

Leena Tiitto

HISTOPATHOLOGICAL
FEATURES IN THE
PROGRESSION OF IDIOPATHIC
PULMONARY FIBROSIS/USUAL
INTERSTITIAL PNEUMONIA
WITH SPECIAL EMPHASIS ON
THE REDOX MODULATING
ENZYMES OF
THE HUMAN LUNG

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LEENA TIITTO

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Abstract

Interstitial lung diseases (ILD), including interstitial pneumonias (IP), represent disorders with variable degrees of parenchymal inflammation and/or fibrosis offer an ideal model to investigate the histopathological features in relation to the course of these diseases. The most common IP is idiopathic pulmonary fibrosis (IPF) with the histological pattern of usual interstitial pneumonia (UIP) exhibiting the histological hallmark of fibroblast foci (FF). Surgical lung biopsy (SLB) is not usually needed for diagnosis of IPF, but the lung biopsy samples taken by SLB confers the diagnosis in atypical cases. The safety of SLB in IPF/UIP has been a controversial issue. The acute exacerbation occasionally occurs during the course of IPF/UIP, but pathological features related to this event are poorly understood.

Recent studies suggest that one important determinant in the pathogenesis of ILDs, as in IPF, is oxidant stress and an imbalance of the redox-state in the lung. Thiol containing redox-regulated proteins which participate in the antioxidant defence of the lung include thiorexin (Trx) and gamma-glutamylcysteine synthetase (γ GCS), also called glutamate-cysteine ligase (GLCL), the rate-limiting enzyme of glutathione (GSH) synthesis.

The goal of this research was to evaluate the safety of SLB and the relationships between the histological findings and the course of IPF/UIP, and to investigate the above mentioned defense mechanisms in a variety of ILDs by means of immunohistochemical analyses, Western Blotting and immunoelectronmicroscopy.

No deaths occurred in the following 30 days after 34 video-assisted thoracoscopic lung biopsy (VATS). The number of FF in the lung sample predicted the survival, but it was not associated with acute exacerbation of IPF/UIP before death. Diffuse alveolar damage was a common feature in autopsy samples. The studied redox regulated defense enzymes were expressed in bronchial epithelium, metaplastic alveolar epithelium and alveolar macrophages, but the fibrotic areas generally showed no expression.

In IPF/UIP VATS is a safe diagnostic method and counting the number of FF represents a reproducible and reliable method for predicting patient survival. Alterations in the redox regulated defense enzymes further point to the importance of oxidant burden in the fibrotic lung.

Keywords: gamma-glutamylcysteine synthetase, idiopathic pulmonary fibrosis, thioredoxin, thioredoxin reductase, thoracoscopic lung biopsy, usual interstitial pneumonia

To Matti

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Oulu, 22.8.2006

Leena Tiitto

Abbreviations

AB-PAS	Alcian blue periodic acid Schiff
AIP	Acute interstitial pneumonia
AP-1	Activator protein-1
ARE	Antioxidant response element
ASK	Apoptosis signal regulating kinase
ATS	American Thoracic Society
BAL	Bronchoalveolar lavage
BALF	Bronchoalveolar lavage fluid
BOOP	Bronchiolitis obliterans organizing pneumonia
CI	Confidence interval
COP	Cryptogenic organizing pneumonia
COPD	Chronic obstructive pulmonary disease
CVD	Collagen vascular diseases
CuSOD	Copper-zinc superoxide dismutase
Grx	Glutaredoxin
DAD	Diffuse alveolar damage
DIP	Desquamative interstitial pneumonia
D _{LCO}	Diffusion capacity for carbon monoxide
GPx	Glutathione peroxidase
ECSOD	Extracellular superoxide dismutase
eNos	Endothelial nitric oxide synthase
ERS	European Respiratory Society
FF	Fibroblast focus
FEV1	Forced expiratory volume
FVC	Forced vital capacity
γGCS	Gamma-glutamylcysteine synthetase
γGCS _h	Gamma-glutamylcysteine synthetase heavy subunit
γGCS _l	Gamma-glutamylcysteine synthetase light subunit
GLCL	Glutamate-cysteine ligase

GPx	Glutathione peroxidase
GSH	Glutathione
GSSG	Glutathione disulfide
HE	Hematoxylin eosin
HO-1	Hemeoxygenase 1
HOCl	Hypochlorous acid
HP	Hypersensitivity pneumonitis
HRCT	High-resolution computed tomography
H ₂ O ₂	Hydrogen peroxide
IFN γ	Interferon-gamma
IFN γ 1b	Interferon-gamma1b
IIP	Idiopathic interstitial pneumonia
IP	Interstitial pneumonia
IPF	Idiopathic pulmonary fibrosis
IL	Interleukin
ILD	Interstitial lung disease
ILD-CVD	Interstitial lung diseases with collagen vascular disease
iNOS	Inducible nitric oxide synthase
IPF	Idiopathic pulmonary fibrosis
kDa	Kilo Dalton
LIP	Lymphoid interstitial pneumonia
MAP	Mitogen activated proteins
MMP	Matrix metalloproteinase
MnSOD	Manganese superoxide dismutase
MPO	Myeloperoxidase
mRNA	Messenger ribonucleic acid
NAC	N-acetylcysteine
NADPH	Nicotinamide-adenine dinucleotide phosphate
NF $\kappa\beta$	Nuclear factor kappa beta
NRF2	Nuclear-factor-E2-related factor
NO	Nitric oxide
NO ₂	Nitrogen dioxide
NOS	Nitric oxide synthetase
NSIP	Nonspecific interstitial pneumonia
O ₂ ⁻	Superoxide anion
OH	Hydroxyl radical
OLB	Open lung biopsy
RB-ILD	Respiratory bronchiolitis associated interstitial lung disease
RNS	Reactive nitrogen species
ROC	Receiver operating characteristics
ROS	Reactive oxygen species
SLB	Surgical lung biopsy

