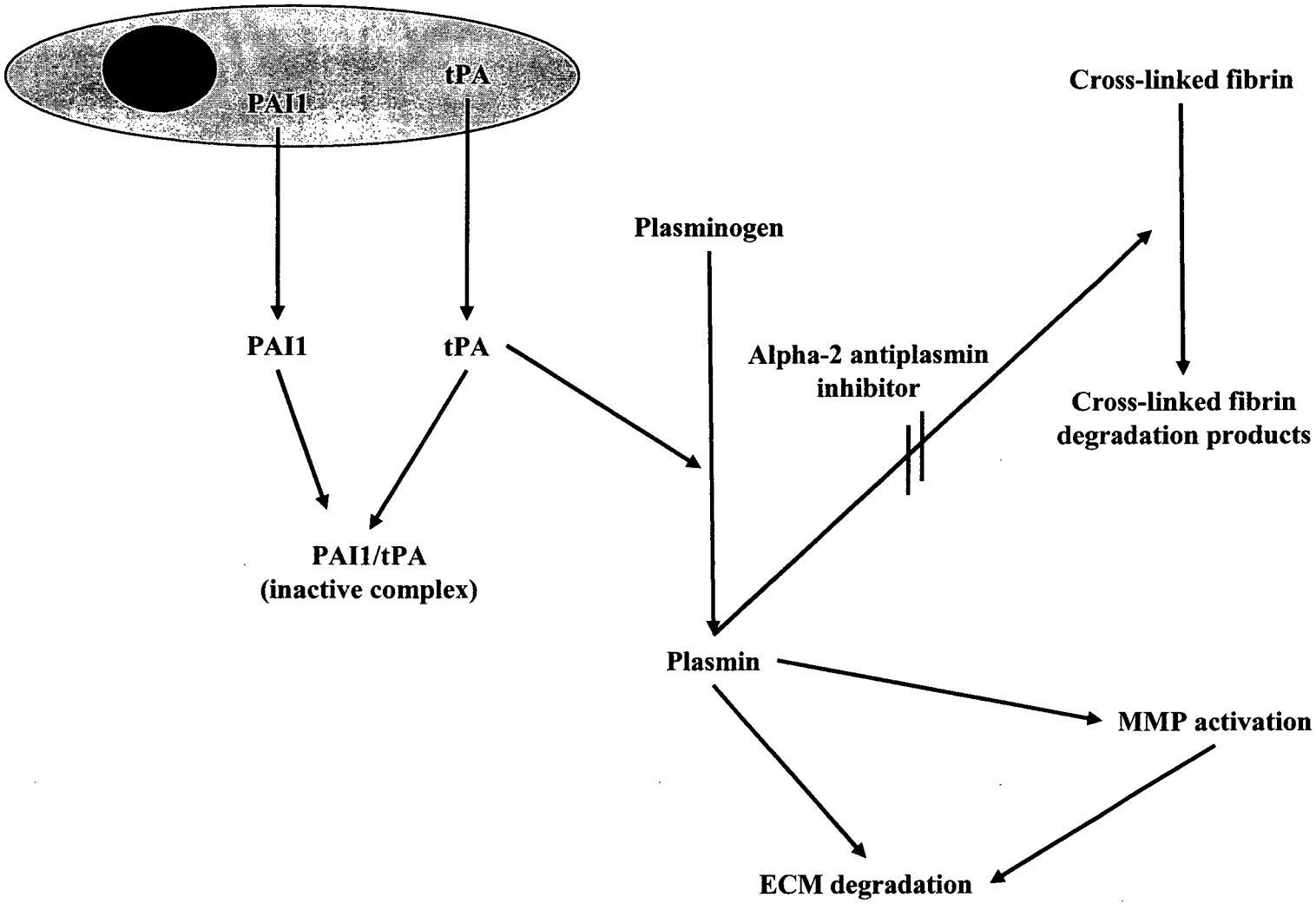


Figure 3.12 Plasminogen system.

The major reaction of the plasminogen system involves the conversion of plasminogen into the active enzyme plasmin. This reaction is mediated by the controlled synthesis and release of both plasminogen activators and inhibitors. Plasmin degrades fibrin, components of the extracellular matrix, and activates matrix metalloproteinases. PAI1 denotes plasminogen activator inhibitor 1; tPA, tissue-type plasminogen activator; ECM, extracellular matrix; and MMP, matrix metalloproteinase.

In addition to its role in thrombus resolution, the plasminogen system also regulates ECM proteolysis. Plasmin enhances proteolytic degradation of the ECM via at least four different mechanisms. First, plasmin directly degrades a number of extracellular matrix proteins, including fibronectin, type IV collagen, and laminin.[290-292] Second, plasmin removes glycoproteins from the ECM that allows subsequent MMP-dependent degradation of elastase and collagen to occur.[293, 294] Third, plasmin activates MMPs directly to degrade ECM components. Pro-MMP1[295] and pro-MMP3[296] are activated by plasmin. MMP3 is a potent activator of MMP9.[297] Fourth, plasmin may contribute to ECM degradation by neutralizing MMP inhibitors. In summary, plasmin directly degrades ECM components and plasmin exerts control over MMP activity at the substrate, activation, and inhibitor levels. In addition, plasmin plays a role in the generation of proinflammatory and profibrotic signals by promoting the activation of cytokines such as IL-1[298] and TGF-beta.[299]

Because of the high concentration of plasminogen in most tissues, the production of a small amount of PA can result in high local concentrations of plasmin; therefore, precise control of PA activity is important. tPA and uPA activity is regulated by three proteinase inhibitors, plasminogen activator inhibitor (PAI) type 1 (PAI1), type 2 (PAI2), and type 3 (PAI3).[300] PAI1 is the primary inhibitor of both tPA and uPA in plasma[301] and the alveolar space.[302] PAI3 activity is associated with the regulation of enzymes involved in fertilization; therefore, it is not relevant to PA regulation in the lung. PAI2, previously termed placental-type inhibitor, has inhibitory activity towards tPA and uPA that is 20- to 100-fold less than observed for PAI1.[303] Unstimulated human monocytes and alveolar macrophages do not express PAI1 or PAI2.[304-306] PAI2 is the only form of PA inhibitor expressed by stimulated human monocytes.[306, 307] Stimulated alveolar macrophages express both PAI1 and PAI2, however, PAI2 mRNA levels are markedly less compared to PAI1 mRNA levels.[307] Furthermore, PAI2 is not generally detectable in human plasma, except during pregnancy.[308, 309]

The plasminogen system has been extensively studied with regard to cardiovascular and cerebrovascular diseases. Outside these fields, there has been increasing evidence suggesting the possible contribution of the plasminogen system to many other important biological processes including tissue remodeling, cell adhesion,

cell migration, cell-cell signaling, tumour invasion and metastasis, ovulation, and wound healing. Although both uPA and tPA are both excellent candidate genes for COPD, uPA was not investigated in this study due to the lack of characterized SNPs that altered uPA expression and/or function at the time of the study. PAI1 and tPA will be discussed further.

PAI1, a single-chain 50 kDa glycoprotein, is a member of the serine proteinase inhibitor superfamily. PAI1 shares 30% homology at the amino acid level with two other members of the serine proteinase inhibitor superfamily, alpha-1 antitrypsin and antithrombin III. All serine proteinase inhibitors share a highly conserved tertiary structure consisting of three beta-sheets (A, B, and C), 7-9 alpha-helices, and an exposed flexible reactive center loop comprising residues P16-P10'. Like other serine proteinase inhibitors, PAI1 forms a one:one complex with its target proteinase resulting in neutralized enzymatic activity. Serine proteinase inhibitors are known as suicide substrate inhibitors because they develop their inhibitory ability only after the initial interactions with the proteinase as a normal substrate.[310, 311] PAI1 is synthesized and secreted by various cell types in the lung including endothelial cells, epithelial cells, alveolar macrophages, fibroblasts, mast cells, and smooth muscle cells as well as hepatocytes and platelets. PAI1 exists in both active and inactive forms in plasma.

Two groups of agents appear to modulate PAI1 activity: 1) those that interact with cells to alter their rate of synthesis of PAI1 and 2) those that interact directly with PAI1 to alter its activity. Protein C,[312] endotoxin,[313, 314] thrombin,[315] TGF-beta,[316] and inflammatory cytokines such as IL-1[317, 318] and TNF-alpha[319] have all been found to modulate PAI1 production. Vitronectin forms a stable complex with the active form of PAI1, which is thought to protect PAI1 from oxidation.[320, 321]

PAI1 is located on chromosome seven (7q21.3-q22). The gene is approximately 12.2 kb long, consists of nine exons and eight introns, and encodes a protein containing 402 amino acids.[322] The PAI1 locus is linked to the cystic fibrosis, the erythropoietin, and the alpha-2 chain of type I collagen loci.[323] Several polymorphisms have been characterized in this gene and are listed in the SeattleSNPs database (http://pga.mbt.washington.edu/). A functional polymorphism in the promoter region of the PAI1 gene (-675/4G→5G) effects the binding of nuclear proteins regulating

transcription[324, 325] and is significantly correlated with the plasma levels of PAI1.[326] The 4G allele is associated with increased gene transcription and higher PAI1 plasma concentrations.[325] The two alleles are almost equally distributed among the Caucasian population. The 4G allele of this common -675/4G→5G promoter polymorphism is associated with myocardial infarction,[325, 327] coronary artery disease,[326, 328] abdominal aortic aneurysms,[329] stroke,[330] obesity,[331] a poor survival rate after severe trauma,[332] meningococcal disease,[333] and asthma.[334] The 5G5G genotype is associated with an increased risk of multiple sclerosis in women.[335]

In humans, elevated plasma levels of PAI1 are associated with myocardial infarction[336, 337] and deep vein thrombosis.[338] Genetically modified mice have provided some insight into the function of PAI1. Transgenic mice overexpressing PAI1 develop deep vein thrombi[339] and vascular fibrinolysis is accelerated in PAI1 deficient mice.[340] In addition, PAI1 is believed to play an important role in a number of plasminogen-dependent proteolytic events outside the vasculature. PAI1 knockout mice do not develop pulmonary fibrosis after lung injury.[341] Furthermore, evidence suggests that in a murine model of chronic asthma, PAI1-deficient mice have increased ECM deposition in the airways because of decreased MMP9 activity and increased fibrinolysis.[342] There is extensive and growing evidence that PAI1 is involved in ovarian follicular rupture[343] as well as angiogenesis and tumour invasion.[344] Further investigations into the biological effects of altered concentrations of PAI1 outside the vasculature are needed.

Human tPA is an extracellular serine proteinase produced by numerous cells types in the lung including endothelial cells, epithelial cells, alveolar macrophages, and smooth muscle cells. Endothelial cells are considered the most important source of tPA in vivo.[345] tPA is released from endothelial cells in a constitutive and regulated fashion. Changes in the plasma level of tPA are mainly achieved by the endothelium through two mechanisms: 1) a rapid (within minutes) regulated release of tPA from a storage pool and 2) a long-term change in the rate of synthesis and constitutive secretion of tPA. Additionally, plasma tPA concentrations are dependent on hepatic clearance and serine proteinase inhibitor-proteinase (e.g. PAI1-tPA) complex formation. In addition to tPA regulation by PAI1, tPA activity may be effected at the level of the formation and resolution of fibrin[346] and by the binding of tPA to cell surfaces or ECM components.[347]

The tPA-mediated pathway is thought to be primarily involved in the resolution of blood clots. Studies suggest that high plasma levels of tPA mark an increased risk of atherothrombotic ischemic events such as myocardial infarction and stroke;[348-350] elevated tPA levels may represent the activation of the endogenous fibrinolytic system in response to the existence of preclinical atherosclerosis. tPA is widely used to treat myocardial infarction and has been approved for treatment of occlusive stroke, however, the therapeutic potential for stroke victims is controversial.[351] tPA plays an important role in the nervous system; tPA is involved in a number of processes including neuronal migration, neurite outgrowth, and neuronal plasticity. It has also been suggested that tPA plays a role in seizures and demyelinating diseases.

Studies on tPA-deficient mice have confirmed the importance of tPA in mediating thrombolysis as tPA knockout mice accumulate fibrin in several organs and have a decreased capacity to cleave exogenous clots.[352, 353] tPA knockouts have also provided insight into the role of tPA in several other pathologic conditions. Studies show that tPA -/- mice have decreased MMP2 and MMP9 activity.[354-356] Knockout mice lacking the tPA gene demonstrate a significant reduction in both the rate and the extent of learning.[357] tPA-/- mice have an earlier dissemination of *Mycobacterium avium* infection from lungs compared with wild-type mice.[358] Neural damage is decreased in tPA(-/-) mice after spinal cord injury.[359]

The >30 kb human gene encoding tPA, PLAT, is located on chromosome eight (8p12) and consists of 14 exons.[360] tPA is synthesized as a 70 kDa single polypeptide chain. Proteolytic cleavage of the single chain protein at a centrally located arginine-isoleucine bond by plasmin gives rise to a two-chain disulfide-linked form, composed of the N-terminally derived heavy chain and the C-terminal light chain. Gene expression is mainly regulated at the level of transcription.[345] The promoter contains two transcription start sites, which are dependent on an initiation element and a TATA box.[360, 361] Additionally, transcription of PLAT is regulated through a multihormone-responsive enhancer located -7.3 kb from the initiator element.[362] Genetic variation at the PLAT locus has been characterized and extensively studied in association with plasma tPA levels.[362-364] Ladenvall and colleagues[363] reported an association between SNPs at the PLAT locus and vascular tPA release. The Alu insertion polymorphism is associated with

vascular tPA release rates. This polymorphism is in linkage disequilibrium with three other SNPs at the PLAT locus: 1) -7351C→T (enhancer), 2) 20099T→C (exon 6), and 3) 27445T→A (intron 10). The enhancer SNP resides within a GC box and the T allele is associated with low vascular tPA release rates due to reduced binding affinity of Sp1 to the T allele. A recent study by the same group indicates that genetic variation at the PLAT locus and the PAI1 -675/4G→5G polymorphism are not strong predictors of plasma tPA levels; therefore, variations in other genes may contribute to this phenotype.[364]

Evidence indicates that events activating coagulation and inflammation are closely related and mutually interdependent. In primitive species, there is only one pathway for both coagulation and inflammation.[365] Therefore, PAI1 and tPA, an inhibitor and activator of the fibrinolytic pathway, may also contribute to an amplified inflammatory response in COPD, however, this remains to be defined.

3.2.2 Genetic susceptibility to chronic obstructive pulmonary disease: contribution of plasminogen activator inhibitor 1 and tissue-type plasminogen activator to rate of decline in lung function in the Lung Health Study participants

3.2.2.1 Introduction

Variations in genes encoding proteins involved in plasmin formation are of interest when investigating genetic risk factors for COPD because of the involvement of plasmin in extracellular matrix proteolysis and the regulation of MMP activity. The 5G allele of the PAI1 -675/4G→5G polymorphism is associated with low PAI1 plasma levels. The C allele of the -7351C→T PLAT polymorphism is associated with increased Sp1 binding affinity.

3.2.2.2 Hypothesis

I hypothesized that polymorphisms associated with decreased PAI1 levels and increased tPA would be associated with a fast decline in lung function.

3.2.2.3 Specific aim

The aim of this study was to investigate the association of PAI1 and PLAT polymorphisms with rate of decline in lung function in smokers. I selected 580 participants in the Lung Health Study who had either a fast decline or no decline in lung function over the five year study period.

3.2.2.4 Methods

Study subjects

Subjects were selected from participants in Phase I of the NHLBI Lung Health Study. The design of this multicentre randomized clinical trial has been described more extensively elsewhere.[280] Study participants were current smokers, 35-60 years of age, with mild to moderate airflow obstruction (FEV1 55–90% predicted and FEV1/FVC $\leq$ 0.70). Exclusion criteria included serious illnesses such as cancer, heart attack or stroke, or other important conditions that required medical treatment. The primary outcome variable was rate of decline in FEV1 over a follow-up period of five years. Of the 3216

continuing smokers in this cohort, 276 were chosen with a fast decline in lung function (decline in percent predicted FEV1>3.0% per year), and 304 were selected with no decline in lung function over the same period (increase in percent predicted FEV1>0.4% per year); all 580 selected participants were Caucasian (white, non-Hispanic). In addition, 27 African American Lung Health Study participants were genotyped; the data were used to determine the prevalence of genotypes across racial groups.

DNA extraction

Genomic DNA was isolated from frozen whole blood with a standard phenol/chloroform protocol.[281]

Plasminogen activator inhibitor 1 promoter polymorphism

Real-time PCR was performed utilizing a LightCycler (Roche Molecular Biochemicals) with hybridization probes to detect variation at position -675 as previously described.[366] A 134 or a 135 bp fragment of the human PAI1 gene was amplified using the sense primer 5'-AGCCAGACAAGGTTGTTGACAC and the antisense primer 5'-CAGAGGACTCTTGGTTTTCCC. The donor probe sequence was 5'-TGACTCCCCCACGTGTCC and the acceptor probe sequence was 5'-ACTCTCTCTGTGCCCCTGAGGGCTCT. The donor probe was labeled at the 3' end with fluorescein and was an 18-mer oligonucleotide complementary to the leading strand of the 5G allele (mismatched when bound to the 4G allele). The 26-mer acceptor probe was labeled at the 5' end with LightCycler Red 640 and modified at the 3' end by phosphorylation to block extension. The donor and acceptor probes were separated by two bases when bound to the template. Both fluorophore-labeled probes were synthesized and purified by reverse-phase HPLC by Operon Technologies, Inc (Alameda, CA). Real-time PCRs were performed in a total reaction volume of 20 μL with 100 ng of genomic DNA, 0.3 μM forward and reverse primers, 0.2 μM donor and acceptor probes, 4 mM Mg^{2+}, and 2 μL LightCycler FastStart DNA Master Hybridization Probes Mix (Roche Molecular Biochemicals). The samples were loaded in the LightCycler glass capillaries, closed, centrifuged, and placed in the LightCycler rotor. The cycling program consisted of 10 minutes of initial denaturation at 95°C and 50 cycles at 95°C for 0 s (ramp rate

20°C/s), 57°C for 5 s (ramp rate 3°C/s), and 72°C for 2 s (ramp rate 20°C/s). The analytical melting program was 94°C for 1 s, cooling to 45°C and holding for 3 minutes, and increasing to 75°C at a ramp rate of 0.2°C/s, with continuous fluorescence acquisition (Figure 3.13).

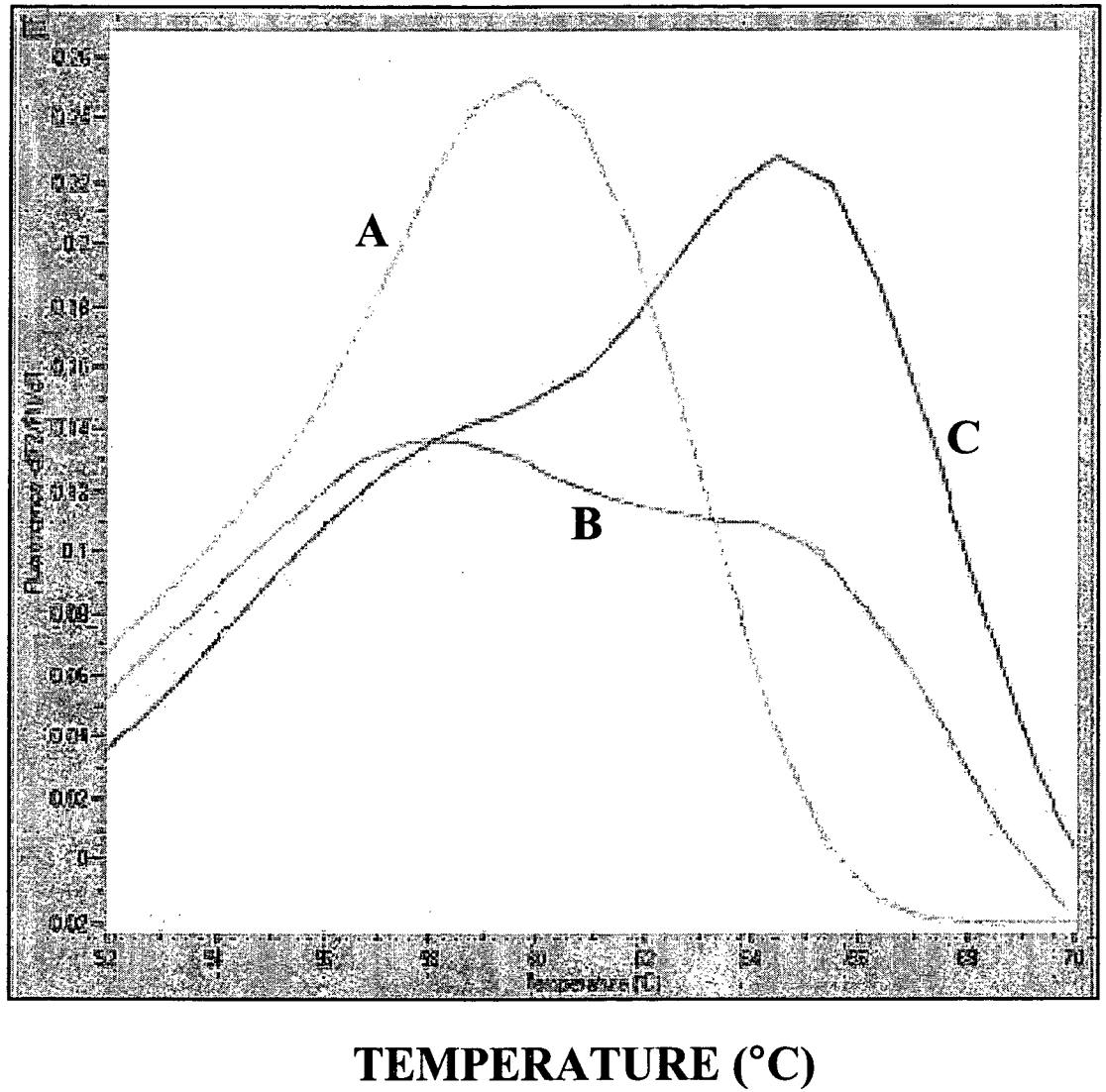


Figure 3.13 Genotyping of the PAI1 -675/4G→5G polymorphism using hybridization probes.

Melting curve analysis was performed immediately after real-time PCR. Fluorescence data were converted to derivative melting curves by plotting the negative derivative of the fluorescence with respect to temperature against temperature [(-dF/dT) vs T]. The derivative melting curve is plotted for a homozygote for the 4G allele (A), a heterozygote for the 4G and 5G alleles (B), and a homozygote for the 5G allele (C). PAI1 denotes plasminogen activator inhibitor 1.

Human tissue-type plasminogen activator enhancer polymorphism

Genotyping of the PLAT -7351C→T enhancer polymorphism was performed by restriction fragment length polymorphism analysis. An amplicon of 311 bp was generated by 30 cycles of PCR using the sense primer 5'-CGTGGGTAACCAGAACTGATG and the antisense primer 5'-ACTGTGATCCCATCACTGCAC. PCRs were performed in a total reaction volume of 20 μL with 100 ng of genomic DNA, 0.5 U *Taq* polymerase (Invitrogen), 10X PCR buffer (Invitrogen), 2.5 mM Mg^{2+}, 0.25 μM forward and reverse primers, and 200 μM dNTPs, with an annealing temperature of 60°C. After PCR, 20 μL of the reaction mixture was digested with 2 U *Fau* I restriction endonuclease (New England BioLabs Inc.) overnight at room temperature. The digest mixture was resolved on a 2.5% agarose gel stained with ethidium bromide. DNA from individuals with the homozygous C genotype (CC) produced two bands, one at 141 bp and one at 170 bp; the homozygous T genotype (TT) produced one band at 311 bp; and the heterozygous genotype (CT) produced all three bands (Figure 3.14).

Template-free controls and known genotype controls were included in each experiment. Genotypes were assigned by two independent investigators who were unaware of the patients' identities and phenotypes. Inconsistencies were resolved by two additional genotyping reactions. Twenty samples were selected at random and sequenced to confirm the genotyping protocols.

Statistical analysis

An association with rate of decline in lung function was assessed by the chi-square test. The associations were also analyzed by multiple logistic regression to adjust for potential confounding factors. Age, sex, smoking history (pack year), methacholine response, and initial level of lung function (pre-bronchodilator FEV1 percent predicted) were included as covariates in the multivariate model. Hardy-Weinberg equilibrium was tested on a contingency table of observed versus predicted genotype frequencies. The JMP Statistics software package (SAS Institute Inc., Cary, NC) was used to manage and analyze the data. Statistical significance was defined at the standard 5% level.