SLE	Systemic lupus erythematosus
SOD	Superoxide dismutase
TIMP	Tissue inhibitor of metalloproteases
TNF α	Tumour necrosis factor alpha
TGF β_1	Transforming growth factor beta ₁
TLB	Transbronchial lung biopsy
Trx	Thioredoxin
TrxR	Thioredoxin reductase
UIP	Usual interstitial pneumonia
VATS	Video-assisted thoracoscopic lung biopsy
VEGF	Vascular endothelial growth factor
VC	Vital capacity

List of original publications

This thesis is based on the following publications, which are referred to in the text by their Roman numerals:

- I Tiitto L, Heiskanen U, Bloigu R, Pääkkö P, Kinnula V & Kaarteenaho-Wiik R (2005) Thoracoscopic lung biopsy is a safe procedure in diagnosing usual interstitial pneumonia. *Chest* 128: 2375–2380.
- II Tiitto L, Bloigu R, Heiskanen U, Pääkkö P, Kinnula V & Kaarteenaho-Wiik R (2006) Fibroblast foci, diffuse alveolar damage and exacerbation of idiopathic pulmonary fibrosis / usual interstitial pneumonia. *Thorax* 12: Epub ahead of print.
- III Tiitto L, Kaarteenaho-Wiik R, Sormunen R, Holmgren A, Pääkkö P, Soini Y & Kinnula V (2003) Expression of the thioredoxin system in interstitial lung disease. *Journal of Pathology* 201: 363–370.
- IV Tiitto L, Peltoniemi M, Kaarteenaho-Wiik R, Soini Y, Pääkkö P, Sormunen R & Kinnula V (2004) Cell specific regulation of gammaglutamylcysteine synthetase in human interstitial lung diseases. *Human Pathology* 35: 832–839.

Contents

Abstract	
Acknowledgements	
Abbreviations	
List of original publications	
Contents	
1 Introduction	17
2 Review of the literature	20
2.1 Interstitial lung diseases	20
2.1.1 Idiopathic pulmonary fibrosis	20
2.1.1.1 Pathology of IPF	20
2.1.1.2 Pathogenesis of IPF	21
2.1.1.3 Survival and treatment of IPF	25
2.1.1.4 Safety of the lung biopsy in UIP	26
2.1.1.5 Acute exacerbation in IPF	26
2.1.2 Desquamative interstitial pneumonia	27
2.1.3 ILD associated with collagen vascular diseases	28
2.1.4 Sarcoidosis	29
2.1.5 Allergic alveolitis	30
2.2 Oxidants and antioxidant-oxidant imbalance in the pathogenesis of interstitial lung diseases	30
2.2.1 Thioredoxin system proteins	33
2.2.2 Glutathione related redox modulating pathways in human lung	35
2.2.2.1 Gamma-glutamylcysteine synthetase	35
3 Aims of the study	37
4 Materials and methods	38
4.1 Clinical information (I, II, III, IV)	38
4.2 Human tissue specimens (I, II)	38
4.3 Human tissue specimens (III, IV)	39
4.4 Lung biopsies (I, II, III, IV)	39
4.5 Bronchoalveolar lavage (III)	40
4.6 Cell culture and exposures (IV)	40
4.7 Histopathological evaluation (I, III, IV)	41

4.8	Histopathological evaluation (II)	41
4.9	Immunohistochemistry and evaluation of immunoreactivity (III, IV)	41
4.10	Immunoelectron microscopy (III, IV)	42
4.11	Statistical analyses (I, II, III, IV)	43
4.12	Ethical considerations (I, II, III, IV)	43
5	Results	44
5.1	Safety of the thoracoscopic and open lung biopsies (I)	44
5.1.1	Clinical findings and symptoms (I, II)	44
5.1.2	Lung biopsy and survival (I)	45
5.2	Fibroblast foci, diffuse alveolar damage and exacerbation of idiopathic pulmonary fibrosis/usual interstitial pneumonia (II)	46
5.2.1	Clinical features (II)	46
5.2.2	Histopathologic findings of lung biopsies and their correlation with survival (II)	47
5.2.3	Histopathologic findings of autopsy samples (II)	47
5.2.4	The associations between FF, DAD and acute exacerbations before death (II)	48
5.3	Expression of the Thioredoxin system in interstitial lung diseases (III)	50
5.3.1	Clinical features (III, IV)	50
5.3.2	Localization for Trx and TrxR by immunohistochemistry (III)	50
5.3.2.1	Trx and TrxR in normal lung (III)	50
5.3.2.2	Trx and TrxR in UIP, DIP and ILD-CVD (III)	50
5.3.2.3	Trx and TrxR in granulomatous diseases (III)	51
5.3.3	Immunohistochemical analyses of BAL samples (III)	52
5.3.4	Ultrastructural distribution of Trx and TrxR in UIP (III)	53
5.4	Cell-specific regulation of gamma-glutamylcysteine synthetase in human interstitial lung diseases (IV)	53
5.4.1	Localization of γ GCSH and γ GCSI by immunohistochemistry (IV)	53
5.4.1.1	γ GCSH and γ GCSI in normal lung (IV)	53
5.4.1.2	γ GCSI and γ GCSH in UIP, DIP and ILD-CVD (IV)	53
5.4.1.3	γ GCSH and γ GCSI in granulomatous diseases (IV)	54
5.4.2	Ultrastructural localization for γ GCSI and γ GCSH in UIP and sarcoidosis (IV)	55
5.4.3	<i>In vitro</i> experiments with A549 cells (IV)	55
6	Discussion	56
6.1	Methodological aspects (I, II, III, IV)	56
6.2	Survival and safety of SLB in usual interstitial pneumonia (I)	57
6.3	Fibroblast foci, diffuse alveolar damage and exacerbation of idiopathic pulmonary fibrosis/usual interstitial pneumonia (II)	59
6.4	The expression of the thioredoxin system in interstitial lung diseases (III)	61
6.5	Cell specific regulation of gamma-glutamylcysteine synthetase in human interstitial lung diseases (IV)	63
7	Conclusions	65
	References	
	Original publications	