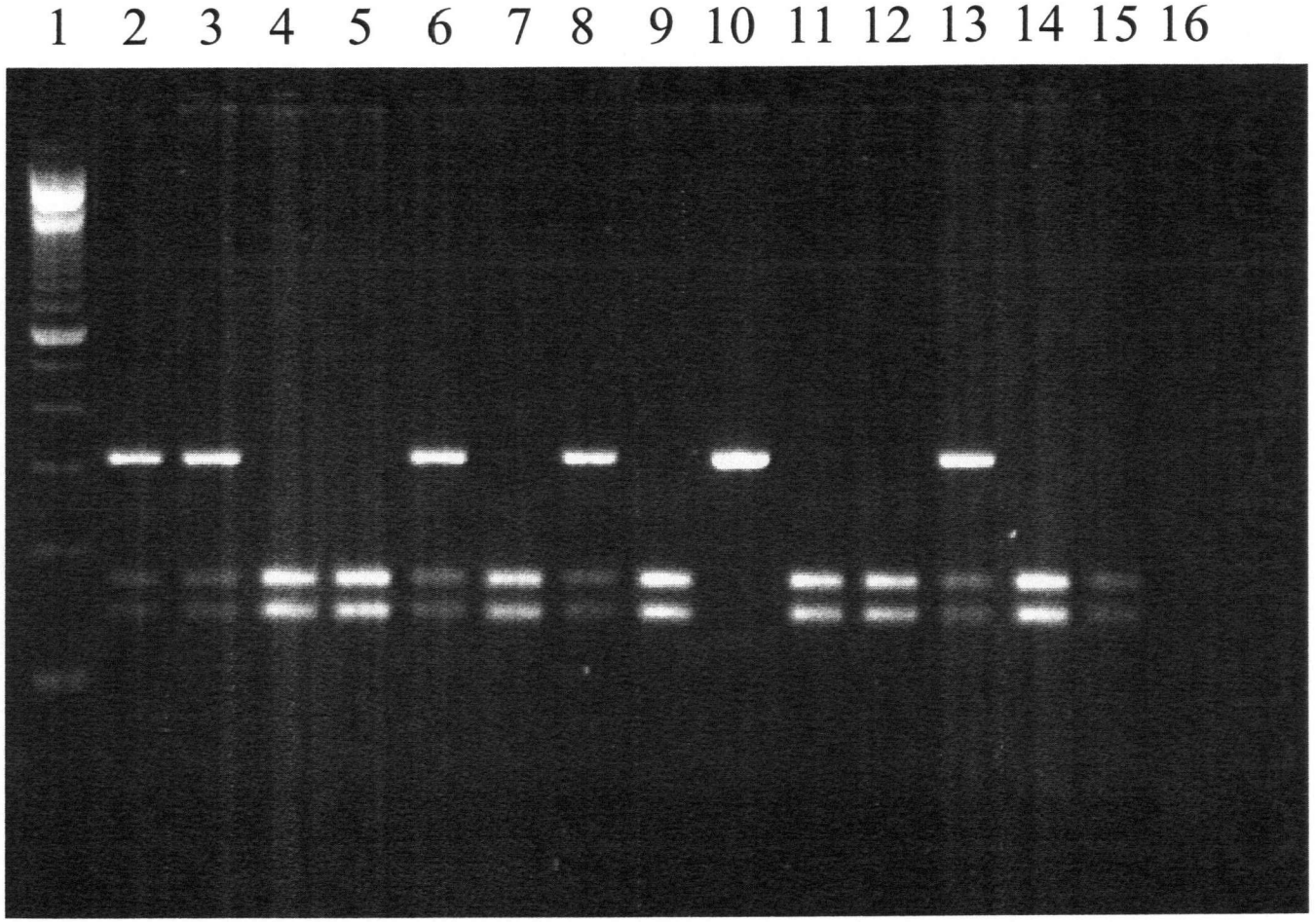


Figure 3.14 Genotyping of the PLAT -7351C→T SNP.

The PCR product was digested with the *Fau* I restriction endonuclease. After digestion, the digest mixture was resolved on a 3% agarose gel stained with ethidium bromide. Lane 1, size marker (100 bp DNA Ladder from New England BioLabs); lanes 2, 3, 6, 8, and 13, heterozygotes for the C and T alleles; lanes 4, 5, 7, 9, 11, 12, 14, and 15, homozygotes for the C allele; lane 10, homozygote for the T allele; and lane 16, negative control, no bands produced. PLAT denotes tissue-type plasminogen activator.

3.2.2.5 Results

General characteristics

The study population consisted of 276 Caucasian (white, non-Hispanic) smokers with a fast decline in lung function and 304 Caucasian smokers with no decline in lung function over the five year study period. The characteristics of the study population are described in Table 3.15. Age, sex, smoking history (pack year), baseline FEV1, and methacholine response of smokers with a fast decline in lung function and smokers with no decline in lung function were significantly different.

Table 3.15 Description of the study population.

Variable	Fast decline (n=276)	No decline (n=304)	p Value*
Age, yr†	49.5 (6.4)	47.6 (6.9)	0.0014
Sex, n (%)	162 men (59)	203 men (67)	0.04
Smoking history, pk yrs†‡	43.0 (19.2)	38.4 (18.2)	0.0016
Baseline FEV1, % predicted†~	72.6 (8.9)	75.6 (8.1)	<0.0001
Methacholine response†∞	-23.0 (32.4)	-7.8 (15.2)	<0.0001

* p Values derived from Wilcoxon test or chi-square analysis.

† Mean (SD).

‡ Number of packs of cigarettes smoked per day x number of years of smoking.

~ Lung function at the start of the study.

∞ Measurement of the responsiveness of the airways to methacholine expressed as percent decline in FEV1 per final cumulative dose of methacholine administered.[368] Methacholine response is strongly associated with rate of decline in lung function.[369, 370]

PAI1 -675/4G→5G polymorphism

Genotype and allele frequencies of the -675/4G→5G polymorphism are given in Table 3.16. The observed distribution of genotypes was consistent with Hardy-Weinberg equilibrium (Table 3.17). Univariate analysis did not suggest any significant association between the -675/4G→5G genotype and rate of decline in lung function. Logistic regression modeling to adjust for confounding variables confirmed the lack of association (data not shown).

Table 3.16 Plasminogen activator inhibitor 1 -675/4G→5G allelic and genotypic frequencies by fast decline in lung function or no decline in lung function status.

	Allele		Genotype		
	4G	5G	4G4G	4G5G	5G5G
Fast decline, n (%)	313 (56.7)	239 (43.3)	92 (33.3)	129 (46.7)	55 (19.9)
No decline, n (%)	324 (53.3)	284 (46.7)	86 (28.3)	152 (50.0)	66 (21.7)
Univariate analysis	χ^2=1.36, p=0.24, OR=0.9 (CI=0.7-1.1)*			p=0.0.42†	

*OR for possession of 5G allele versus 4G allele.

†p value derived from chi-square test.

The abbreviation OR denotes odds ratio; CI = 95% confidence interval.

Table 3.17 Hardy-Weinberg equilibrium shown for all subjects, subjects with a fast decline, and subjects with a slow decline in lung function.

	p Value*
PAI1 -675/4G→5G	
All	0.87
Fast decline	0.73
Slow decline	1.00

* p Value derived from chi-square test.

The abbreviation PAI1 denotes plasminogen activator inhibitor 1.

PLAT -7351C→T polymorphism

Genotype and allele frequencies of the -7351C→T SNP are given in Table 3.18. The observed distribution of genotypes was consistent with Hardy-Weinberg equilibrium (Table 3.19). Univariate analysis did not suggest any significant association between the -7351C→T genotype and rate of decline in lung function. Logistic regression modeling to adjust for confounding variables confirmed the lack of association (data not shown).

Table 3.18 Tissue-type plasminogen activator -7351C→T allelic and genotypic frequencies by fast decline in lung function or no decline in lung function status.

	Allele		Genotype		
	C	T	CC	CT	TT
Fast decline, n (%)	347 (62.9)	205 (37.1)	115 (41.7)	117 (42.4)	44 (15.9)
No decline, n (%)	399 (65.6)	209 (34.4)	135 (44.4)	129 (42.4)	40 (13.2)
Univariate analysis	χ^2=0.96, p=0.33, OR=1.1 (CI=0.9-1.4)*			p=0.60†	

*OR for possession of T allele versus C allele.

†p value derived from chi-square test.

The abbreviation OR denotes odds ratio; CI = 95% confidence interval.

Table 3.19 Hardy-Weinberg equilibrium shown for all subjects, subjects with a fast decline, and subjects with a slow decline in lung function.

	p Value*
PLAT -7351C→T	
All	0.19
Fast decline	0.31
Slow decline	0.58

* p Value derived from chi-square test.

The abbreviation PLAT denotes tissue-type plasminogen activator gene.

Polymorphisms in PAI1 and PLAT in different racial groups

I determined the prevalence of PAI1 (Table 3.20) and PLAT (Table 3.21) genotypes across Caucasian and African American participants of the Lung Health Study and found statistically significant differences between racial groups.

Table 3.20 Plasminogen activator inhibitor 1 genotypic frequency by racial group.

Racial group	n	4G4G	4G5G	5G5G	p Value*
Caucasian	580	30.7	48.5	20.9	0.0005
African American	27	11.1	37.0	51.9	

Overall observed SNP frequency (%).

* p Value derived from chi-square test.

Table 3.21 Tissue-type plasminogen activator genotypic frequency by racial group.

Racial group	n	CC	CT	TT	p Value*
Caucasian	580	43.1	42.4	14.5	0.0060
African American	27	74.1	22.2	3.7	

Overall observed SNP frequency (%).

* p Value derived from chi-square test.

3.2.2.6 Discussion

PAI1 and PLAT may play a role(s) in the pathogenesis of COPD and are excellent candidate genes for a COPD association studies. First, both genes are expressed by numerous cells types in the lung including endothelial cells, epithelial cells, and alveolar macrophages. Second, PAI1 and tPA are involved in plasmin formation and plasmin contributes to extracellular matrix proteolysis. In addition, plasmin regulates MMP1[295] and MMP9[297] activity and both proteinases have been implicated in the pathogenesis of COPD.[367] Finally, PAI1 can be induced by inflammatory cytokines such as IL-1,[317, 318] and TNF-alpha,[319] which are increased in patients with COPD. To my knowledge, PAI1 and PLAT have not been previously investigated as candidate genes for COPD. This is the first time polymorphisms in these genes have been tested for an association with rate of decline in lung function.

Using a population-based test of association, I failed to find associations between an insertion/deletion polymorphism in the PAI1 gene and a SNP in the PLAT gene with an increased rate of decline in lung function in smokers in a relatively large population of outbred Caucasian individuals. Power analysis indicated that this study was not underpowered. It was estimated that for the PAI1 and PLAT polymorphisms, the study design had eighty percent power to detect an association with a relative risk of 1.60 and 1.61, respectively (Table 3.14). In addition, I found that the frequency of these polymorphisms differed significantly across racial groups. I observed as high as 31% genotype frequency differences for the PAI1 -675/4G→5G polymorphism and the PLAT -7351C→T SNP between Caucasian and African American populations.

Although the results of these association studies were negative, these studies were well designed and fulfill the major criteria for robust association studies.[116] These studies have two major strengths. First, these studies are large association studies involving approximately 600 individuals. Second, population stratification can lead to false positive associations. Based on self-reported ancestry I excluded subjects that were not Caucasian (white, non-Hispanic).

In interpreting the data, a few limitations should be considered. In this study only a single polymorphism was investigated in each gene. At times, the results obtained by analyzing single polymorphisms are insufficient. It is widely accepted that haplotypes,

rather than genotypes, are a better way to detect the genetic contribution, especially to multifactorial diseases. In addition, haplotype analysis sometimes demonstrates genetic influences that are not detected by the analysis of individual polymorphisms. A number of SNPs have already been identified in both PAI1 and PLAT making haplotype analysis possible in the future. Although I did not find an association between polymorphisms in the PAI1 and PLAT genes and rate of decline in lung function, these polymorphisms may influence other COPD-related phenotypes. Moreover, I limited the association study to Caucasian subjects and I have shown that the genotype frequencies of the -675/4G→5G polymorphism of the PAI1 gene and the -7351C→T SNP of the PLAT gene differed significantly between ethnic groups. Therefore, it may be of interest to perform an association study with a large number of African American subjects.

In conclusion, this population-based association study suggests that the -675/4G→5G polymorphism of the PAI1 gene and the -7351C→T SNP of the PLAT gene do not influence smokers' susceptibility to lung function decline. In the future, I would like to examine additional genotypes as well as haplotypes in the PAI1 and PLAT genes and determine if they play a pathogenetic role in COPD.

CHAPTER 4. ALVEOLAR MACROPHAGE PROTEINASES

Four classes of proteinases have been identified in humans. Each class of enzymes degrades ECM components with different degrees of specificity.[371] The four classes are distinguished according to the amino acid or chemical group at the catalytic centre of the enzyme. Aspartate and cysteine proteinases primarily catalyze intracellular pathways whereas metalloproteinases and serine proteinases catalyze extracellular pathways, for the most part. The integrity of the ECM results from a dynamic balance between synthesis and degradation of its various components. Excessive or defective ECM turnover may contribute to the pathogenesis of many diseases including emphysema. The importance of alveolar macrophages and alveolar macrophage proteolytic enzymes in the pathogenesis of COPD was discussed in Chapter 1.

4.1 CATHEPSIN OVERVIEW

Cathepsins are members of the papain family of cysteine proteinases. Mature active forms of cathepsins are located predominantly within lysosomes, which led to the view that their function was limited to the terminal digestion of internalized protein following initial degradation of extracellular proteins by proteolytic enzymes.[372] For this reason, the role of cathepsins in the pathobiology of lung disease has been largely ignored. However, since the 1960s when Gross and colleagues[79] found the first reproducible model of emphysema by injecting rat lungs with the plant proteinase papain it has been suggested that cathepsins may be involved in the pathogenesis of emphysema. Recent evidence suggests that cathepsins are involved in proteolytic events at the cell surface and the extracellular space.

Currently, eleven members of the papain family of cysteine proteinases have been identified in humans. Members of this family are characterized by two domains folded together in a V-shaped configuration. At the angle of the V, a cysteine and a histidine residue form the catalytic diad.[373] Cathepsins are synthesized in the endoplasmic reticulum as prepropeptides and then processed to the active form via a propeptide. The prepropeptide consists of a signal peptide, a propeptide, and a catalytic region. The signal peptide targets the cathepsin to the Golgi apparatus. In the Golgi apparatus, it is

glycosylated with high-mannose carbohydrates. These carbohydrates bind to one of the two mannose-6-phosphate receptors and the complex is transported to the prelysosomal compartment. The acidic environment within the compartment causes the dissociation of the enzyme-receptor complex and the activation of the enzyme.

Although members of papain family of cysteine proteinases share strong amino acid homologies in their active site regions, there is only twenty to sixty percent overall amino acid homology among cathepsins.[374] Each cathepsin has unique structural features and patterns of expression suggesting specific roles in the cells and tissues they are expressed in and distinct differences in substrate affinities. Only cathepsin L and cathepsin S, the most closely related members of the family (sixty percent sequence similarity), will be discussed.

Cathepsins L and S are among the most potent elastases known.[375] Cathepsins L and S differ in some enzymatic properties, including substrate specificities and pH stability. They each have unique amino acids near the active site that confers their substrate specificity. Like most cathepsins, cathepsin L is active at an acidic pH and loses its activity quickly at a neutral pH. In contrast, cathepsin S is active at a relatively broad pH range; it is stable and active at both acidic and neutral pHs. In the lung, alveolar macrophages and smooth muscle cells synthesize and secrete cathepsins L and S. Alveolar macrophages are able to release cathepsin L and S into the extracellular space.[376] Cystatin C is the major physiologic inhibitor of cathepsin L and cathepsin S.

Cathepsin L is localized on human chromosome nine (9q21-22). Cathepsin L is initially transcribed as a 43 kDa preprocathepsin L and processed to the active 25 kDa form via a 34 kDa procathepsin L.[377] Mature cathepsin L is stored in lysosomes. The cathepsin L gene is activated by a variety of growth factors and oncogenes; procathepsin L is secreted from various cells under these conditions.[378] The extracellular functions of procathepsin L and the mature enzyme differ. In the presence of glycosaminoglycans, procathepsin L degrades both laminin and fibronectin and is converted to cathepsin L, which degrades type I and IV collagen, laminin, and fibronectin as well as elastin.[378] Cathepsin L is expressed in most tissues.

Cathepsin S is mapped to human chromosome one (1q21). The structure of the cathepsin S gene is similar to that of cathepsin L through the first five exons, the

difference is that cathepsin S has substantially larger introns.[379] The mature form of the enzyme is 28 kDa. Cathepsin S shows a restricted pattern of tissue distribution. It is found in significant quantities in antigen-presenting cells. Cathepsin S degrades fibrillar collagens, fibronectin, and laminin as well as elastase.

Cathepsins contribute to matrix remodeling either intracellularly on phagocytosed matrix molecules or extracellularly after secretion from the lysosome. Collagens can be degraded by MMPs and cathepsins. Extracellular degradation of collagen can be incomplete leaving fragments to be phagocytosed by macrophages, smooth muscle cells, and fibroblasts.[380] Within these cells, the collagen-containing phagosome fuses with lysosomes where collagen degradation is completed by cathepsins. Studies with fibroblasts have demonstrated that cytokines, hormones, and growth factors regulate this process. Uptake of fibrillar collagen can be enhanced by TGF-beta and IL-1alpha; cortisol decreases phagocytosis.[380, 381] Loss of cathepsin activity may contribute to pathologic tissue fibrosis such as in idiopathic pulmonary fibrosis.

Cathepsins released into the extracellular space can degrade matrix proteins extracellularly. Two mechanisms have been proposed for the release of cathepsins into the extracellular space: 1) altered trafficking of the newly formed enzyme and 2) regulated release from endosomes and lysosomes.[382] As most cathepsins are active at acidic pHs, it is important that they be released into an acidic microenvironment in order to maintain their function. Punturieri[376] report that macrophages acidify the pericellular environment during elastinolysis. Some pathologic conditions are associated with acidic extracellular environments due to several factors associated with the whole disease process rather than attributed to individual cells.

The elastinolytic properties of cathepsins L and S may be important in the progression of COPD or more specifically, emphysema. In 1991 Reilly and colleagues[383] demonstrated that alveolar macrophage lysates from cigarette smokers had a sevenfold increase in elastinolytic activity compared with alveolar macrophages from nonsmokers. However, they also found that the levels of active cathepsin L in smokers and nonsmokers were the same and that inhibitors of cathepsin L had little effect on lysate elastinolytic activity. They concluded that an enzyme other than, but similar to cathepsin L, must be responsible for the increase in elastinolytic activity. One year later it was

established that human macrophages express at least two cysteine proteinases, cathepsin L and S, with elastinolytic activity.[379] Furthermore, increased cathepsin L activity is found in alveolar macrophages and BAL fluid from smokers.[384]

Transgenic murine models support the role of cathepsins L and S in the development of emphysema. Induced overexpression of IL-13 in six to eight week old mice results in lesions characteristic of human emphysema.[81] It is demonstrated that IL-13-induced expression of cathepsins and MMPs, including cathepsins L and S, results in the observed emphysematous changes. When mice are given the cysteine proteinase inhibitors E-64 and leupeptin as well as MMP inhibitors, the lung damage is markedly reduced. This study provides strong evidence that both cathepsins and MMPs are involved in the proteolytic degradation of lung ECM. In addition, interferon-gamma is also known to induce the secretion of cathepsins L and S in the adult murine lung where emphysematous changes are observed.[67]

Genetic variations in the genes encoding cathepsins L (CTSL) and S (CTSS) may be important in the progression of emphysema if the polymorphisms result in altered elastinolytic activity. A number of SNPs in CTSL and CTSS are listed in the National Center for Biotechnology Information SNP database (dbSNP) (http://www.ncbi.nlm.nih.gove), however, none of these SNPs have been validated.

To date, there are no CTSL polymorphisms in the literature. A polymorphism in the promoter region of CTSL at position -1622 (C→A) was listed in the dbSNP (rs3118869). I sequenced 20 subjects to validate the SNP. In addition, using PROSCAN version 1.7 (http://bimas.dcrt.nih.gov/molbiol/proscan) I found that this polymorphism occurs in a putative Sp1 binding site.

Cao et al.[385] sequenced all of the coding regions of the CTSS gene and detected one polymorphism at nucleotide -25 (G→A) within the promoter. The functional effect of this polymorphism remains to be determined. No association studies with this polymorphism have been published thus far.

4.2 MATRIX METALLOPROTEINASE OVERVIEW

MMPs comprise a family of more than 20 structurally related proteinases that are involved in numerous cellular processes (Figure 4.1); as a whole they are capable of destroying all of the proteins of the ECM.[386-388] MMPs share a number of common properties: 1) the active enzymes cleave ECM proteins; 2) they contain common amino acid sequences; 3) they are secreted as inactive proenzymes; 4) they are activated by proteolytic cleavage; 5) they contain zinc at their active site and require calcium for stability; 6) they function at a neutral pH; and 7) they are all inhibited by TIMPs.

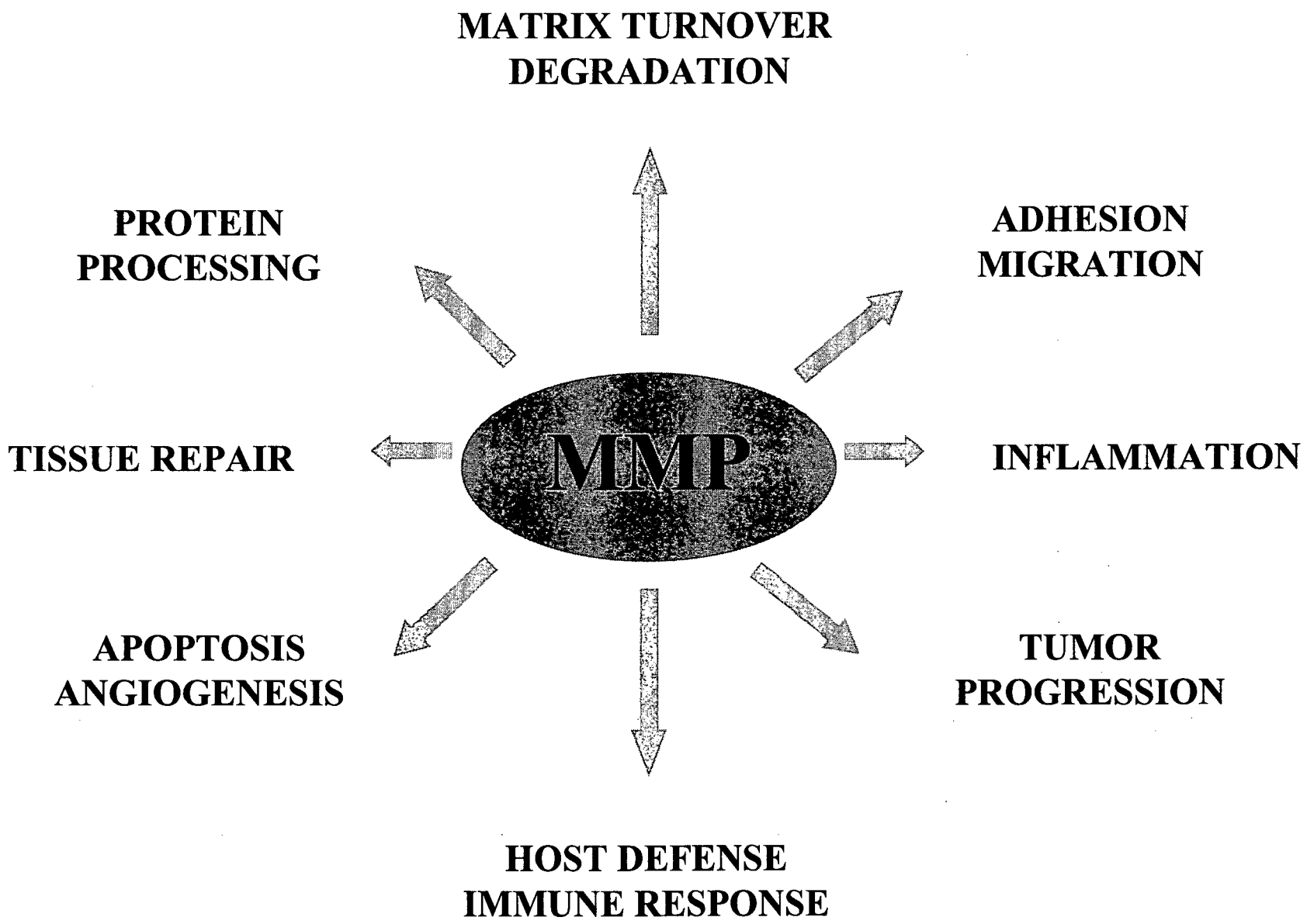


Figure 4.1 Role of matrix metalloproteinases (MMP).