1 Introduction

Interstitial lung diseases (ILD), including interstitial pneumonias (IP), represent disorders with variable parenchymal inflammation and/or fibrosis. The increase in the oxidant burden offers an ideal model to investigate the histopathological features in relation to the course of the diseases and the significance of the antioxidant enzymes *in vivo*. IPs include idiopathic pulmonary fibrosis (IPF)/usual interstitial pneumonia (UIP) a disease with aggressive fibrogenetic remodelling, non-specific interstitial pneumonia (NSIP) with uniform histopathology and a more favourable prognosis, desquamative interstitial pneumonia (DIP), which is a macrophage associated interstitial pneumonia, related to smoking in the majority of cases, cryptogenic organizing pneumonia (COP), rapidly progressive acute interstitial pneumonia (AIP), respiratory bronchiolitis associated interstitial lung disease (RB-ILD) and lymphoid interstitial pneumonia (LIP) with diffuse lymphocytic interstitial infiltrates (American Thoracic Society (ATS) & European Respiratory Society (ERS) 2002). Table 1.

Table 1. Classification of idiopathic interstitial pneumonias modified by American Thoracic Society and European Respiratory Society (ATS & ERS 2002).

Clinical diagnosis	Pathologic pattern
Idiopathic pulmonary fibrosis (IPF)	Usual interstitial pneumonia (UIP)
Nonspecific interstitial pneumonia (NSIP)	Nonspecific interstitial pneumonia (NSIP)
Desquamative interstitial pneumonia (DIP)	Desquamative interstitial pneumonia (DIP)
Cryptogenic organizing pneumonia (COP)	Organizing pneumonia (OP)
Acute interstitial pneumonia (AIP)	Diffuse alveolar damage (DAD)
Respiratory bronchiolitis interstitial lung disease (RBILD)	Respiratory bronchiolitis interstitial lung disease (RBILD)
Lymphoid interstitial pneumonia (LIP)	Lymphoid interstitial pneumonia (LIP)

Granulomatous lung diseases such as sarcoidosis and allergic alveolitis represent ILDs with well preserved lung architecture. IPF with the histopathological finding of UIP is the most common type of idiopathic interstitial pneumonias (IIP). Furthermore a UIP like histopathological pattern can be detected in several connective tissue diseases (Nicholson

et al. 2002a). The prevalence of IPF is estimated at 20 per 100 000 males and 13 per 100 000 females (Coultas *et al.* 1994). In Finland the estimates for prevalence range 16 to 18 per 100 000 population with the familial form of the disease explaining 3.3 % to 3.7 % of the cases (Hodgson *et al.* 2002). The incidence per year is at 10.7 per 100 000 for males and 7.4 per 100 000 for females (Coultas *et al.* 1994). The diagnosis of IPF is based on the clinical findings, the presence of the typical features of IPF in high-resolution computed tomography (HRCT) and exclusion of other diseases, but histopathological samples may be needed in atypical cases (ATS & ERS 2002).

The safety and utility of surgical lung biopsy (SLB) have been debated for over two decades in spite of the fact that verified histopathological diagnosis not only reduces unnecessary “overmedication” but it also helps with other therapeutic decisions and their planning, one example being lung transplantation (Gaensler *et al.* 1980, Utz *et al.* 2001). IPF/UIP has often a chronic and progressive nature, but findings of unpredictable rapid exacerbations that lead to the death of most patients within a few weeks have been described (Martinez *et al.* 2005). The special pathological features associated with these terminal events are poorly understood (Kondoh *et al.* 1993, Ambrosini *et al.* 2003, Rice *et al.* 2003).