MMPs differ in substrate specificity, cellular sources, and inducibility. Based on substrate specificity and primary structure, the MMP family can be loosely divided into four main groups. The four subfamilies are called collagenases, gelatinases, stromelysins, and membrane-type metalloproteinases. Some MMPs are referred to as soluble types, which are secreted into the extracellular space; others are anchored to the cell membrane. Members of the MMP family are produced by a variety of cells after stimulation by different cytokines, hormones, and growth factors.[387] In addition, MMP8 and MMP9 have been found stored within specific granules of the neutrophil.[389] This allows degranulation of pre-stored MMP8 or MMP9 from neutrophils versus de novo production after stimulation, as in other cell types.

Once activated, MMPs are capable of degrading all components of the ECM; therefore, MMP activity must be carefully controlled. MMP activity is regulated at different levels including: 1) the synthesis and secretion of proMMPs;[387] 2) the activation of inactive MMP proenzymes by proteinases, such as plasminogen activator[390] or by other members of the MMP family;[391] and 3) the inhibition of MMP enzymatic activity by TIMPs[388, 392] or reversion-inducing cysteine-rich protein with Kazal motifs[82, 83] or non-specific inhibitors, such as alpha2-macroglobulin.[388]

4.2.1 Matrix metalloproteinases and COPD

Abnormal expression of MMPs has been implicated in numerous disease processes including tumour cell invasion,[393, 394] arthritis,[395, 396] atherosclerosis,[397] arterial aneurysms,[398] and angiogenesis.[399] It is now becoming evident that ECM degradation by MMPs is likely involved in the pathogenesis of COPD.

MMPs may not only be involved in COPD through their direct role in ECM degradation, but also as regulators of inflammation. The truncated form of IL-8 is more active than the full-length form. The MMP9 aminoterminal processing of IL-8 results in a tenfold increase in activity.[400] In addition, the potent proinflammatory mediator, TNF-alpha, is activated by proteolytic cleavage by MMPs.[401]

Based on the observation that alpha-1 antitrypsin deficiency is associated with the early onset of severe emphysema, it is believed that neutrophil elastase plays an important role in COPD. Neutrophils also secrete the proteolytic enzymes, MMP8 and

MMP9. There is some convincing evidence showing the importance neutrophils in the pathogenesis of COPD. However, macrophages are the most abundant inflammatory cells under normal conditions, and in diseased lungs. Macrophages produce large amounts of MMP1, MMP9, and MMP12. Transgenic studies demonstrate that both MMP1[402] and MMP12[80] are important in the development of pulmonary emphysema in mice.

4.2.2 Matrix metalloproteinase 1

The 17 kb MMP1 gene, located on chromosome 11q22-q23,[403] is composed of 10 exons and 9 introns. Among the MMPs, MMP1 is the most highly expressed interstitial collagenase degrading fibrillar collagens, the most abundant proteins in the human body.[404] MMP1 expression is partly regulated by upstream promoter sequences.[405] A common polymorphic site, consisting of an insertion/deletion of a single G nucleotide at position -1607, was identified in the core recognition sequence of the binding sites for transcription factors that modify the level of MMP1 expression (Table 4.1).[406] Promoters containing the 2G allele display significantly higher transcriptional activity than 1G promoters do. An increase in MMP1 transcription is linked to ovarian[407] and endometrial cancer,[408] an overall poor prognosis in colorectal and esophageal cancers,[409, 410] and is thought to be associated with tumour invasion and metastasis.[410-412] Zhu et al.[404] demonstrate that the 2G2G genotype enhances lung cancer susceptibility, especially in current smokers.

Although MMP1 is not an elastinolytic enzyme, studies support a pathogenetic role for this proteinase in both experimental emphysema and human emphysema. Transgenic mice that express a human MMP1 transgene in their lungs develop disruption of the alveolar walls and coalescence of the alveolar spaces, a histological pattern similar to human emphysema.[402] In humans, Finlay et al.[413] report increased levels of MMP1 in BAL fluid and increased levels of MMP1 mRNA in alveolar macrophages from patients with emphysema.[414] Segura-Valdez et al.[415] demonstrate increased MMP1 by histochemistry in lung tissue from patients with emphysema. Imai et al.[416] report that MMP1 RNA, protein, and activity are present in the lung parenchyma of patients with emphysema and not in the lung of normal control subjects.

4.2.3 Matrix metalloproteinase 9

The 3.3 kb MMP9 gene contains 13 exons and 12 introns, and is located on chromosome 20q11.2-q13.1.[417] Compared to other members of the MMP family, MMP9 has three additional exons. The extra exons encode the amino acids of the fibronectin-like domain, which is found only in MMP9 and MMP2.[418] MMP9 is predominantly produced by macrophages but is also found in neutrophil granules. Abnormal expression of MMP9 is shown in tumour metastasis and rheumatoid arthritis.[418-420]

Two common MMP9 promoter polymorphisms effect gene transcription. Zhang et al.[421] showed that a C→T transition polymorphism at position -1562 (C→T) of the MMP9 promoter has a functional effect on transcription and is associated with severity of coronary artery disease (Table 4.1).[422] The T allele is associated with higher levels of expression in a reporter gene assay. There is a CA (cytosine–adenine) microsatellite in the promoter-enhancer region of MMP9 around the -90 region. The length of this CA repeat in the promoter of MMP9 also effects the level of transcription in a reporter gene assay (Table 4.1).[423] The activity of the MMP9 promoter is proportional to the number of CA repeats. A potential explanation for this association is that tracts of CA repeats are able to form left-handed Z-DNA.[424-426] It was proposed that Z-DNA acts as a transcriptional enhancer.[424] The microsatellite allows the DNA to switch to a Z structure, which eases the opening of the double strand of DNA and increases transcription. Thus, longer CA repeats may result in more stable Z-DNA and thus up-regulate gene expression. One allele with more repeats may express more protein than two alleles with shorter repeats.

MMP9 activity is inhibited by forming a 1:1 complex with TIMP1.[427-430] Several studies suggest that the balance between MMP9 and TIMP1 the may be an important determinant of the clinical expression of COPD.[431-433] However, the MMP9 to TIMP1 ratio is decreased in sputum of patients with chronic bronchitis.[432] On the other hand, immunohistochemical studies of emphysematous lungs show an increase in the expression of MMP9 but not of TIMP1.[433] Segura-Valdez et al.[415] found evidence to suggest that there is an upregulation of MMP9 in patients with COPD. Immunohistochemical analysis of COPD lungs show an increase in expression of MMP9, mainly in neutrophils. In the same study, BAL gelatin zymography show intense bands

corresponding to proMMP9. Finlay et al.[414] report increased levels of MMP9 mRNA in macrophages from patients with emphysema.

4.2.4 Matrix metalloproteinase 12

The 13 kb MMP12 gene, located on chromosome 11q22.2-22.3, is composed of 10 exons and 9 introns.[434] MMP12 shares the highly conserved exon size and intron-exon borders with other MMPs, similar to collagenases and stromelysins.[434] MMP12 is most closely related to MMP1 and MMP3, being forty-nine percent identical to each at the amino acid level.[435] MMP12 shares its elastinolytic activity with the gelatinases (MMP2 and MMP9),[436, 437] and to a lesser extent MMP7.[438, 439] MMP12 expression appears to be largely restricted to tissue macrophages.[434] There is a common A→G transition at position -82 (-82A→G) within the MMP12 promoter, which decreases gene transcription and is linked to severity of coronary artery disease (Table 4.1).[440] Joos et al.[181] detected a novel polymorphism in the MMP12 coding region (Asn357Ser) that may effect gene function (Table 4.1).

Studies performed on a MMP12 knockout mouse model provided support for the involvement of elastinolytic enzymes in emphysema.[80] MMP12 knockout mice exposed to cigarette smoke, at the human equivalent of 2,000 cigarettes per day, do not develop emphysematous changes in the lungs as do wild type animals.[80] These data suggest that the lack of the MMP12 enzyme may reduce the susceptibility of the animals to emphysema, however, there is no direct evidence demonstrating that excess elastinolytic activity of MMP12 is critically involved in the pathogenesis of human emphysema. MMP12 is present at very low levels in normal macrophages but expressed in high levels in the alveolar macrophages of cigarette smokers.[431] Finlay and co-workers[413] examined human alveolar macrophages from patients with emphysema and normal volunteers and did not demonstrate increased levels of MMP12 within the lavage fluid of the patients with emphysema.

Table 4.1 Summary of matrix metalloproteinase polymorphisms, allele frequencies in Caucasians, and their functional properties.

MMP Symbol	Polymorphism	Allele frequency	Functional effect
MMP1	-1607/1G→2G	2G: 0.53	2G: increased transcription[406]
MMP9	-1562C→T	T: 0.14-0.19	T: increased transcription[422]
	CA repeat		↑ CA repeat length: increased transcription[423]
MMP12	-82A→G	G: 0.11-0.19	G: decreased transcription[440]
	Asn357Ser	Ser357: 0.05	Not known[181]

The abbreviation MMP denotes matrix metalloproteinase.

4.3 MATRIX METALLOPROTEINASE ASSOCIATION STUDIES

4.3.1 Genetic polymorphisms in MMP1, MMP9, and MMP12 and rate of decline of lung function

The Lung Health Study was conducted by the National Heart, Lung and Blood Institute and was designed to describe the natural history of cigarette smoke-induced COPD.[280] A total of 5,887 male and female smokers were recruited, aged 35 to 60 years, who had signs of early COPD based on lung function measurements. During five years of follow-up, 3,216 subjects continued to smoke and from this group Joos et al.[181] selected the 284 with the fastest rate of decline of FEV1 and the 306 who had the slowest decline (this group actually had a slight increase in their lung function). All of the subjects were Caucasians.

In order to investigate the role of the MMP1, MMP9, and MMP12 polymorphisms in the development of COPD, Joos and coworkers[181] determined the prevalence of five MMP polymorphisms in the continuing smokers chosen from the LHS.

The 2G allele of the MMP1 -1607/1G→2G polymorphism was associated with an increased rate of decline in lung function (p=0.02).[181] Genetic polymorphisms of MMP9 and MMP12 were not individually associated with rate of decline of lung function.[181]

Haplotype analysis of alleles from the MMP1 -1607/1G→2G and MMP12 Asn357Ser polymorphisms revealed an association with rate of decline of lung function (p=0.0007).[181] These data suggest that the polymorphisms in the MMP1 and MMP12 genes investigated by Joos et al.[181] are either causative factors in smoking-related lung injury or are in linkage disequilibrium with causative polymorphisms.

4.3.2 Genetic polymorphism in MMP9 and pulmonary emphysema

Minematsu et al.[183] also examined the association between the MMP9 promoter polymorphism (-1562C→T) and the development of pulmonary emphysema in Japanese habitual smokers. They reported that the T allele frequency was higher in subjects with distinct emphysema on chest CT-scans than in those without it (p=0.02). In addition, a measure of the gas exchange capacity of the lung (diffusing capacity of the lung for carbon monoxide per liter of alveolar volume) was lower (p=0.02) and emphysematous

changes were more conspicuous (p=0.03) in subjects with CT or TT than those with the CC genotype.[183] These data were consistent with the higher level of gene expression associated with the T allele in the in vitro assay.[421] These data from Minematsu et al.[183] suggest that the -1562C→T polymorphism acts as a genetic factor for the development of smoking-induced pulmonary emphysema in Japanese subjects, however, the T allele may not contribute to the disease process, but may be in linkage disequilibrium with the true disease-causing allele.

4.3.3 Summary of matrix metalloproteinase association studies

Joos and coworkers[181] were the first to assess whether the MMP1 and MMP12 genetic polymorphisms influence smokers' susceptibility to COPD. Further studies are needed to validate these findings.

The inconsistent results reported by Joos et al.[181] and Minematsu et al.[183] regarding the MMP9 -1562C→T polymorphism could be due to many factors. Such factors include differences between study populations in ethnic and geographic origin, and the COPD phenotypes tested.

It is important to consider ethnic origin when comparing studies because different ethnic groups will have different genetic backgrounds. The -1562T allele may result in susceptibility to emphysema only through an interaction with other risk alleles and these other risk alleles may be absent, or rare, in the Caucasian population. Therefore, an association may be seen in the Japanese population that is not seen in other ethnic groups.

Geographic origin may also produce conflicting results because it is difficult to control for all known environmental factors that predispose to COPD. These environmental factors may be different in different geographical regions.

Another reason for the apparent discrepancy between the results of Joos et al.[181] and Minematsu et al.[183] could be that these studies utilized very different phenotypes. While measures of lung function correlate with measures of emphysema the correlation is far from perfect. As discussed above, inadequate lung function can result from both emphysema and inflammatory narrowing of the airways. The MMP polymorphisms would be predicted to influence proteolytic destruction of the lung and therefore contribute to the development of emphysema. The emphysema may then result in

decreases in lung function but only indirectly; therefore, it would be more difficult to detect an association with decline of lung function as the phenotype. In support of this idea there was no association of FEV1 with the -1562T allele in the study by Minematsu et al.[183]

4.4 ALVEOLAR MACROPHAGE PROTEINASE GENETIC POLYMORPHISMS AND EXPRESSION IN THE DEVELOPMENT OF PULMONARY EMPHYSEMA

4.4.1 Introduction

Alveolar macrophages produce potent proteolytic enzymes including cathepsin L, S, MMP1, MMP9, and MMP12[376, 441] and a number of studies support the role of these enzymes in the alveolar destruction characteristic of emphysema. Genetic polymorphisms that result in altered expression and/or function of these enzymes may increase smokers' susceptibility to the development of emphysema.

4.4.2 Hypothesis

I hypothesized that in individuals who smoke, CTSL, CTSS, MMP1, MMP9, and MMP12 promoter polymorphisms will be associated with: 1) increased cathepsin and MMP mRNA transcript levels and 2) the development of emphysema.

4.4.3 Specific aims

The aim of this study was to examine the role of alveolar macrophage proteinase genetic polymorphisms in the development of emphysema using human diseased lungs. The experimental design is shown in Figure 4.2. Aim one: to evaluate alveolar macrophages obtained by BAL of resected lung specimens to determine whether CTSL, CTSS, MMP1, MMP9, and MMP12 mRNA transcript levels are related to the cathepsin and MMP promoter polymorphisms. Aim two: to determine whether the promoter polymorphisms are related to the presence and extent of emphysema as assessed by computed tomography (CT) scans. Aim three: to determine whether mRNA expression was related to emphysema.

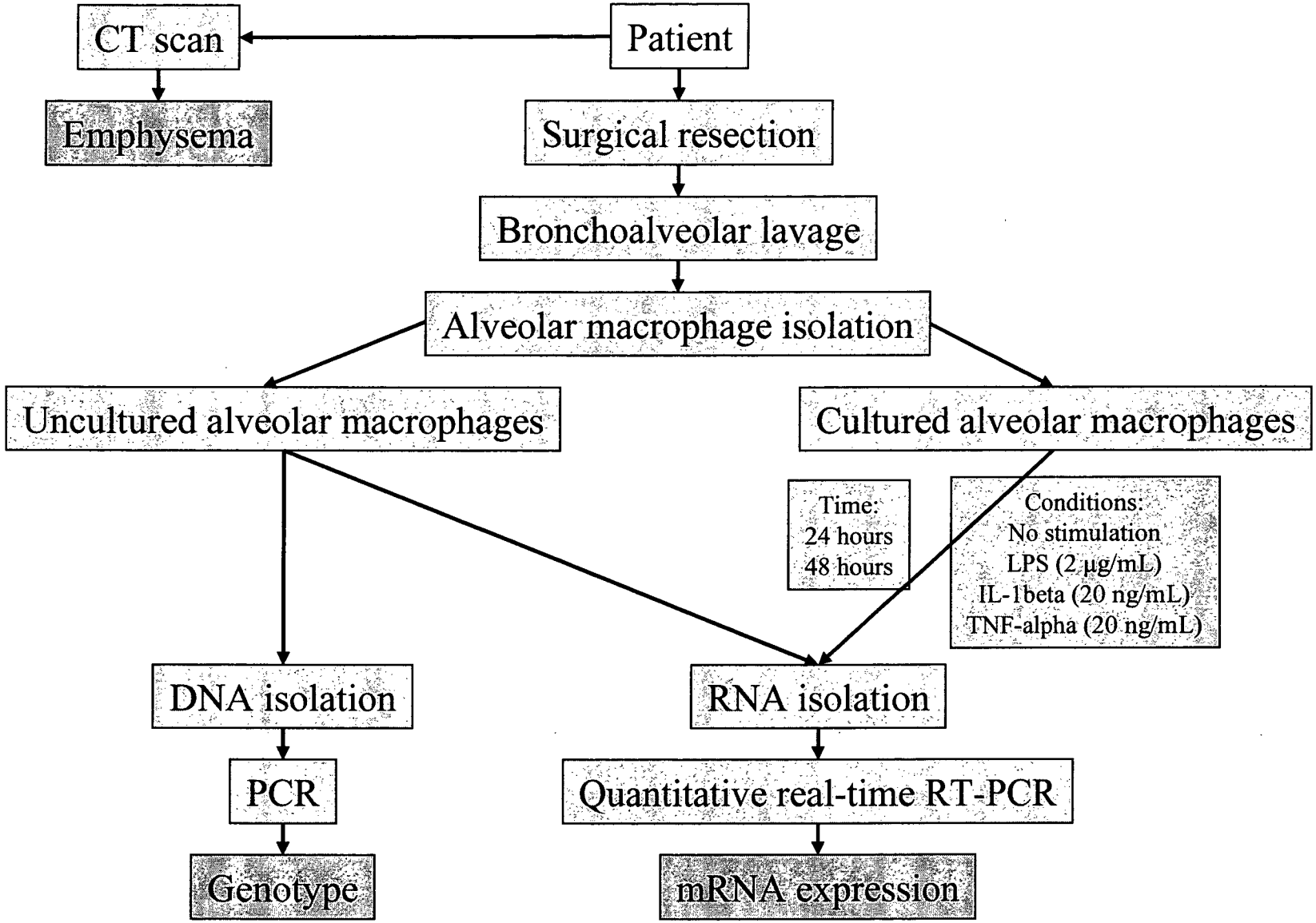


Figure 4.2 Experimental design.

CT denotes computed tomography; LPS, lipopolysaccharide; IL, interleukin; TNF, tumor necrosis factor; PCR, polymerase chain reaction; and RT-PCR, reverse transcriptase-polymerase chain reaction.

4.4.4 Methods

Study subjects

Fifty-four subjects (forty-eight Caucasian, six Asian) who underwent lung resection for a small (<3 cm), Stage I or II, peripheral tumour at Vancouver General Hospital between April 2001 and March 2003 were recruited for the study (Table 4.2 and 4.3). This study was approved by the University of British Columbia/Providence Health Care and Vancouver Hospital and Health Sciences Research Ethics Boards. All subjects provided written informed consent for the use of physiologic data, CT scans, and surgically resected tissue. All subjects completed a questionnaire.

Table 4.2 Characteristics of subjects, smoking history, spirometry, alpha-1 antitrypsin genotype, and cellular content of bronchoalveolar lavage fluid.

Variable	Subjects (n=54)
Age, yr†	66.6 (1.4)
Sex, n (%)	26 men (48)
Smoking history	
Current smoking, n (%)	24 (44)
Former smoking, n (%)	53 (98)
Pack year*†	46.7 (3.8)
Spirometry	
FEV1, % predicted†	85.7 (2.9)
FVC, % predicted†	93.6 (2.0)
FEV1/FVC†	69.7 (1.7)
DL_{CO}, % predicted†	74.5 (2.6)
Alpha-1 antitrypsin genotype, n (%)	
PiS	
MM	46 (85.2)
MS	8 (14.8)
SS	0
PiZ	
MM	54 (100)
MZ	0
ZZ	0
Cell content of BAL fluid†	
Total cell count, x 10^6	270.7 (42.9)
Macrophages, %	80.3 (2.4)
Neutrophils, %	8.5 (1.4)
Lymphocytes, %	11.2 (1.5)

† Mean (SE).

* Number of packs of cigarettes smoked per day x number of years of smoking.

The abbreviation FEV1 denotes forced expiratory volume in one second; FVC, forced vital capacity; DL_{CO}, carbon monoxide diffusing capacity; PiS, S defective allele for alpha-1 antitrypsin deficiency; PiZ, Z defective allele for alpha-1 antitrypsin deficiency; and BAL fluid, bronchoalveolar lavage fluid.

Table 4.3 Characteristics of subjects.

Subject	Age	Sex	Current smoker	Pack year*	FEV1, % predicted	FVC, % predicted	FEV1 / FVC	DL_{CO}, % predicted	A1-AT PiS	A1-AT PiZ
1	77	F	No	53.0	95.0	95.0	68.6	96.0	MM	MM
2	70	M	Yes	46.0	68.0	81.0	66.7	86.0	MM	MM
3	70	M	No	40.0	N/A	N/A	75.2	N/A	MM	MM
4	53	F	Yes	36.0	51.0	91.0	45.1	70.3	MS	MM
5	65	F	No	20.0	79.0	79.0	76.4	77.0	MM	MM
6	72	M	No	54.0	90.0	95.0	72.1	78.0	MM	MM
7	60	F	Yes	50.0	98.0	95.0	100.0	90.1	MS	MM
8	64	M	Yes	55.0	66.0	97.0	53.9	N/A	MM	MM
9	77	F	No	42.0	106.0	93.0	78.0	96.0	MM	MM
10	77	F	No	4.2	97.0	102.0	70.3	69.2	MM	MM
11	46	M	No	24.0	85.0	86.0	80.5	110.0	MM	MM
12	58	F	Yes	52.5	N/A	N/A	N/A	57.0	MM	MM
13	68	F	Yes	52.0	95.9	98.3	77.1	68.9	MM	MM
14	87	M	No	27.0	81.0	88.0	66.3	83.1	MM	MM
15	74	F	No	3.8	97.0	78.0	88.4	68.0	MM	MM
16	57	F	Yes	36.0	88.0	98.0	66.7	N/A	MM	MM
17	50	F	Yes	42.5	90.0	87.0	79.4	N/A	MM	MM
18	74	M	Yes	150.0	N/A	N/A	69.1	N/A	MM	MM
19	71	M	No	69.0	73.0	88.0	70.3	80.0	MM	MM
20	59	M	Yes	70.5	N/A	N/A	N/A	N/A	MM	MM
21	59	F	Yes	40.0	N/A	N/A	75.0	N/A	MM	MM
22	74	F	No	43.0	97.0	98.0	73.3	N/A	MM	MM
23	74	F	Yes	91.5	94.0	83.0	79.0	N/A	MS	MM
24	55	F	Yes	38.0	79.0	92.0	69.9	50.0	MM	MM
25	71	F	Yes	52.0	97.0	104.0	72.0	53.0	MM	MM
26	52	F	Yes	39.0	113.0	113.0	75.3	85.0	MM	MM
27	66	M	No	94.0	56.0	68.0	56.2	N/A	MM	MM
28	55	F	No	70.0	N/A	N/A	N/A	N/A	MM	MM
29	73	M	Yes	61.0	69.0	89.0	59.9	68.0	MS	MM
30	75	M	No	135.0	55.0	76.0	55.5	81.0	MM	MM
31	76	M	Yes	60.0	83.0	101.0	62.9	83.0	MM	MM
32	77	M	No	40.0	94.0	92.0	77.7	67.0	MM	MM
33	68	F	No	61.5	81.0	86.0	73.1	62.0	MM	MM

34	58	F	Yes	37.0	84.0	87.0	76.8	66.0	MM	MM
35	68	M	No	22.0	109.0	108.0	75.5	105.0	MS	MM
36	46	M	Yes	26.0	97.0	108.0	67.9	84.0	MM	MM
37	53	F	Yes	34.0	88.0	102.0	68.9	101.0	MM	MM
38	62	F	No	33.0	N/A	N/A	N/A	72.0	MS	MM
39	63	F	No	50.0	82.0	103.0	62.7	75.0	MM	MM
40	69	F	Yes	44.0	100.0	90.0	79.7	N/A	MM	MM
41	51	F	No	24.0	18.0	64.0	23.2	27.0	MS	MM
42	72	M	No	26.3	68.0	90.0	58.6	39.0	MM	MM
43	73	M	No	105.0	47.0	78.0	45.9	62.0	MM	MM
44	56	M	Yes	42.0	83.0	88.0	67.7	N/A	MM	MM
45	68	M	No	32.0	101.0	107.0	72.9	77.0	MS	MM
46	62	M	No	33.0	97.0	130.0	58.8	69.0	MM	MM
47	88	M	N/A	N/A	81.0	78.0	75.6	75.0	MM	MM
48	83	M	No	5.6	66.0	68.0	73.5	94.0	MM	MM
49	56	M	Yes	42.0	87.0	96.0	72.1	67.0	MM	MM
50	74	F	No	28.5	91.0	114.0	60.6	69.0	MM	MM
51	67	F	Yes	51.0	104.0	109.0	74.6	78.0	MM	MM
52	83	M	No	33.0	125.0	130.0	73.2	69.0	MM	MM
53	71	F	No	34.0	113.0	104.0	76.9	71.0	MM	MM
54	67	M	No	20.0	110.0	90.0	85.2	N/A	MM	MM

* Number of packs of cigarettes smoked per day x number of years of smoking.