ILDs, especially IPF/UIP, represent disorders where free radicals participate in the pathogenesis, i.e. there is an increased oxidant burden (Cantin *et al.* 1987a, Schaberg *et al.* 1993). A free radical is any molecule with one or more unpaired electrons. These can be produced by oxygen metabolism, inflammation or photochemical air pollution etc. (Freeman & Crapo 1982). Reactive oxygen species (ROS) include free radicals like superoxide anion (O_2^-) and hydroxyl radical (OH^\cdot), molecules like hydrogen peroxide (H_2O_2) and ions (Halliwell *et al.* 1992). Nitric oxide (NO) and nitrogen dioxide (NO_2) are also free radicals (Gaston *et al.* 1994). The impacts of ROS are especially important in the lung, because of its great surface area and its exposure to higher concentrations of oxygen than other tissues (Kinnula *et al.* 1995). In addition to the endogenous oxidant stress, the lung is exposed to a large amount of exogenous oxidants like pollutants and cigarette smoke. ROS are known to be associated with the progression of many lung disorders, both in experimental animal models and in humans, including IPF and asthma (Freeman & Crapo 1982, Saleh *et al.* 1997, Kinnula *et al.* 2005). They participate in multiple signalling pathways and in the induction of the cellular antioxidant defense system (Thannikal & Farnburg 2000). Biochemical defense mechanisms of human lung may therefore have a central role, not only in protection against ROS, but also as indicators of oxidant stress, markers of disease activity or repair processes in the diseased lung.

The most widely investigated antioxidant enzymes include the superoxide dismutases (SOD) and the H_2O_2 scavenging enzymes such as catalase and glutathione peroxidases (Halliwell *et al.* 1992, Kinnula & Crapo 2003). In addition to these classical antioxidant enzymes, the cellular antioxidant defense includes thiol (SH) containing proteins such as thioredoxins, thioredoxin peroxidases (peroxiredoxins), glutaredoxins and gamma-glutamylcysteine synthetase (GCS) also called glutamate-cysteine ligase (GLCL) which is the rate-limiting enzyme in the biosynthesis of one major antioxidant in the lung, glutathione (GSH). These thiol-proteins participate in the regulation of the cellular redox state, cell proliferation and the control of apoptosis (Holmgren 1985, Tu & Anders 1998, Rahman & MacNee 2000a).

The purpose of this study was to evaluate the safety of lung biopsy, to examine the prognostic factors and special histopathological features in the most common interstitial pneumonia, IPF/UIP and to investigate the redox regulated thiol proteins, thioredoxin (Trx), thioredoxin reductase (TrxR) and GCS in ILDs.

2 Review of the literature

2.1 Interstitial lung diseases

2.1.1 Idiopathic pulmonary fibrosis

IPF is the most common type of IIP with the histopathological pattern of UIP (Coultas *et al.* 1994, Lettieri *et al.* 2005). The diagnosis of IPF requires exclusion of other known causes of ILD, like drug toxicities, environmental exposures and connective tissue disorders with no features to support an alternative diagnosis from transbronchial lung biopsy (TLB) or bronchoalveolar lavage (BAL) samples (ATS & ERS 2002). The other major criterion for IPF consist of abnormal pulmonary function tests, including evidence of restriction and impaired gas exchange, as well as detecting bibasilar reticular abnormalities with minimal ground glass opacities on HRCT (ATS & ERS 2002).

2.1.1.1 Pathology of IPF

A definite histopathological diagnosis of IPF requires SLB exhibiting an UIP pattern (ATS & ERS 2002). The appearance of UIP is heterogeneous and patchy, with alternating areas of normal lung, fibrosis that causes architectural destruction with honeycombing and only moderate interstitial inflammation (Katzenstein & Myers 1998, ATS & ERS 2002). The fibrosis in UIP is temporally non-uniform, with older, dense scarring, honeycombing and fibroblast foci (FF) scattered at the edges of the scars (ATS & ERS 2002). FF are one of the major features, but not unique, in UIP, consisting of a loose type of fibrosis with myofibroblasts and few collagen fibers in the stroma (Travis *et al.* 2002). The fibrosis extends inwards until the alveolar walls are replaced by the fibrosis, resulting in honeycombing (Heppleston 1956, Liebow 1975). The honeycomb changes are enlarged air spaces lined by bronchiolar and bronchial epithelium, type II pneumocytes and occasionally metaplastic squamous epithelium (Liebow 1975, Travis *et al.* 2002).

The honeycomb cysts are separated by thick walls, which contain collagen with varying degrees of inflammation (Katzenstein & Myers 1998). The septal inflammation is characterized by infiltration of lymphocytes and plasma cells with hyperplastic type II pneumocytes (ATS & ERS 2002, Travis *et al.* 2002).

The studies evaluating the histopathological changes in UIP including the analyses between FF and survival related to IPF have been done by semiquantitative scoring systems (Cherniack *et al.* 1991, Hyde *et al.* 1992, King *et al.* 2001a, Nicholson *et al.* 2002b). Nicholson and colleagues described a correlation between increasing extent of FF score and mortality, and there were also correlations between decreased diffusion capacity for carbon monoxide (D_{LCO}) and forced vital capacity (FVC) with the FF score (Nicholson *et al.* 2002b). King and co-workers showed that in semiquantitative grading of histopathological features in UIP, the factor of granulation/young connective tissue including the grading results of the amount of FF, was associated with survival (King *et al.* 2001a, King *et al.* 2001b).

Squamous metaplastic alveolar epithelium has been observed in either SLB or autopsy samples of IPF patients after acute exacerbation (Ambrosini *et al.* 2003). In addition these types of cells are detected in fibrotic lung tissue related to lung cancer (Myer & Liebow 1965, Haddad & Massaro 1968). In animal models metaplastic squamous cells appeared in bleomycin induced lung injury (Adamsson & Bowden 1979). Fukuda and co-authors induced pulmonary fibrosis in monkeys after paraquat treatment and found fibrotic alveolar walls lined by metaplastic squamous cells, which were related to the regeneration process of the epithelium in the remodelling of alveolar structure (Fukuda *et al.* 1985, Fukuda *et al.* 1989). The presence of squamous metaplasia in alveoli of rodents after viral infections has been reported (Baskerville *et al.* 1974).

Neutrophils and eosinophils represent inflammatory cells observed in lung tissue of IPF/UIP patients (Katzenstein & Myers 1998, Obayashi *et al.* 1997, Travis *et al.* 2002). Increased percentages of eosinophils and especially neutrophils have been reported also in bronchoalveolar lavage fluid (BALF), but the relationships between BALF profiles and clinical outcome as well as to the response to therapy have not revealed any consistent results (Haslam *et al.* 1980, Wells *et al.* 1994, Tabuena *et al.* 2005). The focal accumulation of intra-alveolar macrophages is also an unspecific finding in IPF/UIP, with no proved relationship to the stage of the disease (Katzenstein & Myers 1998, Travis *et al.* 2002, Daniil *et al.* 2005).

2.1.1.2 Pathogenesis of IPF

The mechanisms underlying the development of IPF are unknown, however the current opinion is that repeated or ongoing episodes of acute lung injury are associated with a dysfunctional epithelial repair process and a lack of appropriate re-epithelization. The risk of IPF is consistently increased for a number of environmental and occupational exposures suggesting that diverse epithelial injuries can trigger this disease. The potential role of viruses, like hepatitis C, adeno and Epstein-Barr, as etiological factors is under investigation (Egan *et al.* 1997). Tobin *et al.* revealed that the IPF patients had more gastroesophageal reflux without typical symptoms like heartburn or regurgitation

compared to the patients with ILD other than IPF (Tobin *et al.* 1998). Metal dust is significantly associated with the risk of IPF (Hubbard *et al.* 1996, Baumgartner *et al.* 2000, Miyake *et al.* 2005). Other significant associations include exposures to stone/sand dust, livestock, farming/agricultural areas, hairdressing and raising birds (Baumgartner *et al.* 2000). Ultrastructural studies of patients with IPF have demonstrated alveolar epithelial cell injury and apoptosis, illustrating that these could be important in the pathogenesis of IPF (Katzenstein 1985). In animal models, alveolar epithelial cell damage is sufficient to promote the fibrotic process (Adamson *et al.* in 1988, Dai *et al.* 1998). Maeyma *et al.* reported increased immunoreactivity of proapoptotic proteins in alveolar epithelial cells in a IPF patient (Maeyma *et al.* 2001). The development of experimental fibrosis induced by bleomycin was prevented by inhibiting epithelial cell apoptosis via T lymphocytes and natural killer cells in animal models (Kuwano *et al.* 1999). The loss of integrity of the subepithelial basement membrane, leading to denudation of the basal lamina is another major feature in UIP (Katzenstein 1985). One reason for this could be apoptosis, since alveolar type II cells from lung biopsy samples of IPF/UIP patients undergo programmed cell death (Uhal *et al.* 1998, Lappi-Blanco *et al.* 1999a, Barbas-Filho *et al.* 2001) Figure 1.

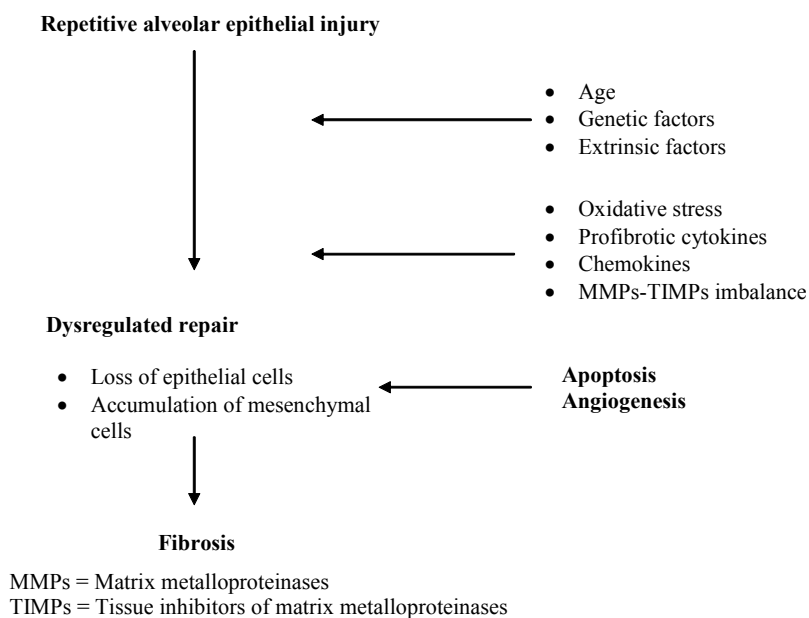


Fig. 1. Progressive pulmonary fibrosis results from alterations in the alveolar microenvironment promoting loss of alveolar cells and accumulation of activated fibroblasts/myofibroblasts. The alterations include activation of profibrotic cytokines, growth factors and chemokines, overproduction of TIMPs relative to MMPs, elevated oxidative stress enhancing epithelial cell apoptosis modified after Thannikal *et al.* (Thannikal *et al.* 2004).