The abbreviation FEV1 denotes forced expiratory volume in one second; FVC, forced vital capacity; DL_{CO}, carbon monoxide diffusing capacity; A1-AT, alpha-1 antitrypsin; PiS, S defective allele for alpha-1 antitrypsin deficiency; and PiZ, Z defective allele for alpha-1 antitrypsin deficiency.

Bronchoalveolar lavage

Lung tissue was obtained from patients undergoing surgical resection for carcinoma. The peripheral location of the tumour allowed lavage from unaffected lung segments. BAL was performed within 1 h after the completion of lung resection. BAL was performed under sterile conditions. A 20-gauge catheter was inserted into an airway supplying an uninvolved (by tumour) segment or lobe. The tissue was instilled with 60 mL of cold saline and BAL fluid was aspirated back. The tissue was gently massaged to help recover the instilled saline. This procedure was repeated five times. Aspirated BAL fluid was collected in sterile plastic bottles. Recovery was between 250 and 300 mL. The BAL fluid was filtered through sterile gauze to remove debris and mucus, and the filtrate was centrifuged (250 *g*, 10 min). Cytospin preparations were made for differential cell counts. Cells were centrifuged (500 rpm, 5 minutes) using a Shandon II cytocentrifuge (Shandon, Pittsburgh, PA) and air dried for 1 h at room temperature. Differential counts were determined on Diff-Quik stained preparations. Cell viability was consistently >90% as assessed using trypan blue exclusion.

Alveolar macrophage culture

Alveolar macrophages were separated by Ficoll-Hypaque density centrifugation. Cells were washed twice with RPMI 1640 (Gibco, Burlington, ON) and resuspended in RPMI 1640 at a final concentration of 10^6 alveolar macrophages/mL. Cells were plated onto 6-well plates (Costar, Cambridge, MA) at a density of 4 million cells/well. Following adherence at 37°C in a humidified incubator (95% air, 5% CO_2 vol/vol) for 2 h, cells were washed two times with RPMI 1640 to remove non-adherent cells before stimulation. Only alveolar macrophages will adhere to cell culture plates; neutrophils do not adhere. Fresh Macrophage-SFM (Gibco), supplemented with 2 mM L-glutamine and penicillin-streptomycin (100 U/mL-100 μg/mL; Gibco), was added to the adhered cells (>99% alveolar macrophages in all cases). Alveolar macrophages were then cultured for a further 24 h or 48 h in the presence of medium alone, medium + lipopolysaccharide (LPS) (2 μg/mL), medium + IL-1beta (20 ng/mL), or medium + TNF-alpha (20 ng/mL). LPS (*Escherichia coli*) was from Sigma (St. Louis, MO). Recombinant human IL-1beta and TNF-alpha were purchased from R&D Laboratories (Minneapolis, MN). Alveolar

macrophages are relatively difficult to maintain in culture; short-term (up to 72 hours) maintenance is recommended.[442] Cell culture time, stimulants, and stimulant concentrations were based on previous work.[443-445]

RNA extraction and cDNA synthesis

Total RNA was isolated from alveolar macrophages using RNeasy Mini Kit according to the manufacturer's protocol (Qiagen). All preparations were treated with RNase-free DNase (Qiagen) to remove genomic DNA. 0.5 to 1 μg of RNA was reverse transcribed in a total volume of 20 μl in the presence of 200 U SuperScript RNase H- Reverse Transcriptase (Invitrogen, Carlsbad, CA), 40 U RNaseOUT Recombinant Ribonuclease Inhibitor (Invitrogen), and 0.5 μg Oligo(dT) Primer (Invitrogen) according to the manufacturer's instructions.

SYBR-green quantitative real-time reverse transcriptase–polymerase chain reaction

Quantitative two-step real-time RT-PCR was performed using a LightCycler (Roche Molecular Biochemicals) in order to assess CTSL, CTSS, MMP1, MMP9, and MMP12 expression in alveolar macrophages. Beta actin (ACTB) was used as a housekeeping gene. A complete overview of the primers, annealing temperatures, and single detection temperatures used to amplify the RT-generated cDNAs are presented in Table 4.4. Primer pairs were designed to flank an intron-containing sequence. All PCR conditions (except MMP12) used 3 mM Mg^{2+}, 0.3 μM forward and reverse primers, and 2 μL of LightCycler FastStart DNA Master SYBR Green I Mix (Roche Molecular Biochemicals) in a final volume of 20 μL. MMP12 used 5 mM Mg^{2+} and 0.1 μM forward and reverse primers. The samples were loaded in the LightCycler glass capillaries, closed, centrifuged, and placed in the LightCycler rotor. The cycling program consisted of 10 minutes of initial denaturation at 95°C and 45 cycles at 95°C for 5 s, T_A°C for 5 s, 72°C for 20 s, and single detection for 1 s with a single fluorescence acquisition (ramp rates 20°C/s). The analytical melting program was 95°C for 0 s and 65°C for 15 s, increasing to 95°C at a ramp rate of 0.2°C/s, with continuous fluorescence acquisition. Each sample was run in triplicate.

Table 4.4 Primers and conditions for RT-PCR experiments.

Gene	Primers	Annealing Temperature	Single Detection	Fragment Length
ACTB	5'-CACCCTGAAGTACCCCATC	56°C	85°C	226 bp
	5'-TAGCACACGCTGGATAGCAAC			
CTSL	5'-TCACAGTTTAGAGGCACAGTGG	54°C	80°C	241 bp
	5'-CTTCCTGGGCTTACGGTTTT			
CTSS	5'-TTGCCTGATTCTGTGGACTG	56°C	82°C	205 bp
	5'-CACCATTGCAGCCTTTGTTTCC			
MMP1	5'-ACAAACCCCAAAAGCGTGT	53°C	79°C	197 bp
	5'-CTCTGTCGGCAAATTCGTA			
MMP9	5'-AGACACCTCTGCCCTCACC	56°C	85°C	202 bp
	5'-ACGCATCTCTGCCACCCGA			
MMP12	5'-ATGATGAAAGGAGACAGATGATGG	54°C	77°C	203 bp
	5'-ACAACCAAACCAGCTATTGC			

The abbreviation ACTB denotes beta actin; CTSL, cathepsin L; CTSS, cathepsin S; MMP1, matrix metalloproteinase 1; MMP9, matrix metalloproteinase 9; MMP12, matrix metalloproteinase 12; bp, base pairs; and RT-PCR, reverse transcriptase–polymerase chain reaction.

A standard curve was included in each run. Standards were prepared by cloning the target sequence into plasmid DNA. The data were analyzed by using the second-derivative maximum of each amplification reaction and relating it to its respective standard curve. The results from the quantitative PCR were expressed as the ratio of the mean target gene measurements to the mean housekeeping gene value for a given sample (Target:Reference) (Equation A). In addition, the results were analyzed according to the method of Muller et al.,[446] with a slight modification. Relative gene expression was determined using the following formulas:

(A) Relative expression level = Copies of target / Copies of reference

(B) Relative expression level = $(E_{reference})^{CTreference,\ mean} / (E_{target})^{CTtarget,\ mean}$

$E = 2$

(C) Relative expression level = $(E_{reference})^{CTreference,\ mean} / (E_{target})^{CTtarget,\ mean}$

$A = B$

where $E_{reference}$ is the PCR amplification efficiency of the housekeeping gene, E_{target} is the PCR amplification efficiency of the target gene, $CT_{reference}$ is the threshold cycle of the PCR amplification of the housekeeping gene, and CT_{target} is the threshold cycle of the PCR amplification of the target gene. Equation B assumes that both the target gene and reference gene are amplifying with the maximum efficiency, E=2 for both. Most of the time, a PCR reaction is not amplifying with the maximum efficiency due to factors such as high G-C content, and the amplification efficiencies of the target gene and the reference gene differ. Differences in PCR efficiency, as little as 0.05, between a target and housekeeping gene can result in a more than 2-fold difference in the final result after 30 PCR cycles.[447] Equation C is not based on the assumption that the PCR amplification efficiencies of the target gene and reference gene are maximal (E=2) or equal to each other. All of the data reported were analyzed using Equation C, efficiency-corrected relative gene expression.

Genotyping

Genomic DNA was isolated from alveolar macrophages using DNeasy Tissue Kit according to the manufacturer's protocol (Qiagen). Template-free controls and known genotype controls were included in each experiment. Genotypes were assigned by two independent investigators who were unaware of the patients' identities and phenotypes. Inconsistencies were resolved by two additional genotyping reactions. Five samples were selected at random and sequenced to confirm each genotyping protocol (except for the CTSL -1622A→C polymorphism, which was detected through sequencing).

CTSS -25G→A. An amplicon of 164 bp was generated by 40 cycles of PCR using the sense primer 5'-ACCTCATGTGACAAGTTCCAAT and the antisense primer 5'-ACCAAATGGGAGAAAAAGAACA as previously described.[385] PCRs were performed in a total reaction volume of 20 μL with 100 ng of genomic DNA, 0.5 U HotStar *Taq* (Qiagen), 10X PCR buffer (Qiagen), 1.5 mM Mg^{2+}, 0.5 μM forward and reverse primers, and 200 μM dNTPs, with an annealing temperature of 55°C. After PCR, 20 μL of the reaction mixture was digested with 2 U *Sfc* I restriction endonuclease (New England BioLabs Inc.). The digest mixture was resolved on a 3% agarose gel stained with ethidium bromide. DNA from individuals with the homozygous G genotype (GG) produced one band at 164 bp; the homozygous A genotype (AA) produced two bands at 104 bp and at 60 bp; and the heterozygous genotype (GA) produced all three bands (Figure 4.3).

MMP9 -1562C→T. Genotyping was performed as previously described.[181] An amplicon of 435 bp was generated by 35 cycles of PCR using the sense primer 5'-GCCTGGCACATAGTAGGCCC and the antisense primer 5'-CTTCCTAGCCAGCCGGCATC. PCRs were performed in a total reaction volume of 20 μL with 100 ng of genomic DNA, 0.5 U HotStar *Taq* (Qiagen), 10X PCR buffer (Qiagen), 1.5 mM Mg^{2+}, 0.5 μM forward and reverse primers, and 200 μM dNTPs, with an annealing temperature of 60°C. After PCR, 20 μL of the reaction mixture was digested with 5 U *Sph* I restriction endonuclease (New England BioLabs Inc.). The digest mixture was resolved on a 2% agarose gel stained with ethidium bromide. DNA from individuals

with the homozygous C genotype (CC) produced one band at 435 bp; the homozygous T genotype (TT) produced two bands at 188 bp and 247 bp; and the heterozygous genotype (CT) produced all three bands (Figure 4.4).

MMP12 -82A→G. Genotyping was performed as previously described.[181] An amplicon of 199 bp was generated by 40 cycles of PCR using the sense primer 5'-GAGATAGTCAAGGGATGATATCAGC and the antisense primer 5'-AAGAGCTCCAGAAGCAGTGG. PCRs were performed in a total reaction volume of 20 μL with 100 ng of genomic DNA, 0.5 U *Taq* polymerase (Invitrogen), 10X PCR buffer (Invitrogen), 2 mM Mg^{2+}, 1 μM forward and reverse primers, and 200 μM dNTPs, with an annealing temperature of 65°C. After PCR, 20 μL of the reaction mixture was digested with 10 U *Pvu* II restriction endonuclease (New England BioLabs Inc.). The digest mixture was resolved on a 3% agarose gel stained with ethidium bromide. DNA from individuals with the homozygous A genotype (AA) produced one band at 199 bp; the homozygous G genotype (GG) produced two bands at 175 bp and 24 bp; and the heterozygous genotype (AG) produced all three bands (Figure 4.5).

Alpha-1 antitrypsin. Multiplex PCR- restriction fragment length polymorphism analysis was used to detect the alpha-1 antitrypsin S and Z mutations as previously described by our laboratory.[186, 448] An amplicon of 98 bp was generated using the sense primer EX35 5'- GAGGGGAAACTACAGCACCTCG and the antisense primer EX33 5'-ACCCTCAGGTTGGGGAATCACC. Another amplicon of 144 bp was generated using the sense primer EX55 5'- TAAGGCTGTGCTGACCATCGTC and the antisense primer BYZ 5'- GGAGACTTGGTATTTTGTTCAATC. 35 cycles of PCR were performed in a total reaction volume of 20 μL with 100 ng of genomic DNA, 0.5 U *Taq* polymerase (Invitrogen), 10X PCR buffer (Invitrogen), 1.5 mM Mg^{2+}, 1 μM EX35 and EX33 primers, 0.5 μM EX55 and BYZ primers, and 200 μM dNTPs, with an annealing temperature of 58°C. After PCR, 20 μL of the reaction mixture was digested with 10 U *Taq* I restriction endonuclease (New England BioLabs Inc.). The digest mixture was resolved on a 2.5% agarose gel stained with ethidium bromide. For the alpha-1 antitrypsin S mutation, DNA from individuals with the homozygous M genotype (MM)

produced two bands, at 20 bp and 78 bp; the homozygous S genotype (SS) produced one band at 98 bp; and the heterozygous genotype (MS) produced all three bands. For the alpha-1 antitrypsin Z mutation, DNA from individuals with the homozygous M genotype (MM) produced two bands, at 21 bp and 123 bp; the homozygous Z genotype (ZZ) produced one band at 144 bp; and the heterozygous genotype (MZ) produced all three bands (Figure 4.6).

MMP1 -1607/1G→2G. Genotyping of the MMP1 single nucleotide insertion polymorphism was performed using hybridization probes and melting curve analysis using a LightCycler (Roche Molecular Biochemicals). A 254 or a 255 bp fragment was amplified using the sense primer 5'-ATGCCTCTGAGAAGAGGAT and the antisense primer 5'-ACCTTTCCCACTGTATCAG. The donor probe sequence was 5'-TTGATTTGAGATAAGTCATATCCTTTCTAAT and the acceptor probe sequence was 5'-TTAACTACAATTTCCTCATCTAAGTGGCAT. The 31-mer donor probe was labeled at the 3' end with fluorescein. The 30-mer acceptor probe was labeled at the 5' end with LightCycler Red 640 and modified at the 3' end by phosphorylation to block extension; the acceptor probe was complementary to the leading strand of the 2G allele (mismatched when bound to the 1G allele). The donor and acceptor probes were separated by three bases when bound to the template. Both fluorophore-labeled probes were synthesized and purified by reverse-phase HPLC by TIB Molbiol LLC (Adelphia, NJ). Real-time PCR were performed in a total reaction volume of 20 μL with 100 ng of genomic DNA, 0.3 μM forward and reverse primers, 0.2 μM donor probe and 0.4 μM acceptor probe, 3 mM Mg^{2+}, and 2 μL LightCycler-FastStart DNA Master Hybridization Probes (Roche Molecular Biochemicals). The samples were loaded in the LightCycler glass capillaries, closed, centrifuged, and placed in the LightCycler rotor. The cycling program consisted of 10 minutes of initial denaturation at 95°C and 50 cycles at 95°C for 0 s (ramp rate 20°C/s), 54°C for 10 s (ramp rate 3°C/s), and 72°C for 10 s (ramp rate 20°C/s). The analytical melting program was 95°C for 10 s and 45°C for 20 s, increasing to 85°C at a ramp rate of 0.1°C/s, with continuous fluorescence acquisition (Figure 4.7).

MMP9 CA repeat. GeneScan analysis was used to determine the number of CA repeats in the promoter of the MMP9 gene. Thirty-five cycles of PCR were performed using an unlabeled forward primer 5'-GAGGTGGTGTAAGCCCTTTCTCATGC and a fluorescently labeled (TAMRA) reverse primer 5'-ATGGTGAGGGCAGAGGTGTCTGACT. PCRs were performed in a total reaction volume of 20 μL with 100 ng of genomic DNA, 0.5 U *Taq* polymerase (Qiagen), 10X PCR buffer (Qiagen), 1.5 mM Mg^{2+}, 0.5 μM forward and reverse primers, and 200 μM dNTPs, with an annealing temperature of 62°C (Figure 4.8). Following PCR amplification, products were mixed with an internal standard (GeneScan-350 ROX, Applied Biosystems, Mississauga, ON). The alleles were separated on an ABI PRISM 3700 multi-capillary electrophoresis system (Applied Biosystems) and allele determination was performed using GeneScan analysis software (Applied Biosystems) (Figure 4.9). DNA standards containing 14, 15, 20, 21, and 23 CA repeats were identified by direct sequencing and were included as positive controls in each experiment. Alleles were defined as: small (less than 16 CA repeats) and large (greater than or equal to 17 CA repeats).[181]

CTSL -1622A→C. The CTSL promoter polymorphism was detected through direct sequencing of a PCR fragment. An amplicon of 176 bp was generated by 30 cycles of PCR using the sense primer 5'-CAGGAGCCACTCGCCCAGTG and the antisense primer 5'-CTGGCGCGGTTCGTGGCTTG. PCRs were performed in a total reaction volume of 20 μL with 100 ng of genomic DNA, 0.5 U HotStar *Taq* (Qiagen), 10X PCR buffer (Qiagen), 1.5 mM Mg^{2+}, 0.5 μM forward and reverse primers, and 200 μM dNTPs, with an annealing temperature of 64°C. After PCR, the PCR products were purified using QIAquick PCR Purification Kit according to the manufacturer's protocol (Qiagen) and then sequenced using the sense primer 5'-CAGGAGCCACTCGCCCAGTG. Cycle sequencing was performed using Applied Biosystems chemistries (ABI BigDye™ v3.1 Terminator Chemistry) and equipment (ABI PRISM 377) (Figure 4.10).

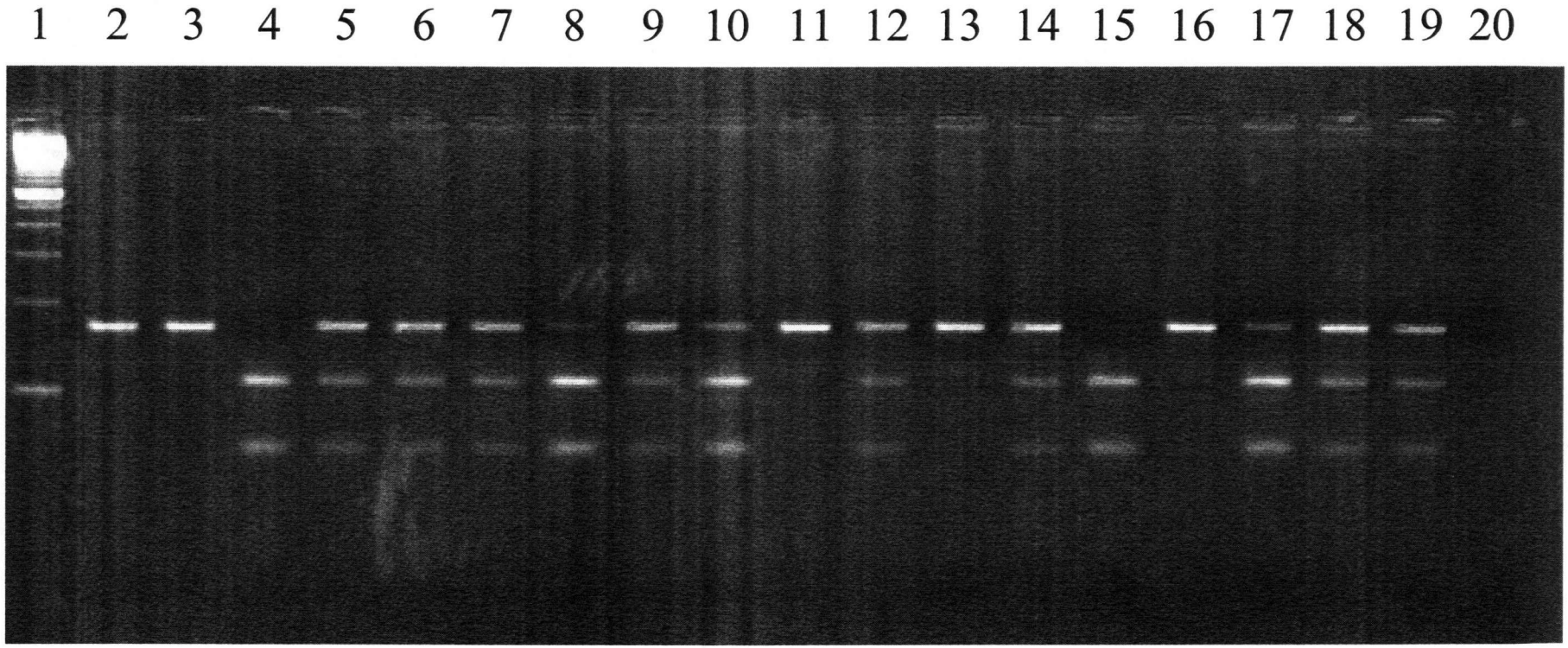


Figure 4.3 Genotyping of the CTSS -25G→A SNP.

The PCR product was digested with the *Sfc* I restriction endonuclease. After digestion, the digest mixture was resolved on a 2.5% agarose gel stained with ethidium bromide. Lane 1, size marker (100 bp DNA Ladder from New England BioLabs); lanes 2, 3, 11, 13, and 16, homozygotes for the G allele; lanes 4 and 15, homozygotes for the A allele; lanes 5-10, 12, 14, and 17-19, heterozygotes for the G and A alleles; and lane 20, negative control, no bands produced. CTSS denotes cathepsin S.

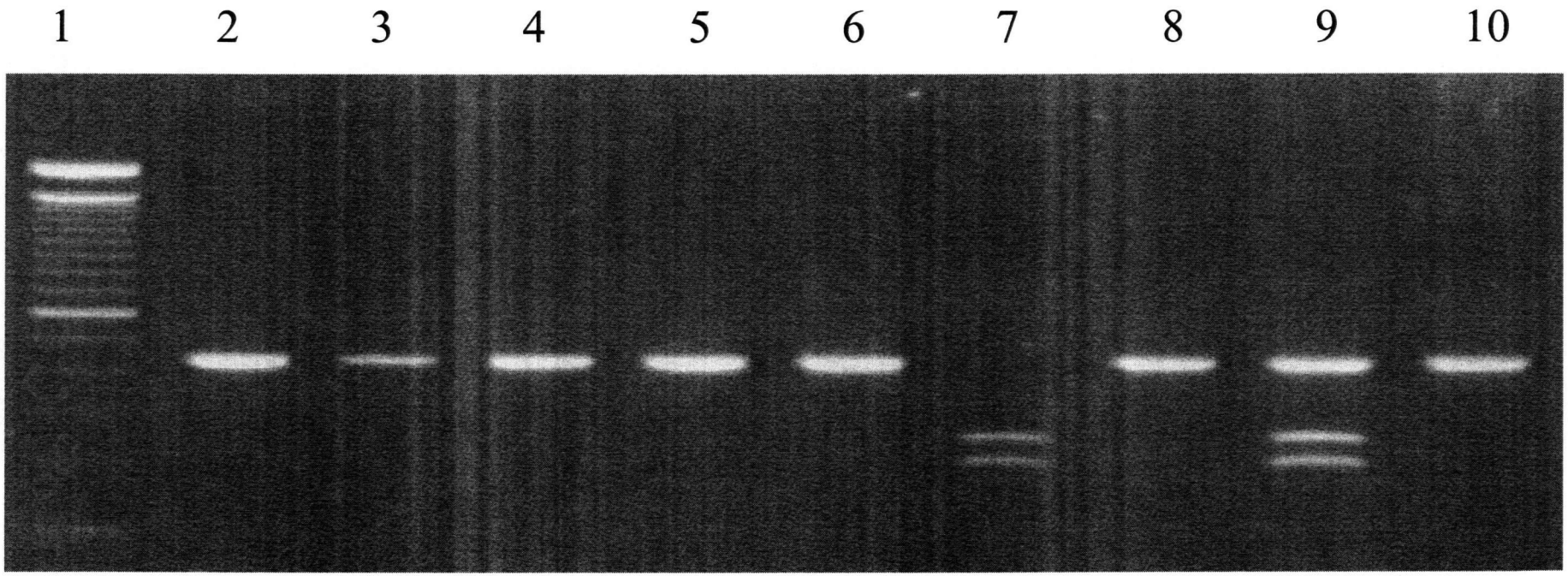


Figure 4.4 Genotyping of the MMP9 -1562C→T SNP.