Accelerated epithelial cell proliferation with hyperplastic alveolar type II cells is prevalent after injury (Honda *et al.* 2002, Qunn *et al.* 2002). In IPF, the re-epithelisation

can be heterogenous and delayed or disturbed, which may contribute to fibroblast proliferation (Lappi-Blanco *et al.* 2002). FF are the primary sites of ongoing injury and repair, where fibroblasts increase their collagen synthesis in association with myofibroblasts (Kuhn *et al.* 1989, Katzenstein & Myers 1998). Collagen and elastin fiber density is higher in UIP than in normal lung (Negri *et al.* 2000). One of these extracellular matrix glycoproteins is tenascin-C which exhibits increased expression underneath the metaplastic bronchiolar epithelium and patients with this characteristic have shortened survival compared to those with lower amount of tenascin-C at injury sites (Kaarteenaho-Wiik *et al.* 1996). The number of myofibroblast is increased in bleomycin induced lung injury, contributing to the synthesis of new collagen (Zhang *et al.* 1994). *In vitro* studies suggest that fibroblasts/myofibroblasts from IPF lung exhibit altered phenotypes and growth rates with a tendency to undergo spontaneous apoptosis (Raghu *et al.* 1988, Ramos *et al.* 2001).

Epithelial cells in IPF are the main sources of cytokines and transforming growth factors such as transforming growth factor beta₁ (TGF γ_1) and tumour necrosis factor alpha (TNF α) (Khalil *et al.* 1991, Nash *et al.* 1993). Khalil *et al.* documented enhanced expression of cytokine TGF γ_1 by immunohistochemistry in lung tissue of IPF patients and Piquet *et al.* also detected elevated expression of TNF (Khalil *et al.* 1991, Piquet *et al.* 1993). TGF γ_1 functions as a chemoattractant for the fibroblasts involved in cellular proliferation and differentiation (Raghow *et al.* 1987). Hagimoto *et al.* described the effect of TGF γ_1 to promote epithelial cell apoptosis *in vitro* and TGF γ_1 induces also the extracellular matrix production by fibroblasts (Khalil *et al.* 1991, Hagimoto *et al.* 2002, Hetzel *et al.* 2005). TNF is a cytokine with both inflammatory and fibrogenic activities (Piquet *et al.* 1990, Takizawa 1998). It enhances also apoptosis in alveolar epithelial cells (Wang *et al.* 2000). Figure 1.

Increased angiogenic activity has been described in the lung tissue of IPF patients (Keane *et al.* 1997, Lappi-Blanco *et al.* 1999b). Lappi-Blanco *et al.* documented increased expression of vascular endothelial growth factor (VEGF) in the areas of undergoing fibrogenesis in IPF patients, emphasizing the role of angiogenesis in the pathogenesis of IPF (Lappi-Blanco *et al.* 2002). Figure 1.

The loss of the protective basement membrane leads to exposure to oxidative injury. In addition pulmonary inflammatory cells of IPF patients generate higher levels of oxidants than control patients emphasizing the importance of cellular redox imbalance in pulmonary fibrosis (Cantin *et al.* 1987a, Kinnula & Crapo 2003, Kinnula *et al.* 2005). Mitochondria are the most important cellular source of ROS and Kuwano *et al.* studied the susceptibility of mitochondria to oxidative stress and they found that mitochondrial generation of ROS in lung epithelial cells from IPF may be associated with apoptosis of these cells (Richter 1992, Kuwano *et al.* 2002, Kuwano *et al.* 2003). Montuschi *et al.* reported also 5-fold elevated levels of 8-isoprostane, a marker of oxidative stress, in BALF samples of IPF patients compared to normal subjects (Montuschi *et al.* 1998). ROS enhances the release of TGF γ_1 from human alveolar epithelial cells *in vitro*, which further induces human lung fibroblasts to produce oxidants (Thannikal & Fanburg 1995, Bellocq *et al.* 1999). Inflammatory cells, bronchial epithelial cells and endothelial cells all produce nitric oxide (NO) via two enzymes, inducible NO synthase (iNOS) or endothelial NO synthase (eNOS), leading to cell damage (Gaston *et al.* 1994, Kinnula *et al.* 1995). In UIP iNOS is expressed in alveolar macrophages and alveolar epithelium (Lakari *et al.*

2002). These studies suggest that the change in the redox state of the lung might be one of the “initiative triggers”, resulting in alveolar epithelial cell injury in IPF.

SODs are one of the most significant and best characterised enzyme systems for protecting the lung against oxidant stress by converting superoxide radicals to H_2O_2 (Crapo & Tieney 1974, Kinnula *et al.* 1995, Kinnula & Crapo 2003). In particular, mitochondrial manganese SOD (MnSOD) together with the H_2O_2 scavenger, catalase, are expressed in acute fibromyxoid lesions of UIP, which consist of myofibroblasts and fibroblasts (Lakari *et al.* 2000). Alveolar epithelial type II cells, which are resistant to oxidant stress, and alveolar macrophages express both these enzymes at high levels in contrast to alveolar type I cells which are sensitive to the injury evoked by oxidative stress (Kinnula *et al.* 1995, Pietarinen-Runtti *et al.* 2000, Kinnula & Crapo 2003).