The PCR product was digested with the *Sph* I restriction endonuclease. After digestion, the digest mixture was resolved on a 2% agarose gel stained with ethidium bromide. Lane 1, size marker (100 bp DNA Ladder from New England BioLabs); lanes 2-6, 8, and 10, homozygotes for the C allele; lane 7, homozygote for the T allele; and lane 9, heterozygote for the C and T alleles. MMP9 denotes matrix metalloproteinase 9.

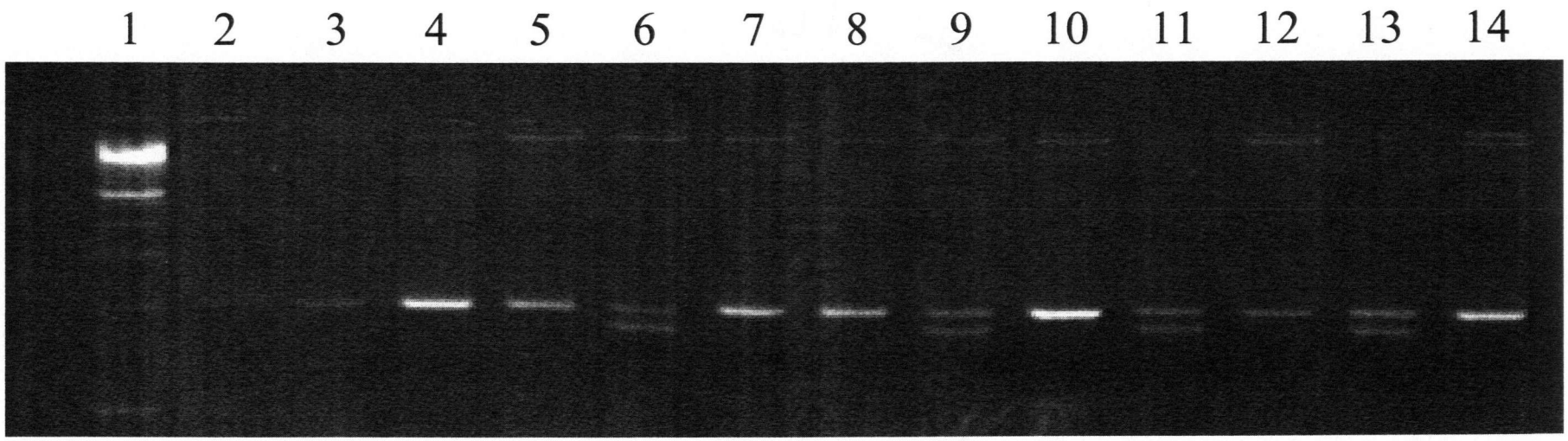


Figure 4.5 Genotyping of the MMP12 -82A→G SNP.

The PCR product was digested with the *Pvu* II restriction endonuclease. After digestion, the digest mixture was resolved on a 3% agarose gel stained with ethidium bromide. Lane 1, size marker (100 bp DNA Ladder from New England BioLabs); lanes 2-5, 7, 8, 10, 12, and 14, homozygotes for the A allele; and lanes 6, 9, 11, and 13, heterozygotes for the A and G alleles. MMP12 denotes matrix metalloproteinase 12.

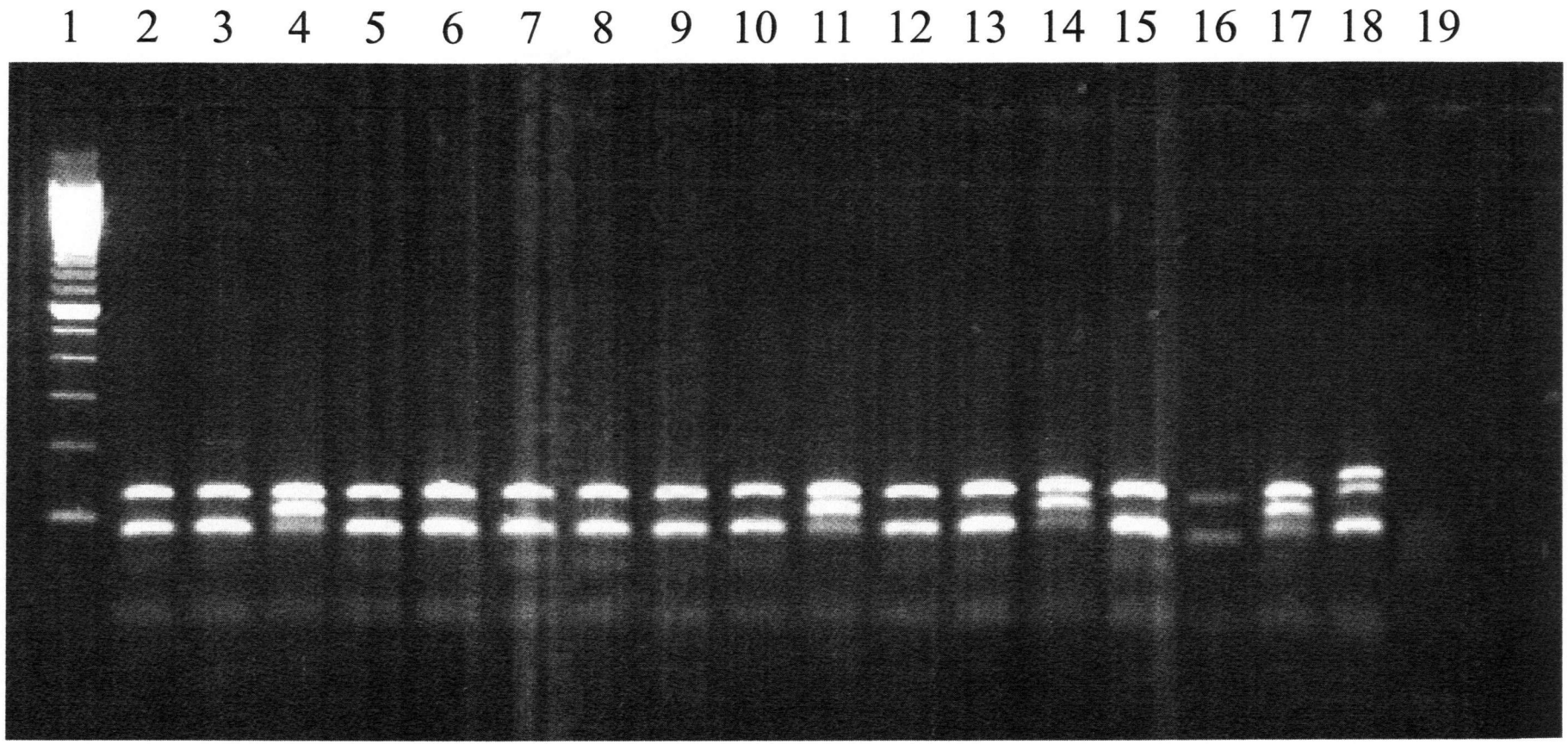


Figure 4.6 Genotyping of the alpha-1 antitrypsin PiS and PiZ mutations.

Multiplex PCR was performed and the PCR product was digested with the *Taq* I restriction endonuclease. After digestion, the digest mixture was resolved on a 2.5% agarose gel stained with ethidium bromide. Lane 1, size marker (100 bp DNA Ladder from New England BioLabs); lanes 2, 3, 5-10, 12, 13, and 15, individuals with the SS (PiS) and MM (PiZ) genotypes; lanes 4, 11, 14, and 17, individuals with the MS (PiS) and MM (PiZ) genotypes; lane 16, individual with the MM (PiS) and MM (PiZ) genotype; lane 18, individual with the MM (PiS) and MZ (PiZ) genotypes; and lane 19, negative control, no bands produced.

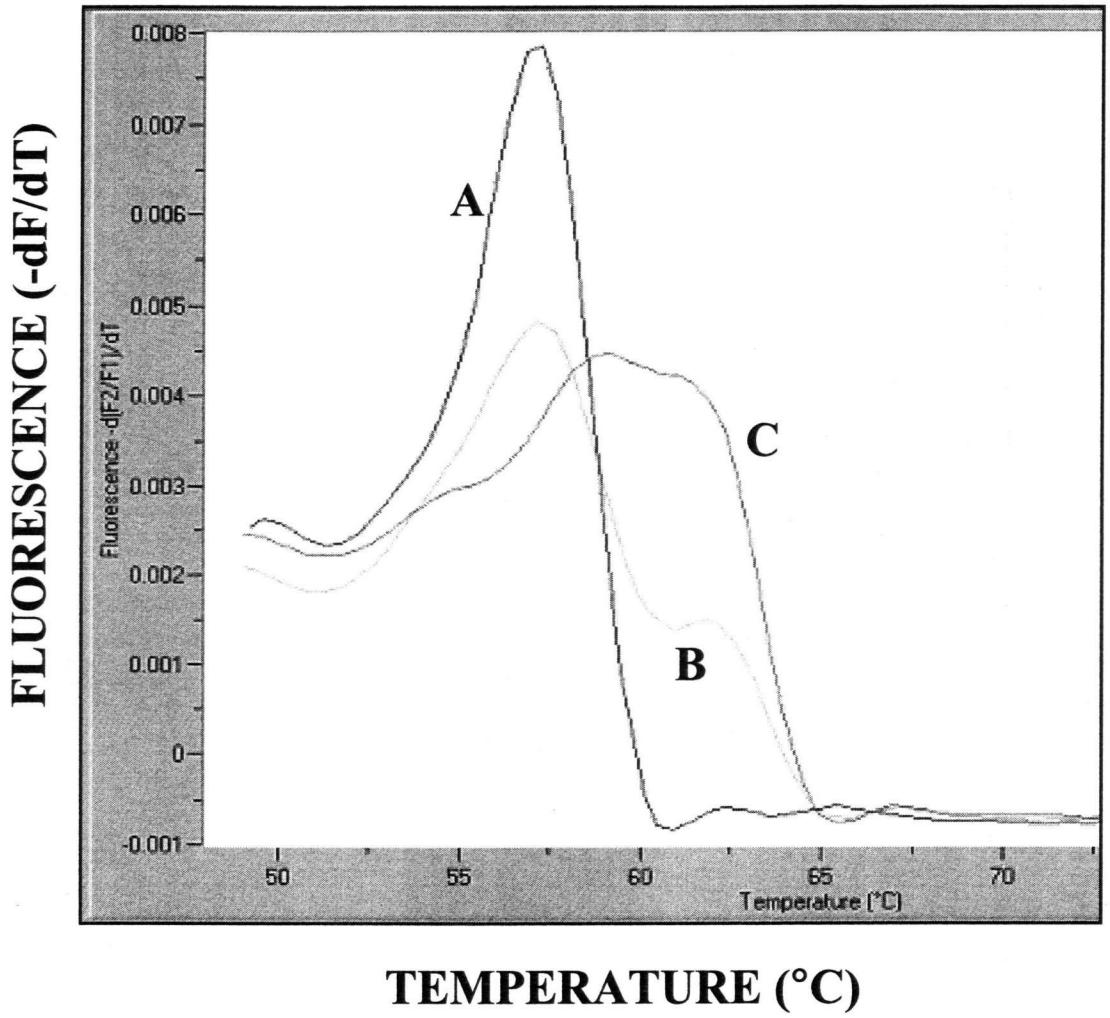


Figure 4.7 Genotyping of the MMP1 -1607/1G→2G polymorphism using hybridization probes.

Melting curve analysis was performed immediately after real-time PCR. Fluorescence data were converted to derivative melting curves by plotting the negative derivative of the fluorescence with respect to temperature against temperature [(dF/dT) vs T]. The derivative melting curve is plotted for a homozygote for the 1G allele (A), a heterozygote for the 1G and 2G alleles (B), and a homozygote for the 2G allele (C). MMP1 denotes matrix metalloproteinase 1.

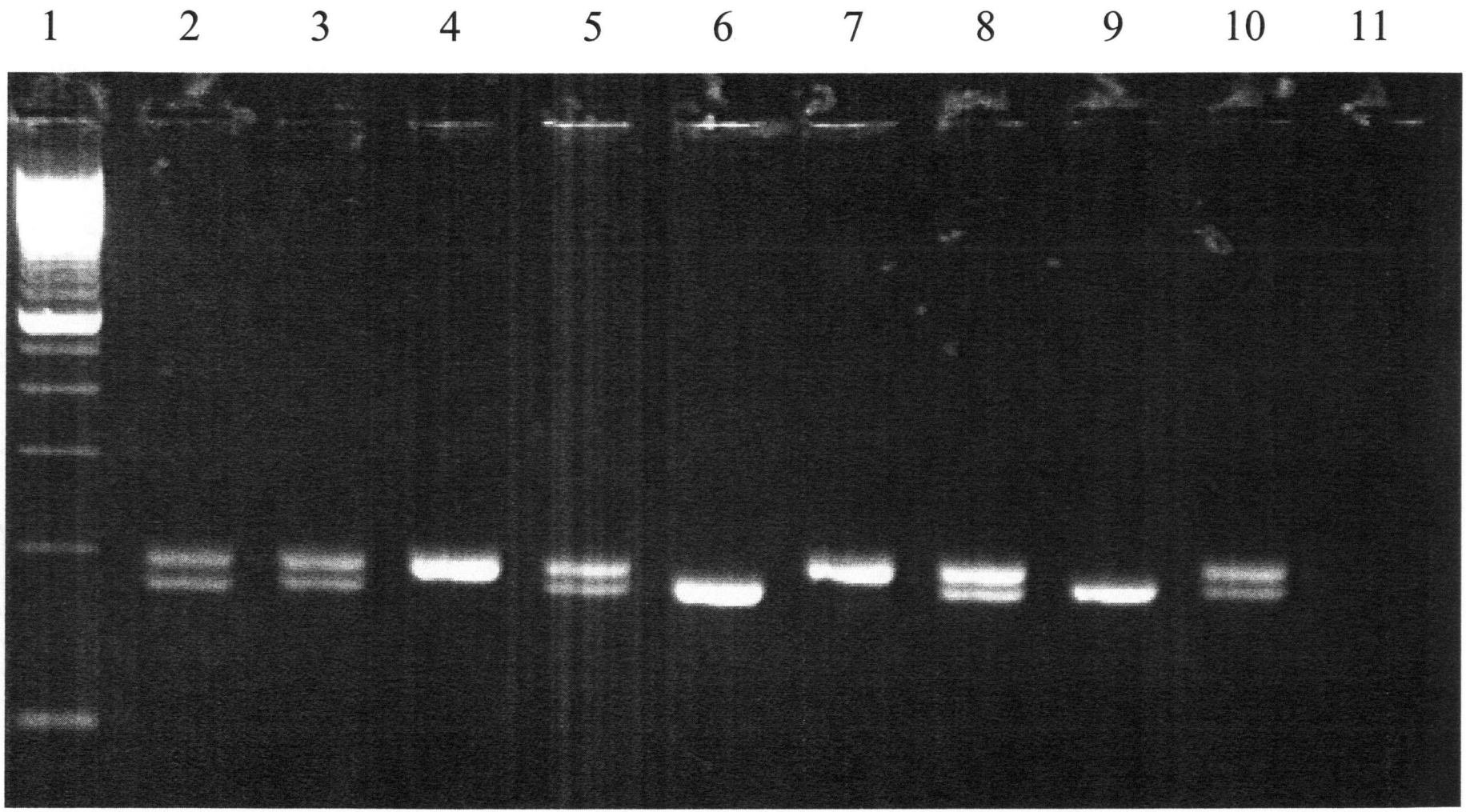


Figure 4.8 MMP9 CA repeat PCR.

The MMP9 CA repeat PCR was confirmed by cycle sequencing. Lane 1, size marker (100 bp DNA Ladder from New England BioLabs); lanes 2 and 3, heterozygotes for the 15 and 23 alleles; lanes 4 and 7, homozygotes for the 22 allele; lanes 5, 8, and 10, heterozygotes for the 14 and 21 alleles; lanes 6 and 9, homozygotes for the 14 allele; and lane 11, negative control, no bands produced. MMP9 denotes matrix metalloproteinase 9.

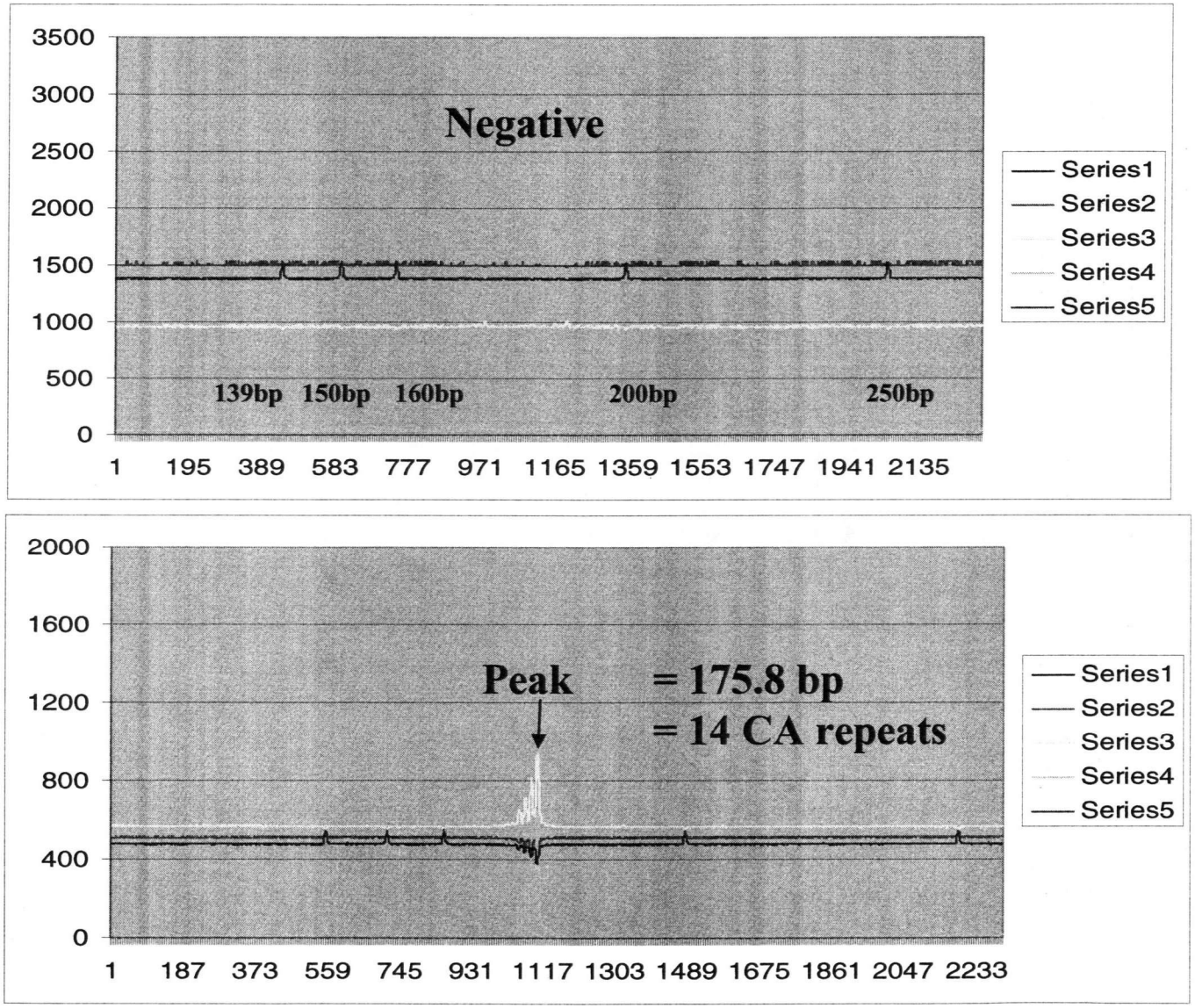


Figure 4.9 Determination of MMP9 CA repeat length using GeneScan analysis software.
MMP9 denotes matrix metalloproteinase 9; and bp, base pairs.

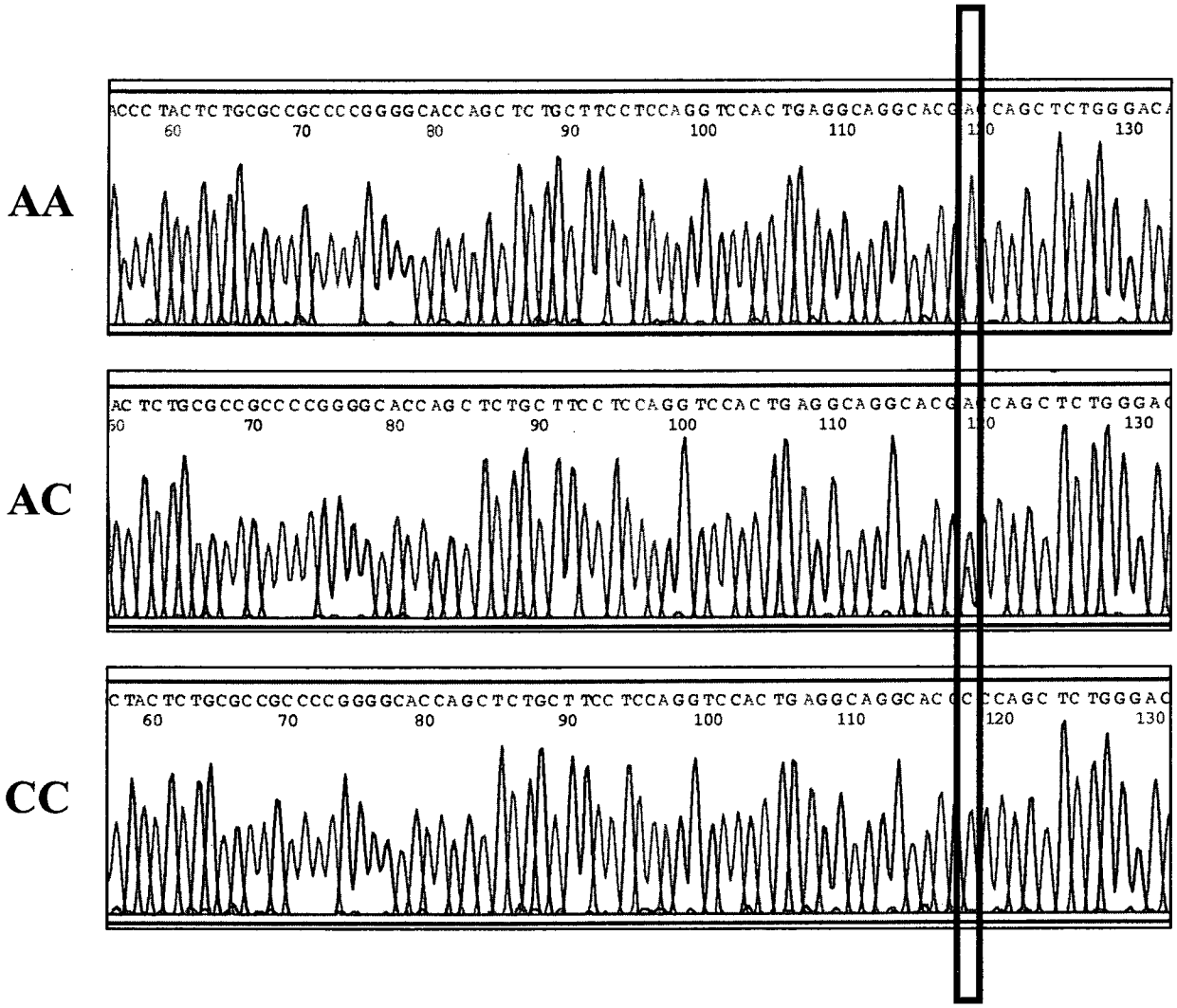
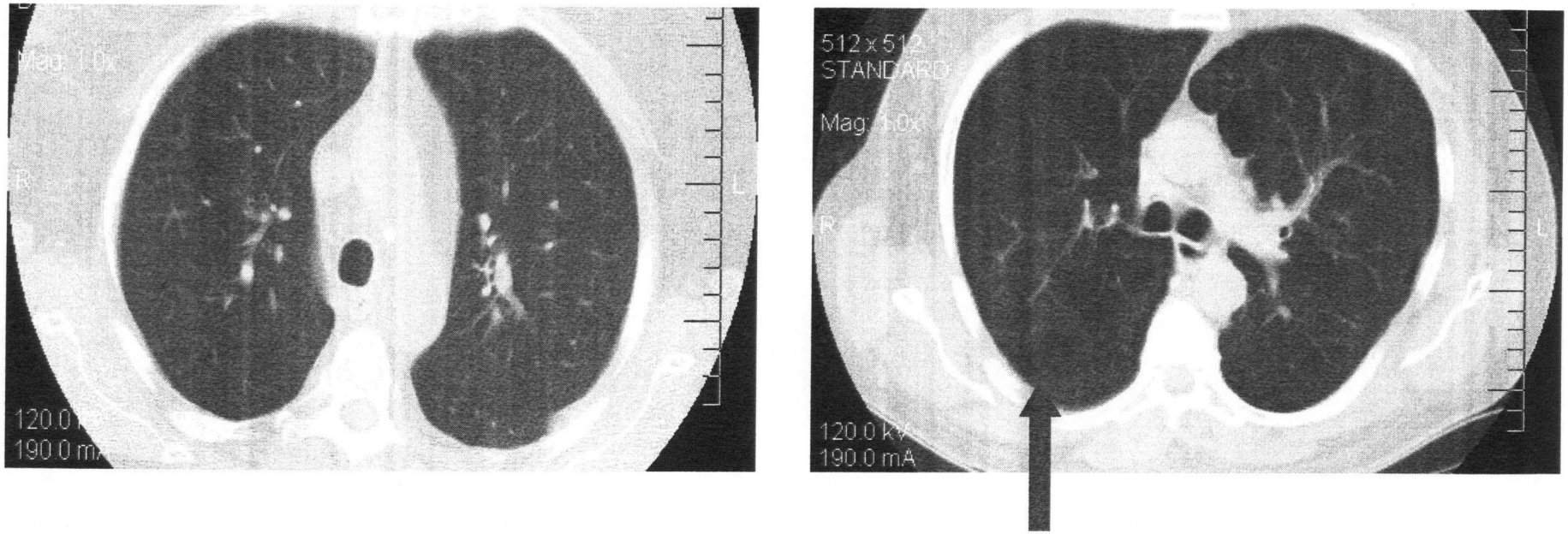


Figure 4.10 Genotyping of the CTSL -1622A→C SNP by cycle sequencing.
CTSL denotes cathepsin L.

Computed tomographic morphometry

Qualitative analysis. A qualitative analysis was performed on the CT scans by two independent observers. The observers graded the extent of emphysema using a 6 point grading scale (0: no emphysema and 5: >75% emphysema). The observers also commented on the distribution of disease, predominate type (centrilobular or panlobular), the presence of bronchiectasis as well as any other observable lung disease. Figure 4.11 shows a CT scan from a patient with no emphysema (left) and a patient with emphysema (right). I was only able to attain CT scans from 43 cases for qualitative analysis.

Quantitative analysis. A quantitative analysis of the lung parenchyma was performed using a previously described technique.[449, 450] Briefly, the lung parenchyma was segmented from the chest wall and large central blood vessels using a contour-following algorithm. Lung volume was calculated by summing the number of voxels in all slices and multiplying by the voxel volume. The CT density of the lung (g/ml) was calculated from x-ray attenuation of each of the CT voxels.[451, 452] Lung weight and air volume were calculated from the lung volume and density measurements.[449, 450] Emphysema was defined using a density mask cut-off of -855 HU (small emphysematous lesions) and -950 HU (large emphysematous lesions), which are appropriate for this CT acquisition technique.[453] CT scans from 21 cases were available for quantitative analysis.



Normal **Emphysema**

Figure 4.11 Assessment of emphysema by chest computed tomography scan.

Statistical analysis

Genotype versus expression and genotype versus emphysema results were compared by analysis of variance and repeated measures analysis of variance where appropriate. Linear regression analysis was used to evaluate the expression versus emphysema data. Age, sex, and smoking history (pack year) were included as covariates in a multiple regression model. Hardy-Weinberg equilibrium was assessed using a goodness of fit chi-square test for the microsatellite marker, with the p-value estimated by simulation, and using the exact test for the biallelic markers. P values less than 0.05 were considered statistically significant. Hardy-Weinberg equilibrium statistics were calculated using the R-genetics package (http://cran.r-project.org/, Authors of the package are Gregory Warnes and Friedrich Leisch). All other statistics were calculated with SAS software version 8.02 (SAS Institute Inc., Cary, NC).

4.4.5 Results

Hardy-Weinberg equilibrium

Genotypic frequencies were consistent with Hardy-Weinberg equilibrium, except for the CTSS -25G→A polymorphism (Table 4.5).

Table 4.5 Hardy-Weinberg equilibrium.

Polymorphism	p Value*
CTSL -1622A→C	0.41
CTSS -25G→A	0.009
MMP1 -1607/1G→2G	0.09
MMP9 -1562C→T	0.67
MMP9 CA repeat	0.79
MMP12 -82A→G	0.58

* p Values derived from chi-square test.

The abbreviation CTSL denotes cathepsin L; CTSS, cathepsin S; and MMP, matrix metalloproteinase.

Linkage disequilibrium

The MMP9 -1562C→T SNP and the MMP9 CA repeat are in complete linkage disequilibrium (D/Dmax=1.0, r^2=0.21, p<0.0001).

Confirmation of primer specificity

All primer pairs were designed to be intron spanning. Specificity of RT-PCR products was documented with gel electrophoresis and resulted in a single product with the desired length (ACTB, 226 bp; CTSL, 241 bp; CTSS, 205 bp; MMP1, 197 bp; MMP9, 202 bp; and MMP12, 203 bp). In addition, a LightCycler melting curve analysis was performed which resulted in single product specific melting temperatures as follows (Figure 4.12): ACTB, 90°C; CTSL, 85°C; CTSS, 87°C; MMP1, 84°C; MMP9, 90°C; and MMP12, 82°C. No primer-dimers were generated during the real-time PCR amplification cycles (Figure 4.12).

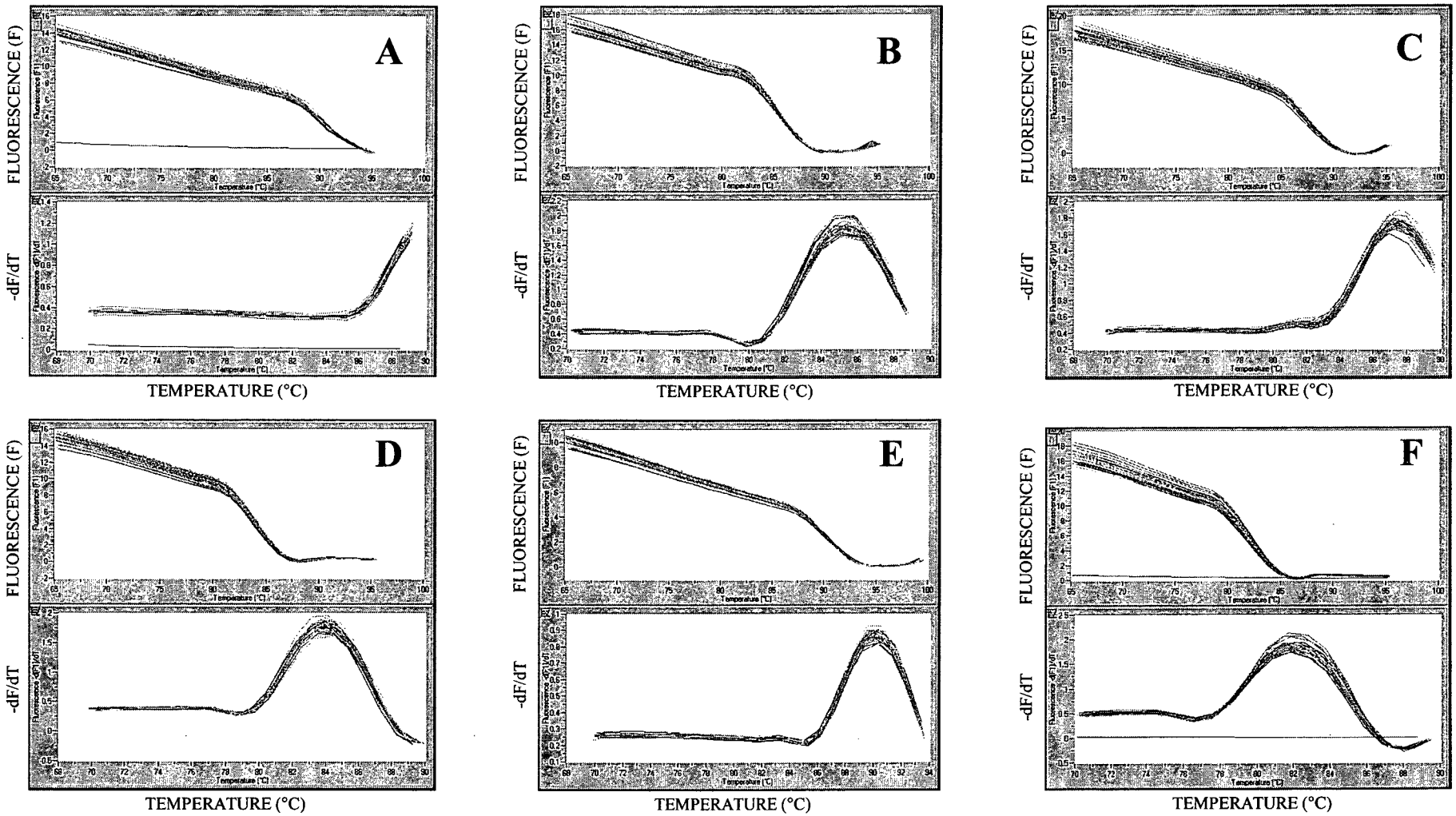
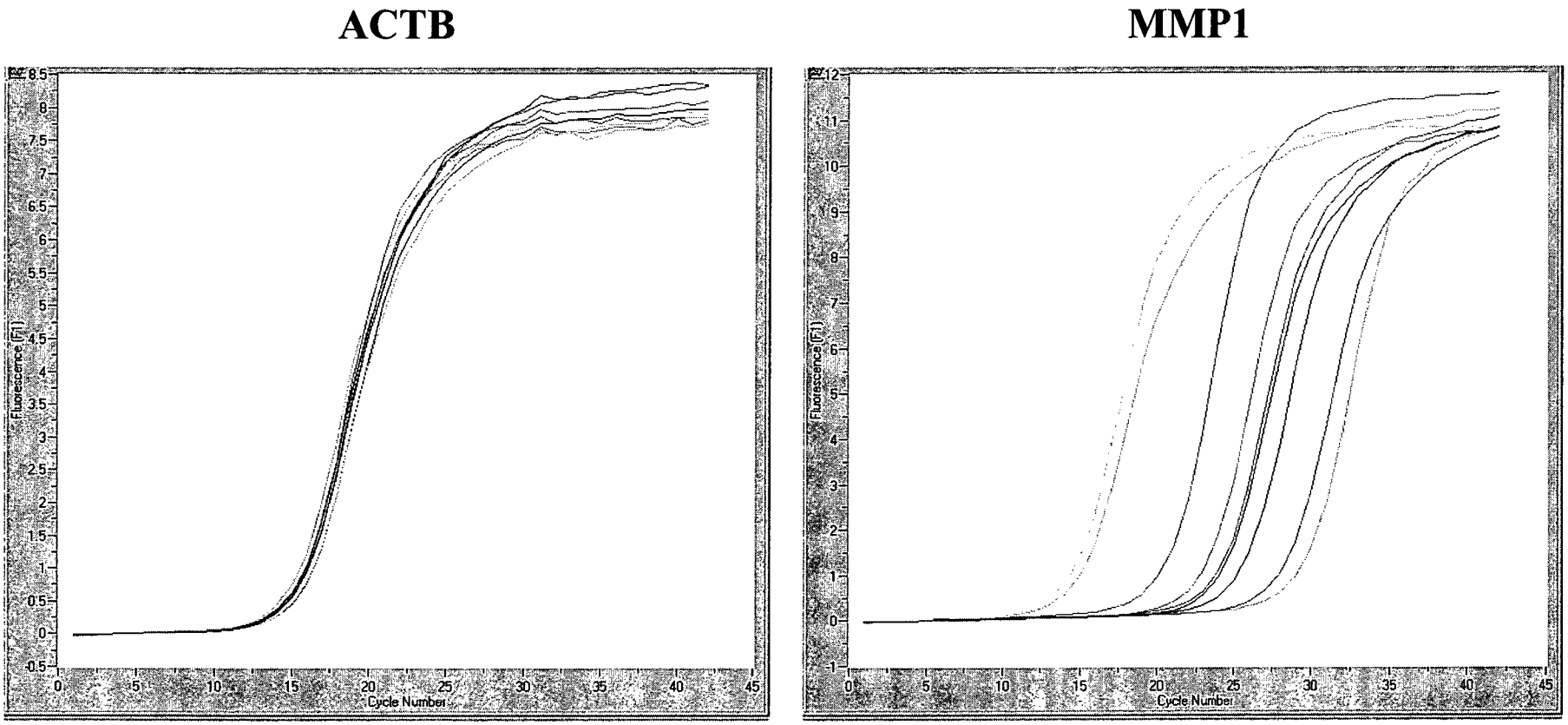


Figure 4.12 Melting curve analysis.

Panel A, ACTB; Panel B, CTSL; Panel C, CTSS; Panel D, MMP1; Panel E, MMP9; and Panel F, MMP12. ACTB denotes beta actin; CTSL, cathepsin L; CTSS, cathepsin S; and MMP, matrix metalloproteinase.

Housekeeping gene verification

ACTB was used as a housekeeping gene. Figure 4.13 shows the ACTB and MMP1 amplification curves for all conditions in the same subject. ACTB expression was stable under all experimental conditions and amplified within the cycle range of the target genes.



Legend: Uncultured; no stimulation; LPS (2 μg/mL); IL1 (20 ng/mL); TNF (20 ng/mL).

Figure 4.13 ACTB and MMP1 amplification curves.

ACTB and MMP1 amplification curves for the same subject are shown. The curves represent uncultured alveolar macrophages plus the four culture conditions at 24 and 48 hours. ACTB denotes beta actin; MMP1, matrix metalloproteinase 1; LPS, lipopolysaccharide; IL1, IL-1beta; and TNF, TNF-alpha.

Expression analysis

SYBR-green quantitative real-time RT-PCR was performed to assess CTSL, CTSS, MMP1, MMP9, and MMP12 mRNA levels in alveolar macrophages. Expression was analyzed in uncultured alveolar macrophages (time zero; 0H) and after cell culture for 24 and 48 hours under four different culture conditions: 1) no stimulation (NS); 2) LPS (2 μg/mL) stimulation; 3) IL-1beta (20 ng/mL) stimulation; and 4) TNF-alpha (20 ng/mL) stimulation.

The patterns of mRNA expression for each gene, under all culture conditions at 24 and 48 hours, are shown in Figures 4.14 to 4.18. Alveolar macrophages expressed CTSL and CTSS mRNA in the greatest amounts.

LPS and IL-1beta caused significant stimulation of CTSL expression at 24 hours compared with the 24 hour time control (NS24H) ($p=0.0001$ and $p=0.002$, respectively). There was no significant difference between TNF-alpha stimulated expression of CTSL at 24 hours and the time control ($p=0.62$). LPS stimulation caused a significant increase and TNF-alpha stimulation caused significant decrease in expression of CTSL at 48 hours relative to the 48 hour time control (NS48H) ($p=0.0018$ and $p=0.0003$, respectively). There was no significant difference between IL-1beta stimulated expression at 48 hours and the time control ($p=0.59$). For all four culture conditions (NS, LPS, IL-1beta, and TNF-alpha), expression of CTSL at 24 hours was significantly greater relative to expression at 48 hours ($p=0.002$, $p=0.0007$, $p=0.0008$, and $p=0.001$, respectively).

LPS stimulation caused a significant decrease and TNF-alpha stimulation caused a significant increase in stimulation of CTSS expression at 24 hours compared with the 24 hour time control ($p=0.0004$ and $p=0.01$, respectively). There was no significant difference between IL-1beta stimulated expression of CTSS at 24 hours and the time control ($p=0.44$). At 48 hours, LPS and IL-1beta stimulated expression of CTSS were both significantly different than the 48 hour time control ($p=0.0001$, and $p=0.04$, respectively); TNF-alpha stimulated expression at 48 hours was not significantly different than the time control ($p=0.07$). The expression of CTSS in the non-stimulated time control alveolar macrophages was not significantly different at 24 and 48 hours ($p=0.83$). LPS, IL-1beta, and TNF-alpha stimulated expression of CTSS at 24 hours were all significantly greater than at 48 hours ($p=0.01$, $p=0.0002$, and $p=0.0007$, respectively).

MMP1 expression showed the largest response to the different culture conditions. Expression was increased in response to the stimuli (LPS, IL-1beta, and TNF-alpha) at 24 hours ($p<0.0001$, $p=0.02$, and $p=0.0004$, respectively) and at 48 hours ($p=0.0001$, $p=0.02$ and 0.001, respectively). In addition, expression decreased significantly from 24 to 48 hours for all conditions (NS, LPS, IL-1beta, and TNF-alpha) ($p<0.0001$, $p=0.008$, $p=0.01$, and $p=0.0001$, respectively).

The level of expression of MMP9 by alveolar macrophages stimulated with LPS, IL-1beta, and TNF-alpha was significantly greater than the 24 hour time control ($p=0.01$, $p=0.002$, and $p=0.0009$, respectively) and the 48 hour time control ($p=0.003$, $p=0.004$, $p<0.0001$, respectively). LPS stimulated expression of MMP9 increased significantly from 24 to 48 hours ($p=0.01$). The level of expression of MMP9 in the non-stimulated time control alveolar macrophages was not significantly different at 24 and 48 hours ($p=0.08$). IL-1beta and TNF-alpha stimulated expression of MMP9 decreased from 24 to 48 hours ($p=0.03$ and $p=0.04$, respectively).

LPS caused markedly increased expression of MMP12 compared with the respective time control at 24 hours ($p<0.0001$) and 48 hours ($p=0.0002$). IL-1beta stimulated expression of MMP12 at 48 hours was greater than the 48 hour time control ($p=0.02$). At 24 hours, there was no significant difference between IL-1beta stimulated expression and the time control ($p=0.30$). TNF-alpha stimulated expression did not differ significantly from the 24 hour or 48 hour time controls ($p=0.46$ and $p=0.47$, respectively). There was no significant difference between expression at 24 hours compared with 48 hours for any of the four culture conditions.

Figure 4.19 shows the level of expression for all five genes in uncultured alveolar macrophages (0H), non-stimulated time control alveolar macrophages at 24 hours (NS24H), and non-stimulated time control alveolar macrophages at 48 hours (NS48H). The level of expression in uncultured alveolar macrophages was significantly less than expression levels in the non-stimulated time control alveolar macrophages at both 24 and 48 hours, for all genes ($p<0.05$).

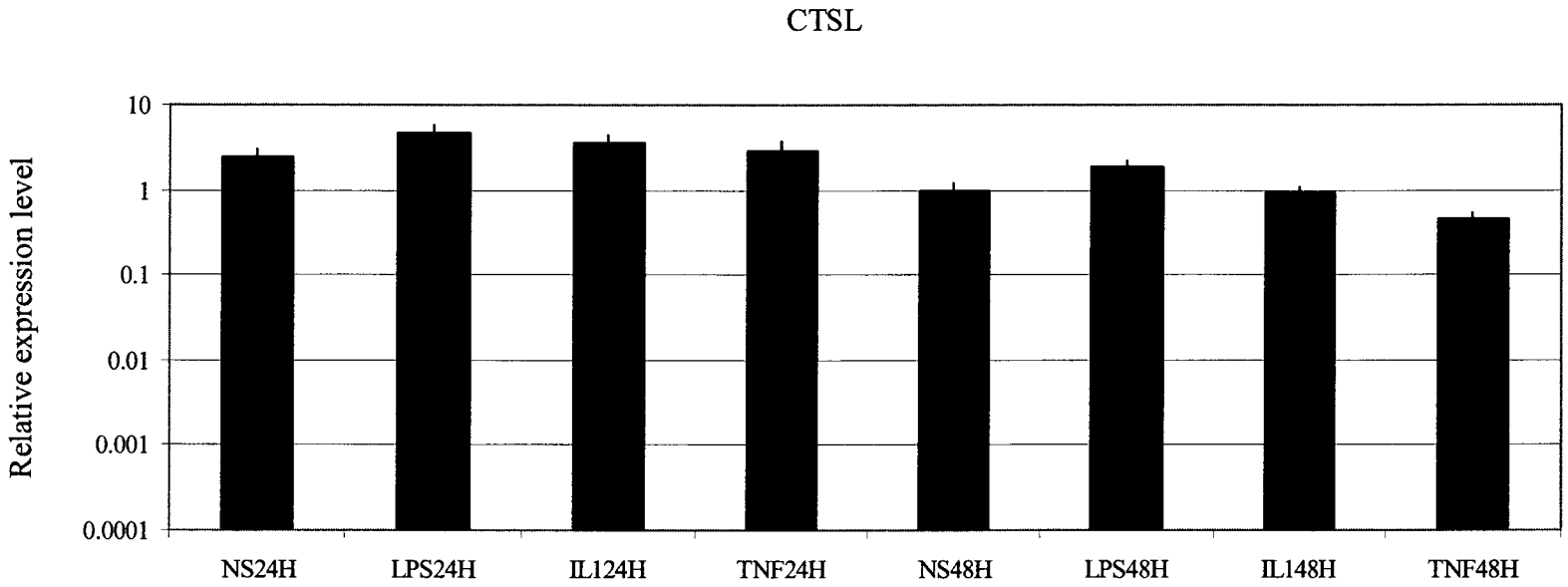


Figure 4.14 Relative CTSL expression.

Expression is shown for the four culture conditions at 24 and 48 hours. The four conditions are: 1) no stimulation (NS); 2)lipopolysaccharide (20 µg/mL) stimulation (LPS); 3) IL-1beta (20 ng/mL) stimulation (IL1); and 4) TNF-alpha (20 ng/mL) stimulation (TNF). Means plus-minus standard error are shown. CTSL denotes cathepsin L. (* $p < 0.05$).