Neutrophils together with macrophages and lymphocytes dominate the inflammatory cell population in IPF (Reynolds *et al.* 1977, Katzenstein & Myers 1998, Daniil *et al.* 2005, Tabuena *et al.* 2005). Activated neutrophils generate high levels of oxidants, causing cell damage (Cantin *et al.* 1987a, Weiss 1989, Halliwell *et al.* 1992). Myeloperoxidase (MPO) is a protein produced by neutrophils and mononuclear phagocytes (Clark *et al.* 1976). It can interact with H_2O_2 , forming highly toxic compounds (Clark *et al.* 1976). IPF patients have elevated MPO concentrations in their BALF samples indicating a local neutrophilic activation, which might be associated with epithelial cell injury in this disorder (Cantin *et al.* 1987a, Hällgren *et al.* 1989). Neutrophils and macrophages are also the sources of matrix metalloproteinases (MPPs) in lung inflammation and injury (Lemjabbar *et al.* 1999, Shapiro & Senior 1999). MPPs are mediators of matrix degradation and are capable of degrading extracellular matrix components (Birkedal-Hansen *et al.* 1993). Two of them, collagenase-1 (MMP1) and matrilysin (MMP7) exhibit increased immunoreactivity in fibrotic lung, furthermore, matrilysin knockout mice were protected from pulmonary fibrosis in response to intratracheal administration of bleomycin (Zuo *et al.* 2002). MMPs also modulate the activity of growth factors and cytokines (Winkler & Fowlkes 2002). Additionally, fibroblasts express monocyte/neutrophil chemotactic activity by altering their synthesis of proinflammatory cytokines and chemokines (Hogaboam *et al.* 1999). MMPs activity is blocked by specific inhibitors like tissue inhibitor of metalloproteases (TIMPs) among others (Birkedal-Hansen *et al.* 1993). Figure 1.

It was previously thought that IPF was a result of chronic inflammation, which was partly based on the expression of inflammatory cells in BALF of IPF patients, however, treatment with corticosteroids has shown no significant long-term therapeutic efficacy (Merrill & Reynolds 1983, Flaherty *et al.* 2001). The possible role of the inflammatory response in modulating tissue injury and/or fibrosis in IPF/UIP needs to be studied further, since Daniil *et al.* did document an association between a lymphoid subpopulation in lung tissue and functional parameters of the disease severity (Gross & Hunninghake 2001, Daniil *et al.* 2005).

Familial clustering of individuals with IPF has brought on the attempts to define gene expressions profiling this disease. The mutation in the gene encoding surfactant protein C, hydrophobic protein that enhances the surface tension in lung, was found in familial cases of ILDs (Nogee *et al.* 2001). Hodgson *et al.* revealed a novel candidate gene, ELMOD2, for susceptibility in familial IPF (Hodgson *et al.* 2006).

These are just some of the mechanisms leading to fibrosis accompanied by a progressive deterioration of lung function parameters and a restriction or impaired gas exchange, which ultimately leads to respiratory insufficiency and death.

2.1.1.3 Survival and treatment of IPF

The prognosis of this disease is poor with a median survival of only 2.8 years after diagnosis (Bjoraker *et al.* 1998). One out of two patients died within 5 years after the onset of dyspnoea (du Bois & Wells 2001). Several studies have described various prognostic factors related to survival, including age at the time of diagnosis, smoking status, response to initial steroid therapy and the finding of pulmonary hypertension on chest radiography (Tukiainen *et al.* 1983, Gay *et al.* 1998, King *et al.* 2001b). Severity of lung volume reduction and the desaturation during the 6 minutes walking test predict the survival as well as the extent of reticulation and honeycombing on HRCT (Flaherty *et al.* 2003, Hallstrand *et al.* 2005, Lynch *et al.* 2005).

There are no unequivocal therapies for IPF. Corticosteroids are the mainstay of therapy, but there are no reports indicating that corticosteroids would alter the survival after 3 years (Flaherty *et al.* 2001). Cyclophosphamide, a highly toxic immunosuppressor, has been used in combination with corticosteroids, although there is no statistically significant data of any favourable effect on pulmonary function tests or survival (Johnson *et al.* 1989, Collard *et al.* 2004). The combination of corticosteroid and the less toxic azathioprine indicated some marginal benefit in one study (Raghu *et al.* 1991). The consensus statement recommends this combination therapy as first line therapy for IPF (ATS & ERS 2002). One report on therapy with interferon-gamma1b (IFN γ 1b), a cytokine having antifibrotic effect, suggests that the treatment might have some benefits on survival among patients with mild or moderate forms of the disease (Raghu *et al.* 2004). However acute pulmonary failure leading to death, which were temporally related to the IFN γ 1b -therapy, have also been reported (Honore *et al.* 2003). Trial of pirfenidone, a novel compound with anti-inflammatory, antioxidant and antifibrotic effects, showed that the treatment group had no acute exacerbations of IPF and less decrease in the change of vital capacity (VC), although treated patients had significantly more adverse effects (Azuma *et al.* 2005). At present, N-acetylcysteine (NAC) is the only therapy that has been shown to slow down the deterioration of pulmonary functions in IPF (Behr *et al.* 1997, Demedts *et al.* 2005). NAC is a cysteine donating thiol compound and it acts as a cellular precursor of GSH releasing cysteine as a deacetylation product (Rahman & MacNee 2000b). This emphasizes the importance of the ROS/antioxidant imbalance in the pathogenesis of IPF and the possibility that thiol compounds have a protective role in this disease. Lung transplantation supports survival, but only few patients are eligible for transplantation (Hosenpud *et al.* 1998).