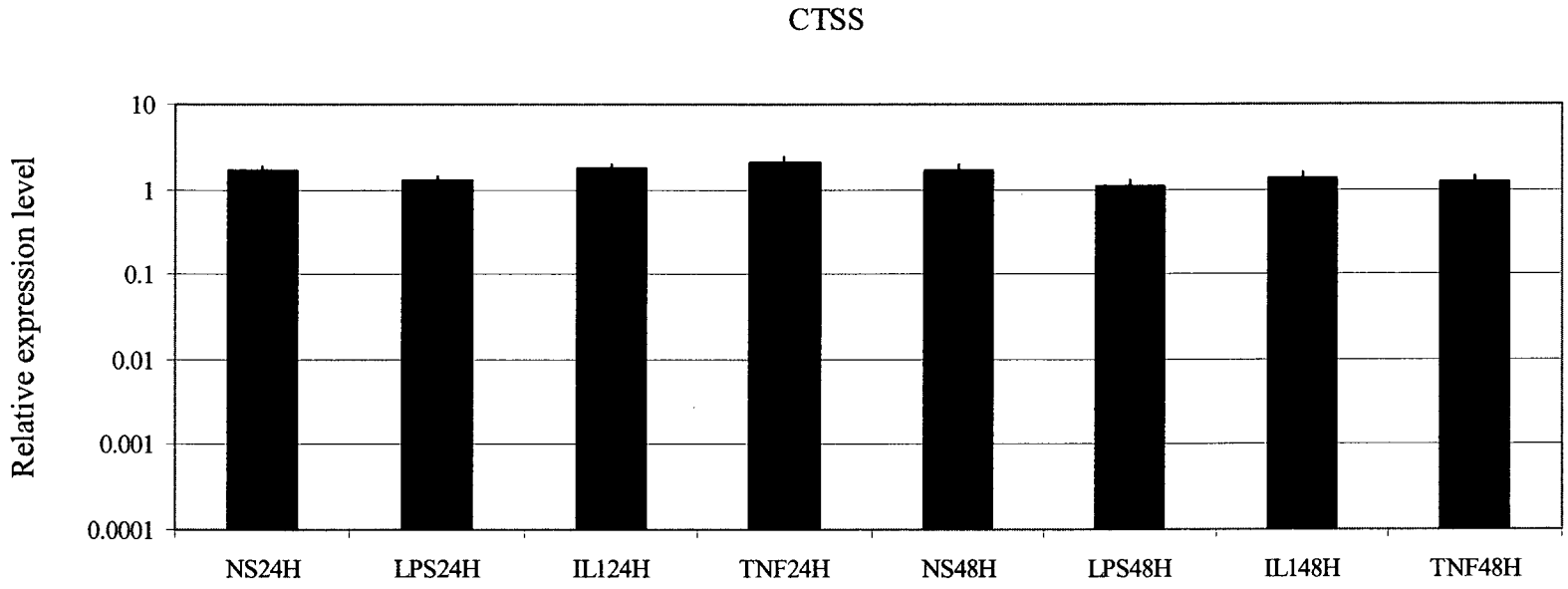


Figure 4.15 Relative CTSS expression.

Expression is shown for the four culture conditions at 24 and 48 hours. The four conditions are: 1) no stimulation (NS); 2)lipopolysaccharide (20 µg/mL) stimulation (LPS); 3) IL-1beta (20 ng/mL) stimulation (IL1); and 4) TNF-alpha (20 ng/mL) stimulation (TNF). Means plus-minus standard error are shown. CTSS denotes cathepsin S. (* $p < 0.05$).

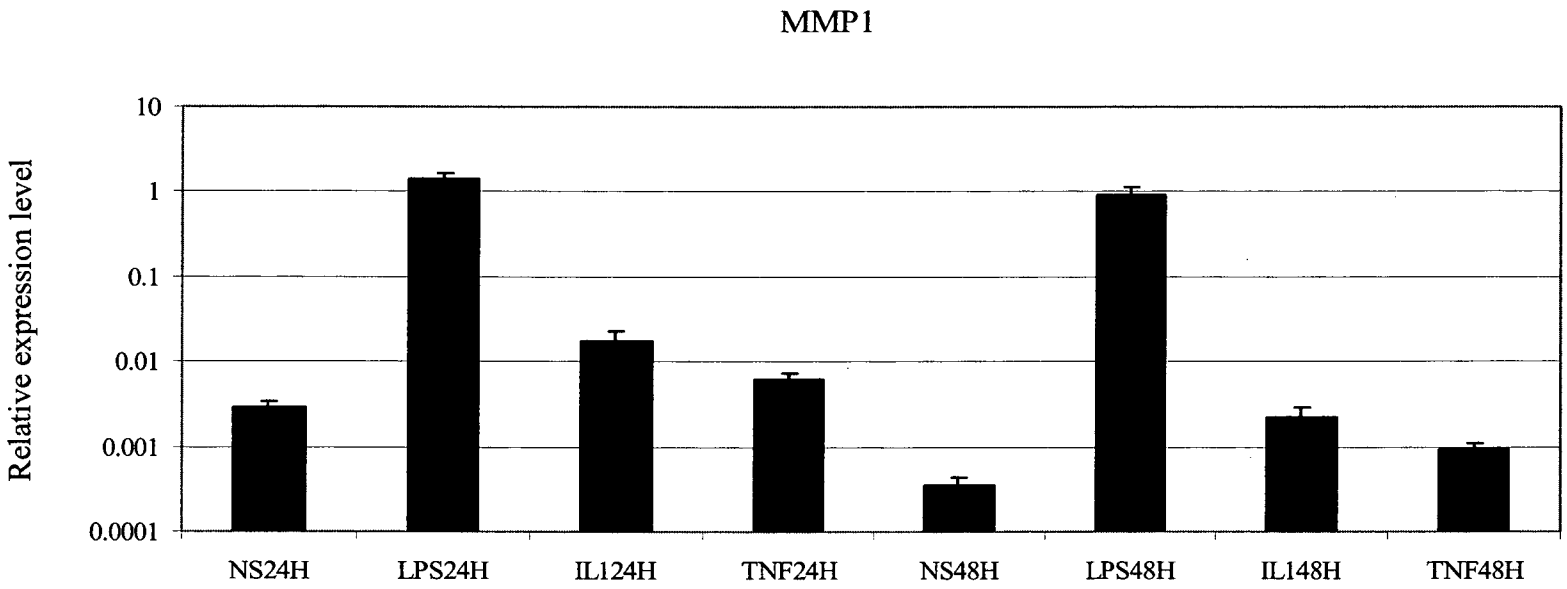


Figure 4.16 Relative MMP1 expression.

Expression is shown for the four culture conditions at 24 and 48 hours. The four conditions are: 1) no stimulation (NS); 2)lipopolysaccharide (20 µg/mL) stimulation (LPS); 3) IL-1beta (20 ng/mL) stimulation (IL1); and 4) TNF-alpha (20 ng/mL) stimulation (TNF). Means plus-minus standard error are shown. MMP1 denotes matrix metalloproteinase 1. (* $p < 0.05$).

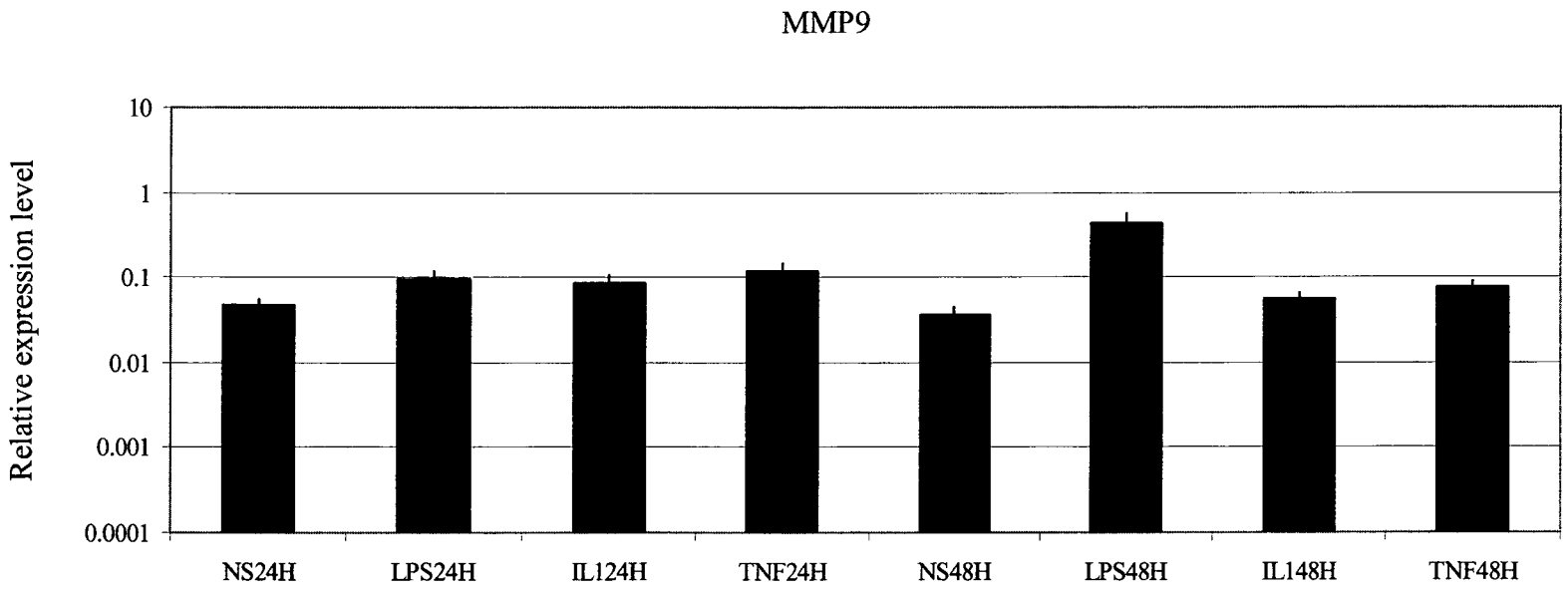


Figure 4.17 Relative MMP9 expression.

Expression is shown for the four culture conditions at 24 and 48 hours. The four conditions are: 1) no stimulation (NS); 2)lipopolysaccharide (20 µg/mL) stimulation (LPS); 3) IL-1beta (20 ng/mL) stimulation (IL1); and 4) TNF-alpha (20 ng/mL) stimulation (TNF). Means plus-minus standard error are shown. MMP9 denotes matrix metalloproteinase 9. (* $p < 0.05$).

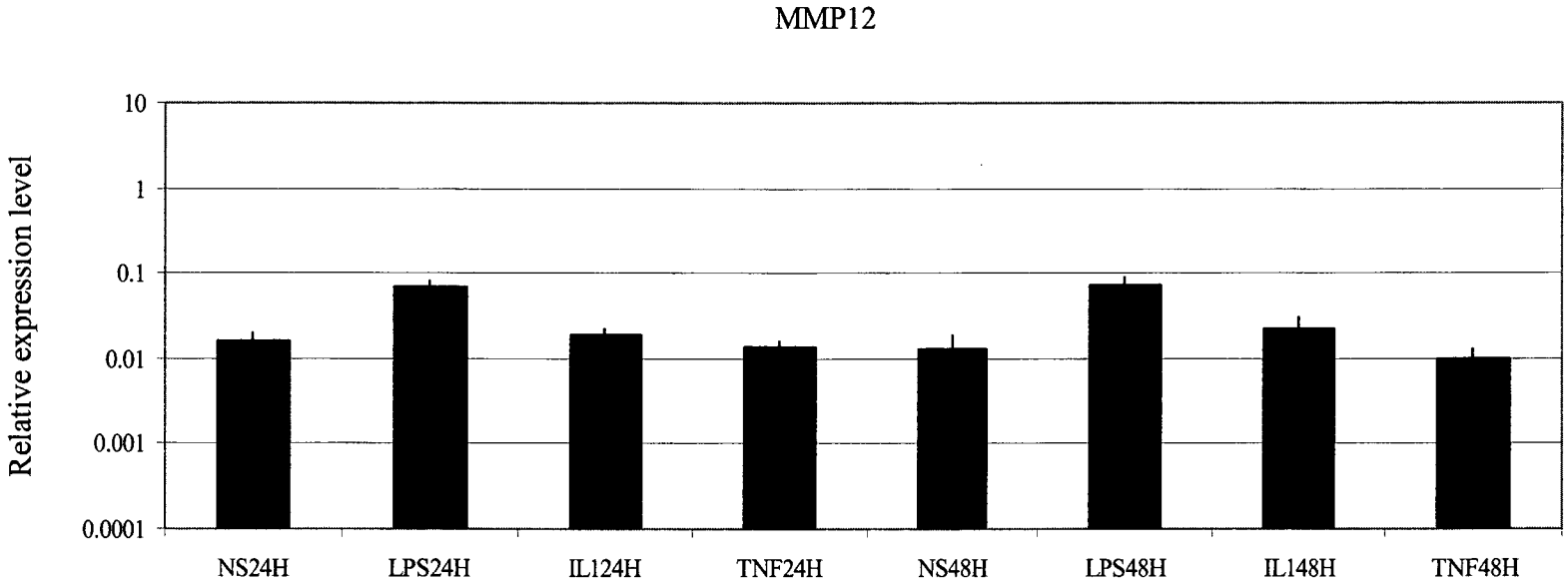


Figure 4.18 Relative MMP12 expression.

Expression is shown for the four culture conditions at 24 and 48 hours. The four conditions are: 1) no stimulation (NS); 2)lipopolysaccharide (20 µg/mL) stimulation (LPS); 3) IL-1beta (20 ng/mL) stimulation (IL1); and 4) TNF-alpha (20 ng/mL) stimulation (TNF). Means plus-minus standard error are shown. MMP12 denotes matrix metalloproteinase 12. (* $p < 0.05$).

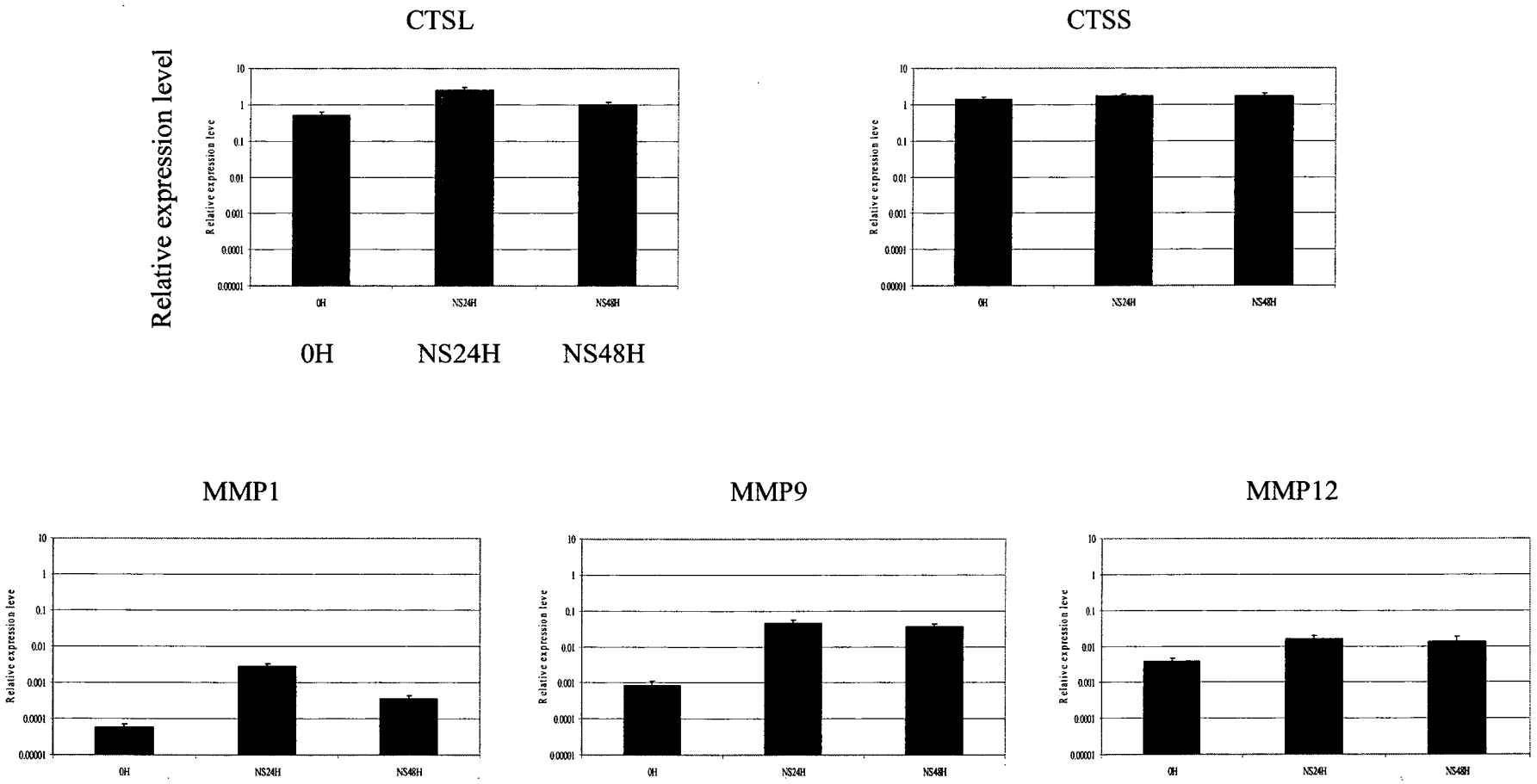


Figure 4.19 Relative gene expression for uncultured cells and non-stimulated cells at 24 and 48 hours.

Means plus-minus standard error are shown. CTSL denotes cathepsin L; CTSS, cathepsin S; MMP, matrix metalloproteinase; 0H, uncultured alveolar macrophages; NS24H, non-stimulated cells at 24 hours; and NS48H, non-stimulated cells at 48 hours. (* $p < 0.05$).

The relationship between expression and genotype

CTSL, CTSS, MMP1, MMP9, and MMP12 mRNA levels in uncultured alveolar macrophages did not differ significantly in any of the genotypic groups (Table 4.6). Codominant disease models were used to analyze all of the data sets. When appropriate, dominant and recessive disease models were also analyzed (data not shown). Conclusions were the same under all three models at a 0.05 p value cutoff (Table 4.7). Both time and stimulus were significant factors involved in expression (Tables 4.7, 4.8, 4.9, 4.11, and 4.14), other than for analysis of the effect of the MMP9 CA repeat, which showed that time was not a significant factor influencing expression (Table 4.13).

The results suggest that the CTSL -1622A→C (Table 4.7), CTSS -25G→A (Table 4.8), MMP9 CA repeat (Table 4.13), and MMP12 -82A→G (Table 4.14) polymorphisms did not have a significant effect on the level of expression of the gene product.

Multivariate analysis showed that the MMP1 -1607/1G→2G genotype had an effect on MMP1 mRNA expression that reached borderline significance (Table 4.9). Univariate analysis of the effect of the MMP1 -1607/1G→2G genotype showed that this trend was present under all culture conditions. At 24 hours, the LPS expression was significantly different between genotypic classes, and the non-stimulated and TNF-alpha stimulated expression levels were both borderline significant (Table 4.10). At 48 hours, non-stimulated and IL-1beta stimulated expression levels showed borderline significance between genotypic classes (Table 4.10). Individuals homozygous for the 1G allele tend to have greater MMP1 expression compared with 2G2G individuals.

The MMP9 -1562C→T SNP significantly influenced expression and the genotype/time interaction was borderline significant (Table 4.11). Univariate analysis showed that at 48 hours, the level of MMP9 expression by the non-stimulated alveolar macrophages was significantly different between genotypic classes. The effect of genotype on the LPS, IL-1beta, and TNF-alpha stimulated expression were marginally significant at 24 and 48 hours (Table 4.12). Heterozygous individuals had markedly increased expression compared with homozygous CC individuals. Mean MMP9 expression at 24 hours for the CC and CT genotypic classes is shown in Figure 4.20. (Only one TT individual was included in the study; therefore, the individual with the TT genotype was not included in this analysis).

Table 4.6 Genotypic effect on expression in uncultured alveolar macrophages.

Polymorphism	p Value*
CTSL -1622A→C	0.4385
CTSS -25G→A	0.7096
MMP1 -1607/1G→2G	0.2912
MMP9 -1562C→T	0.6152
MMP9 CA repeat	0.5805
MMP12 -82A→G	0.6486

* p Values derived from analysis of variance.

The abbreviation CTSL denotes cathepsin L; CTSS, cathepsin S; and MMP, matrix metalloproteinase.

Table 4.7 Analysis of CTSL expression.

	p Value* Codominant	*p Value* Dominant*	*p Value* Recessive*
Genotype	0.9401	*0.7343*	*0.8568*
Time	<0.0001	*<0.0001*	*<0.0001*
Condition	<0.0001	*<0.0001*	*<0.0001*
Genotype*time	0.7687	*0.9493*	*0.4921*
Genotype*condition	0.4380	*0.6428*	*0.2536*

*p Values derived from repeated measures analysis of variance.

The abbreviation CTSL denotes cathepsin L.

Table 4.8 Analysis of CTSS expression.

	p Value*
Genotype	0.9052
Time	0.0392
Condition	0.0015
Genotype*time	0.2483
Genotype*condition	0.9104

*p Values derived from repeated measures analysis of variance.

The abbreviation CTSS denotes cathepsin S.

Table 4.9 Analysis of MMP1 expression.

	p Value*
Genotype	0.0794
Time	<0.0001
Condition	<0.0001
Genotype*time	0.7365
Genotype*condition	0.2245

*p Values derived from repeated measures analysis of variance.

The abbreviation MMP1 denotes matrix metalloproteinase 1.

Table 4.10 Genotypic effect on MMP1 expression.

Time	Condition	p Value*
0	Uncultured	0.2912
24	NS	0.1183
	LPS	0.0340
	IL1	0.3162
	TNF	0.1546
48	NS	0.0565
	LPS	0.2919
	IL1	0.0574
	TNF	0.4017

* p Values derived from analysis of variance.

The abbreviation MMP1 denotes matrix metalloproteinase 1.

Table 4.11 Analysis of MMP9 expression (SNP).

	p Value*
Genotype	0.0423
Time	<0.0001
Condition	<0.0001
Genotype*time	0.0824
Genotype*condition	0.8286

*p Values derived from repeated measures analysis of variance.

The abbreviation MMP9 denotes matrix metalloproteinase 9.

Table 4.12 Genotypic (SNP) effect on MMP9 expression.

Time	Condition	p Value*
0	Uncultured	0.6152
24	NS	0.2075
	LPS	0.1708
	IL1	0.1194
	TNF	0.0580
48	NS	0.0194
	LPS	0.0903
	IL1	0.1335
	TNF	0.1652

* p Values derived from analysis of variance.

The abbreviation MMP9 denotes matrix metalloproteinase 9.

Table 4.13 Analysis of MMP9 expression (CA repeat).

	p Value*
Genotype	0.9877
Time	0.2402
Condition	<0.0001
Genotype*time	0.8395
Genotype*condition	0.1455

*p Values derived from repeated measures analysis of variance.

The abbreviation MMP9 denotes matrix metalloproteinase 9.

Table 4.14 Analysis of MMP12 expression.

	p Value*
Genotype	0.9775
Time	0.0015
Condition	<0.0001
Genotype*time	0.3349
Genotype*condition	0.3927

*p Values derived from repeated measures analysis of variance.

The abbreviation MMP12 denotes matrix metalloproteinase 12.

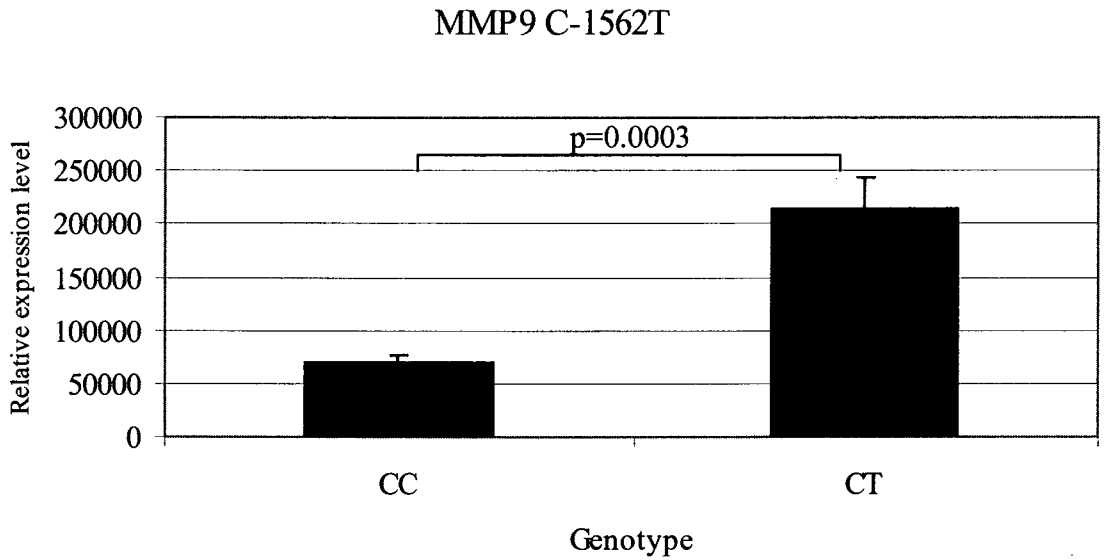


Figure 4.20 MMP9 mean expression at 24 hours by genotypic class.

Means plus-minus standard error are shown. MMP9 denotes matrix metalloproteinase 9.

The relationship between expression and emphysema

I examined the relationship between the level of gene expression for all products in the uncultured alveolar macrophages (time zero) and the mean expression level of all four culture conditions combined at 24 hours with both qualitative and quantitative measures of emphysema. Quantitative measures of emphysema included: 1) small plus large emphysematous lesions (S+L) and 2) large emphysematous lesions only (L).

The level of MMP1 expression in the uncultured cells was significantly related to all three measures of emphysema (Table 4.15). Multiple regression modeling to adjust for covariates confirmed this finding (Table 4.16). Increased levels of MMP1 mRNA were associated with more severe emphysema ($p=0.002$).

The level of expression of CTSL mRNA in the uncultured cells was significantly related to the quantitative measure of large emphysematous lesions but the relationship to the combined lesions (S+L) was only borderline significant (Table 4.15). After adjusting for covariates, these relationships were borderline significant (Table 4.16). Patients with increased CTSL mRNA levels had higher emphysema scores.

Mean MMP1 mRNA expression at 24 hours was significantly related to quantitative (L) measure of emphysema and a marginally significant relationship was found with quantitative (S+L) measure (Table 4.17). However, after adjustment for covariates, these findings were not confirmed (Table 4.18).

The relationship between mean levels of MMP12 mRNA expression at 24 hours and both quantitative measures of emphysema was borderline significant (Table 4.17). Multiple regression modeling to adjust for covariates confirmed these finding (Table 4.18). Patients with greater MMP12 expression had higher emphysema scores.

Table 4.15 Emphysema analysis with expression in uncultured alveolar macrophages.

Gene	Qualitative (n=43)		Quantitative (n=21) (Small plus large lesions)		Quantitative (n=21) (Large lesions)	
	RSquare	p Value*	RSquare	p Value*	RSquare	p Value*
CTSL	0.0022	0.7644	0.1632	0.0693	0.2284	0.0284
CTSS	0.0071	0.5903	0.0136	0.6148	0.0141	0.6078
MMP1	0.1219	0.0218	0.3747	0.0032	0.3173	0.0078
MMP9	0.0344	0.2339	0.0231	0.5104	0.0055	0.7490
MMP12	0.0045	0.6686	0.1146	0.1333	0.0425	0.3702

*p Values derived from linear regression analysis.

The abbreviation CTSL denotes cathepsin L; CTSS, cathepsin S; and MMP, matrix metalloproteinase.

Table 4.16 Emphysema analysis with expression in uncultured alveolar macrophages, including covariates.

Gene	Qualitative (n=43)	Quantitative (n=21) (Small plus large lesions)	Quantitative (n=21) (Large lesions)
	p Value*	p Value*	p Value*
CTSL	0.8277	0.2727	0.1192
CTSS	0.5050	0.6403	0.4665
MMP1	0.0302	0.0040	0.0001
MMP9	0.3530	0.5703	0.9107
MMP12	0.5343	0.0639	0.0641

*p Values derived from multiple linear regression analysis (covariates: age, sex, and smoking history).

The abbreviation CTSL denotes cathepsin L; CTSS, cathepsin S; and MMP, matrix metalloproteinase.

Table 4.17 Emphysema analysis with mean 24 hour expression.

Gene	Qualitative (n=43)		Quantitative (n=21) (Small plus large lesions)		Quantitative (n=21) (Large lesions)	
	RSquare	p Value*	RSquare	p Value*	RSquare	p Value*
CTSL	0.0016	0.8014	0.0302	0.4636	0.0323	0.4484
CTSS	0.0008	0.8623	0.0071	0.7245	0.0092	0.6874
MMP1	0.0050	0.6553	0.1314	0.1163	0.3021	0.0121
MMP9	0.0032	0.7208	<0.0001	0.9974	0.0201	0.5507
MMP12	<0.0001	0.9714	0.1245	0.1271	0.1345	0.1117

*p Values derived from linear regression analysis.

The abbreviation CTSL denotes cathepsin L; CTSS, cathepsin S; and MMP, matrix metalloproteinase.

Table 4.18 Emphysema analysis with mean 24 hour expression, including covariates.

Gene	Qualitative (n=43)	Quantitative (n=21) (Small plus large lesions)	Quantitative (n=21) (Large lesions)
	p Value*	p Value*	p Value*
CTSL	0.9341	0.6389	0.5661
CTSS	0.9479	0.6755	0.5430
MMP1	0.5631	0.9237	0.9136
MMP9	0.9017	0.9196	0.6192
MMP12	0.9520	0.2437	0.0974

*p Values derived from multiple linear regression analysis (covariates: age, sex, and smoking history).

The abbreviation CTSL denotes cathepsin L; CTSS, cathepsin S; and MMP, matrix metalloproteinase.

The relationship between genotype and emphysema

The MMP9 CA repeat was significantly related to the qualitative measure of emphysema (Table 4.19). The L allele is associated with more severe emphysema (p=0.007). In addition, the MMP9 -1562C→T SNP was a borderline significant factor involved in emphysema as determined by qualitative and quantitative (S+L) assessment (Table 4.19). Patients with the CT genotype tend to have higher emphysema scores. Multiple regression modeling to adjust for covariates confirmed all of the above findings (Table 4.20). The CTSL, CTSS, MMP1, and MMP12 promoter polymorphisms were not associated with emphysematous changes in the lung.

Table 4.19 Genotype versus emphysema analysis.

Polymorphism	Qualitative (n=43)	Quantitative (n=21) (Small plus large lesions)	Quantitative (n=21) (Large lesions)
	p Value*	p Value*	p Value*
CTSL -1622A→C	0.3296	0.3739	0.2539
CTSS -25G→A	0.6667	0.4231	0.9606
MMP1 -1607/1G→2G	0.4152	0.9698	0.9231
MMP9 -1562C→T	0.0744	0.0618	0.3712
MMP9 CA repeat	0.0146	0.8238	0.9288
MMP12 -82A→G	0.1485	0.3598	0.3990

* p Values derived from one-way analysis of variance.

The abbreviation CTSL denotes cathepsin L; CTSS, cathepsin S; and MMP, matrix metalloproteinase.

Table 4.20 Genotype versus emphysema analysis, including covariates.

Polymorphism	Qualitative (n=43)	Quantitative (n=21) (Small plus large lesions)	Quantitative (n=21) (Large lesions)
	p Value*	p Value*	p Value*
CTSL -1622A→C	0.3258	0.8150	0.8766
CTSS -25G→A	0.7152	0.1647	0.2711
MMP1 -1607/1G→2G	0.4474	0.4443	0.1194
MMP9 -1562C→T	0.0528	0.0682	0.2181
MMP9 CA repeat	0.0305	0.4039	0.4374
MMP12 -82A→G	0.1538	0.8340	0.9018

* p Values derived from one-way analysis of variance (covariates: age, sex, and smoking history).

The abbreviation CTSL denotes cathepsin L; CTSS, cathepsin S; and MMP, matrix metalloproteinase.

Mean expression at 12 hours compared with 24 and 48 hours

Figure 4.21 shows the mean expression at 12, 24, and 48 hours for all five genes. The CTSL and CTSS mean 12 hour expression was significantly increased compared with the mean expression at 48 hours ($p<0.0001$ and $p=0.002$, respectively). There was no significant difference between the mean expression at 12 hours and the mean 24 hour expression for CTSL and CTSS ($p=0.30$ and $p=0.24$, respectively). MMP1 mean expression at 12 hours was significantly less than the 24 hour mean expression ($p=0.001$). There was no significant difference between the mean 12 and 48 hour MMP1 expression ($p=0.25$). MMP9 mean 12 hour expression was significantly decreased compared with the mean expression at both 24 and 48 hours ($p<0.0001$ and $p=0.02$, respectively). MMP12 mean 12 hour expression was significantly decreased compared with the mean expression at both 24 and 48 hours ($p=0.0002$ and $p=0.0003$, respectively).

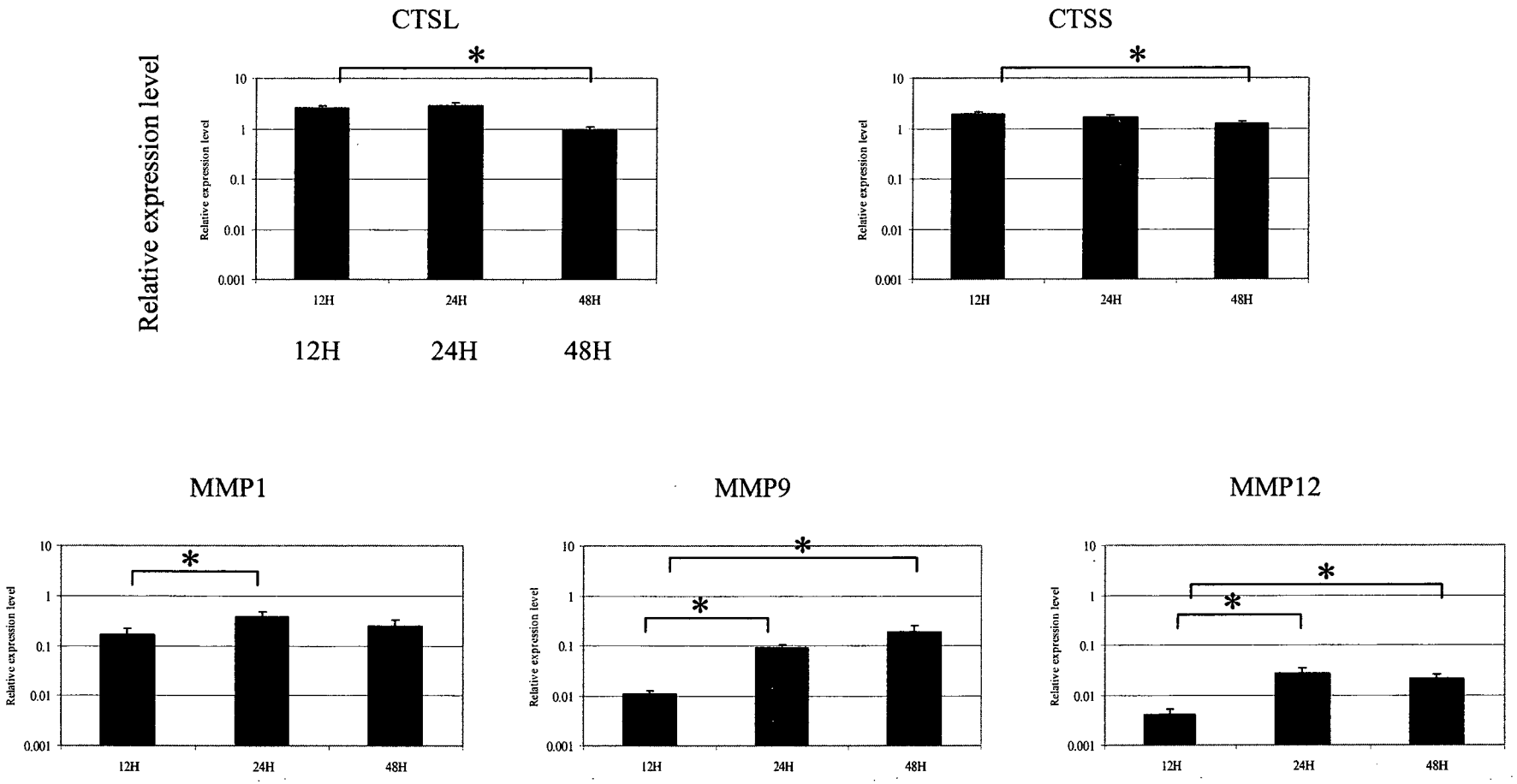


Figure 4.21 Mean expression level at 12 hours compared to 24 and 48 hours.

Means plus-minus standard error are shown. CTSL denotes cathepsin L; CTSS, cathepsin S; MMP, matrix metalloproteinase; NS24H, no stimulation at 24 hours; and NS48H, no stimulation at 48 hours. (* $p < 0.05$).

4.4.6 Discussion

I studied patients undergoing surgical resection for lung carcinoma because I wanted to find out whether promoter polymorphisms in five proteinases produced by alveolar macrophages were related to alveolar macrophage mRNA expression and the development of emphysema. I also wanted to determine whether mRNA expression of these five proteinases was related to emphysema. It took two years to recruit fifty-four subjects for this study.

The study was originally designed in order to assess mRNA expression as well as protein level and function in cultured alveolar macrophages; therefore, cells were cultured for 24 and 48 hours. However, I was concerned that the mRNA levels might peak before 24 hours. After confirming that the alveolar macrophage recovery was sufficient to include another time point, I decided to culture cells for 12 hours, in addition to both 24 and 48 hours. The inclusion of the 12 hour time point started at case number twenty-three; enough alveolar macrophages were recovered to include the 12 hour time point for twenty-three out of a total of thirty-one subsequent cases. I excluded the 12 hour mRNA assessment from the analysis for three reasons. First and foremost, the 12 hour time point was included after the commencement of the study. Second, only a small number of cases had data at the 12 hour time point. Finally, the analysis revealed that CTSL and CTSS mRNA levels assessed at 12 hours were not significantly different compared with 24 hour levels and for MMP1, MMP9, and MMP12, expression at 12 hours was significantly less than the expression levels at 24 hours.

The data indicated that the MMP9 -1562C→T SNP significantly influenced MMP9 mRNA expression in alveolar macrophages. Previously, in a reporter gene assay, the T allele was associated with higher levels of expression.[422] I found that heterozygous individuals had increased expression compared with the CC homozygotes, which is consistent with the *in vitro* study. My study only included one TT homozygous individual; therefore, I could not examine this relationship further.

The second conclusion that can be drawn from the data is that uncultured alveolar macrophage MMP1 expression and mean 24 hour MMP1 expression and uncultured alveolar macrophage CTSL expression were significantly related to emphysema suggesting that these proteinases may play a role in the development of emphysema.

Expression levels in uncultured alveolar macrophages were not different between any of the genotypic classes, however, CTSL mRNA expression in uncultured alveolar macrophages was significantly related to emphysema. This suggests that the CTSL -1622A→C SNP was not important in regulating CTSL mRNA expression related to the development of emphysema. Uncultured and 24 hour mean MMP1 expression were significantly related to emphysema and the results suggest that the expression of MMP1 at 24 hours may be influenced by the -1607/1G→2G polymorphism. Alternatives to a causal relationship explaining the correlations between elevated CTSL and MMP1 mRNA expression and emphysema include: 1) proteinases elevations may be secondary to other events that actually trigger emphysema and 2) alveolar macrophages may respond to the emphysema and/or lung cancer microenvironment by increasing the expression of these proteinases.

Last, the data presented suggest that the MMP9 CA repeat was significantly related to the qualitative measure of emphysema. This is the first report of an association between this polymorphism and emphysema. Longer CA repeats were associated with increased emphysema. Studies have shown that dinucleotide repeats can influence the transcription or translation of genes. This is true for the MMP9 microsatellite. The length of the MMP9 CA repeat was shown to effect the level of transcription in a reporter gene assay; longer repeats resulted in up-regulated gene expression.[423] I failed to find a significant difference between MMP9 expression levels in the CA repeat genotypic groups. The identification of an association between an allele and a trait does not necessarily imply a causal relationship. The MMP9 microsatellite might be in linkage disequilibrium with the true causal polymorphism. The MMP9 SNP and CA repeat are in complete linkage disequilibrium. The MMP9 -1562C→T SNP significantly influenced MMP9 expression levels and was a borderline significant factor involved in emphysema suggesting that this may be the causal polymorphism. The T allele tended to be associated with increased emphysema. However, no relationship was found between MMP9 mRNA levels and emphysema. MMP9 might play a role in emphysema through a mechanism other than altered mRNA expression. Altered protein levels or function may account for the difference. To take this study further, it would be necessary to determine

if MMP9 protein levels or activity are related to the development of emphysema in these subjects.

In addition to the above-mentioned statistically significant findings, a number of borderline significant results were found. All of the results were based on a small population and this study had limited power to detect differences. Furthermore, contrary to some widely held opinions, a statistically significant effect based on a small sample is more remarkable than a statistically significant effect based on a large sample because large sample sizes can demonstrate effects that are not scientifically important.[289] Therefore, the borderline significant results warrant further investigation. Furthermore, the robustness of the results is highlighted by the fact that the results were consistent using different and independent methods of emphysema assessment. In the future I would like to confirm these findings in a study consisting of nonsmokers, and smokers with and without emphysema, who are carcinoma free.

The CTSS -25G→A polymorphism was in Hardy-Weinberg disequilibrium. The observed proportion of heterozygotes was larger that expected and the observed proportions of homozygotes were smaller than expected. The functional effect of the CTSS -25G→A polymorphism is unknown. One study showed that patients with lung cancer, who have low levels of cathepsin S in tumours and lung parenchyma, have a significantly higher risk of death than those with high levels of cathepsin S.[454] If this polymorphism influenced transcription, the genotypic frequency may be altered in the subjects. I would expect to see an excess of heterozygotes because one homozygous class is underrepresented due to death or inoperable tumours, which is consistent with the results. Individuals with the allele that led to low levels of cathepsin S may not have been suitable candidates for surgical resection due the involvement of cathepsin S in malignant progression. However, the results indicate that the CTSS -25G→A polymorphism does not effect CTSS expression levels in alveolar macrophages. I ruled out the possibility of genotyping errors at the CTSS locus by using both DNA sequencing and RFLP analysis.

The use of lung tissue from patients with lung cancer added a potential bias to this study. This was especially a concern for MMP1 given the fact that the MMP1 -1607/1G→2G polymorphism has been reported to be associated with the development of lung cancer. Zhu et al.[404] found that the 2G2G genotype was significantly associated with

lung cancer and that these individuals developed lung cancer at an earlier age. If the genotype was associated with lung cancer risk, then I might have expected the sample to be enriched for the risk genotype. Alternatively, if the genotype affected severity of disease, thereby making the tumour inoperable, I would have expected the sample to be enriched for the genotype that decreased severity. To explore this selection bias, I examined the observed genotype frequencies and compared them to those expected under Hardy-Weinberg equilibrium. The MMP1 polymorphism was in Hardy-Weinberg equilibrium (p=0.09), however, I observed fewer than expected individuals with the 2G2G genotype. These individuals would be underrepresented in this study if their lung cancer was inoperable. The possibility of genotyping errors at the MMP1 locus was ruled out by using both DNA sequencing and mutation detection with the LightCycler system. Furthermore, the population frequency was consistent with a previous study[181] (p=0.14). I did not know the age of lung cancer diagnosis in the subjects, but I did have age at surgery. The mean age at surgery for the 1G genotype was 70, for the 1G2G genotype it was 65, and for the 2G genotype, it was 68. I was examining how genotype was related to expression; therefore, even if there was a bias in the way the samples were collected, I would still expect to see the relationship between genotype and expression. Although this is probably a reasonable assumption, scenarios where bias could effect the results are still possible. With all of the potential biases mentioned, I was still able to detect borderline significant results suggesting that further analysis might be interesting. In order to avoid potential bias caused by the MMP1 polymorphism being associated with lung cancer, the MMP1 results should be confirmed in a population free from lung cancer. In order to do this, patients with emphysema (and no other observable lung disease) and healthy controls could be recruited to undergo bronchoalveolar lavage.

I was not able to obtain CT scans for all fifty-four subjects. Patients had CT scans before surgery and locating the scans for analysis proved to be difficult. Patients' physicians were contacted a minimum of two times in order to obtain the scan, however, in some instances attempts to obtain the CT scan failed. Although the reason for the missing data is unlikely to bias the results, it still leaves very small sample sizes within genotypic classes and this could lead to negative results simply because there are few observations. In addition, the statistical tests selected to examine the association between

expression and genotype or genotype and emphysema have limited power to detect differences.

It is common practice to use only one housekeeping gene for normalization. However, the use of a single housekeeping gene has been criticized. Vandesompele et al.[455] reported that the use of a single gene for normalization led to errors. In order to provide an accurate normalization factor, the use of the geometric mean of multiple carefully selected housekeeping genes was recommended. Some may view the conventional use of a single gene for normalization as another weakness of this study. However, since the level of expression of the housekeeping gene was not influenced by any of the culture conditions and the expression level of the housekeeping gene was similar to the target genes, I think that an appropriate housekeeping gene was selected for this study.

In summary, this study was the first to examine the relationship between alveolar macrophage proteinase promoter polymorphisms, gene expression, and emphysema in human subjects. The main findings of this study were that: 1) the MMP9 -1562C→T SNP significantly influenced MMP9 mRNA expression in alveolar macrophages; 2) MMP1 mRNA levels in uncultured alveolar macrophages and mean MMP1 expression levels at 24 hours and CTSL expression levels in uncultured alveolar macrophages were significantly related to emphysema; and 3) the MMP9 CA repeat was significantly related to the qualitative measure of emphysema.

CHAPTER 5. SIGNIFICANCE OF FINDINGS

The ultimate goal of these studies was to enhance the current understanding of the cellular and molecular mechanisms involved in the pathogenesis of COPD and aid in the development of new and more effective therapies.

Polymorphisms and haplotypes may be helpful for predicting COPD susceptibility and thus preventing the progression of the disease by early intervention. The alpha- and beta-defensins, plasminogen activator inhibitor 1, and tissue-type plasminogen activator polymorphisms studied did not influence smokers' susceptibility to lung function decline, however, as COPD is a complex disease, other COPD phenotypes may be influenced by variations in these genes.

An abnormal increase in proteinases is thought to play a key role in emphysema. The two main proteinase producing cell types in the lung are neutrophils and macrophages, but macrophages are by far the most abundant cell type in bronchoalveolar spaces, particularly in cigarette smokers. Hautamaki et al.[80] clearly demonstrated that macrophages have the capacity to cause emphysema. Furthermore, the IL-13 model of emphysema provides strong evidence that the macrophage alone can cause emphysematous changes.[81] Alveolar macrophage-derived proteolytic enzymes include cathepsin L, cathepsin S, MMP1, MMP9, and MMP12 and smaller amounts of MMP3 and MMP7.[441]

A number of studies suggest that cathepsins and MMPs are involved in alveolar destruction in emphysema. Cathepsins L and S, the two most potent elastases are produced by alveolar macrophages and compared with nonsmokers, smokers have increased levels of cathepsin L in their BAL fluid.[384]

Overexpression of MMP1 in transgenic mice caused pulmonary emphysema similar to that seen in humans.[402] For the first time, this study showed that collagen degradation plays a role in the development of emphysema. MMP12 knock out mice did not develop emphysema when exposed to cigarette smoke.[80] MMP12 is present at very low levels in normal macrophages but expressed in high levels in the alveolar macrophages of cigarette smokers.[431] Finlay and co-workers[413] examined human alveolar macrophages from patients with emphysema and normal volunteers and did not

demonstrated increased levels of MMP12 within the lavage fluid of the patients with emphysema. Increased mRNA expression of MMP1 and MMP9 was found in patients with COPD.[414] In addition, human emphysematous lungs showed an increase in the expression of MMP9.[433] Furthermore, Elias and coworkers[81] demonstrated that a number of cathepsins including cathepsins L and S as well as a number of MMPs including MMP9 and MMP12 are important in the IL-13-mediated emphysema in the murine lung. In summary, animal models indicate that cathepsins L and S as well as MMP1, MMP9, and MMP12, and human studies suggest that cathepsin L, MMP1, MMP9, and MMP12 are key players in the pathogenesis of emphysema. Further investigation is needed in order to elucidate the role of cathepsins and MMPs in the pathogenesis of emphysema in humans.

This is the first study to relate genotype to mRNA levels and to the development of emphysema in human alveolar macrophages in order to investigate the cathepsin and MMP abnormalities that generate emphysematous changes in the lung. In accordance with previous studies, this study demonstrated that alveolar macrophages produce large amounts of mRNA for the two most potent elastases, cathepsins L and S, and smaller mRNA quantities of MMP9, MMP1, and MMP12. In addition, the proinflammatory mediators LPS, IL-1beta, and TNF-alpha modulated the expression of cathepsins and MMPs by alveolar macrophages.

In summary, alveolar macrophages express a number of enzymes involved in pulmonary proteolysis and alter their expression in response to proinflammatory mediators making them key players in the pathogenesis of emphysema in humans. Both the MMP9 SNP and CA repeat were associated with the development of emphysema and may represent important makers of emphysema susceptibility. MMP1, CTSL, and MMP12 mRNA expression was related to the development of emphysema, further confirming the role of both elastin and collagen degradation in the development of human pulmonary emphysema. Future studies are needed to determine the therapeutic potential of blockers of these alveolar macrophage derived proteinases.

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