2.1.1.4 Safety of the lung biopsy in UIP

SLB is needed for diagnosing IPF when there are non-specific findings from radiographs, spirometry or clinical examination. The majority of IPF is diagnosed without biopsy, but the clinical diagnosis has proved to be accurate in only two thirds of the suspected IPF cases (Raghu *et al.* 1999). In a study of 91 new patients suspected of having IPF, histological verification of UIP was documented in 54 cases, i.e. 59 % (Hunninghake *et al.* 2001). In another study of 26 patients, who met ATS/ERS criteria for IPF, 14 individuals had UIP in their SLB samples, so the accuracy of these criteria was 54 % (ATS & ERS 2002, Peckham *et al.* 2004).

Histological samples are taken either by open lung biopsy (OLB) or VATS. The safety of performing SLB in ILD, especially IPF, has been controversial. Gaensler *et al.* studied 502 OLBs including 64 UIP cases and reported that 1 death attributable to carcinoma occurred within 30 days after the operation (Gaensler & Carrington 1980). The highest reported short-term mortality (i.e. in 30 days followed by biopsy) of UIP patients is 21.7 % (Utz *et al.* 2001). Ayed *et al.* compared the safety of OLB and VATS in diagnosing 61 ILD cases, one patient died 21 days after the operation due to progressive pulmonary fibrosis and one other patient died 60 days after the procedure due to malignancy (Ayed & Raghunathan 2000). The studies judging the safety of VATS as a method in diagnosing ILD have shown similar results. Zeghi *et al.* described 19 IPF patients in a material consisting of 64 ILD cases and found 2 short-term deaths related to IPF, one patient had also leukemia (Zeghi *et al.* 1998). Yamaguchi *et al.* found no operative mortality after VATS in 30 ILD patients including 12 IPF cases (Yamaguchi *et al.* 2004). Ooi *et al.* studied 55 ILD patients who underwent VATS and found one in-hospital death due to pulmonary malignancy and post-operative adult respiratory distress syndrome (Ooi *et al.* 2005). Furthermore, one in every four (27.1 %) of the patients had histopathological findings different to their pre-operative clinico-radiological diagnosis (Ooi *et al.* 2005).

Those patients with immunodeficiency and those who require mechanical ventilation (MV) preoperatively have increased risk of death following SLB. Lettieri *et al.* studied 83 ILD cases including patients who needed MV or who were immunosuppressed prior to SLB and found that the short-term mortality among 42 IPF patients was 3 subjects, but this included only one immunocompetent patient who was not receiving MV (Lettieri *et al.* 2005).

In view of the heterogeneity of the previous results considering the safety of SLB in diagnosing UIP, this issue needs to be addressed in a large enough patient group, focusing especially on IPF/UIP. Furthermore, the lung biopsy samples are not only essential in improving the diagnostic accuracy, but they are also valuable for the research purposes which is an important issue in studying a disease with an unknown etiology.

2.1.1.5 Acute exacerbation in IPF

IPF/UIP has often a chronic and progressive nature, but findings of unpredictable rapid exacerbations that lead to death of many patients within a few weeks have been described (Hamman & Rich 1935, Kondoh *et al.* 1993, Akira *et al.* 1997, Ambrosini *et al.* 2003,

Martinez *et al.* 2005, Parambil *et al.* 2005). The criteria of acute exacerbation of IPF, which is also known as accelerated deterioration of IPF, include exacerbation of dyspnoea within one month, new diffuse pulmonary opacities on chest radiography, a decrease in arterial oxygen tension (PaO_2) by more than 10 mmHg (i.e. 1.33 kPa) under similar conditions and the absence of apparent infection agents and heart failure (Akira *et al.* 1997). Kohdoh *et al.* described deterioration of hypoxemia using the ratio of PaO_2 and fractional concentration of oxygen in inspired gas (FIO_2) (Kohdoh *et al.* 1993).

There are no definite predicting factors that contribute to the acceleration of IPF, and the distinct pathological features associated with these terminal events are also poorly understood (Kohdoh *et al.* 1993, Ambrosini *et al.* 2003, Rice *et al.* 2003). Previous studies have described the histopathology of diffuse alveolar damage (DAD) in the lung samples of UIP patients consisting of variable numbers of autopsy samples included in the materials (Saydain *et al.* 2002, Ambrosini *et al.* 2003, Rice *et al.* 2003, Parambil *et al.* 2005). The histopathology of DAD includes hyaline membranes as a hallmark, scant interstitial infiltrates of mononuclear cell in the exudative phase and fibroblast proliferation with pneumocyte II hyperplasia in the organizing phase (Hamman & Rich 1935, Travis *et al.* 2002). The studies investigating DAD and UIP have reported variable results. Saydain *et al.* studied IPF patients admitted to an intensive care unit (ICU) and found that in 9 lung samples including 2 autopsy specimens UIP was present in every case and DAD in 6 cases, but the writers did not describe a detailed analysis of pre- and postmortem lung samples (Saydain *et al.* 2002). Ambrosini *et al.* studied 5 cases of acute exacerbation of IPF including 3 SLBs and autopsy samples from all those 4 patients who died and detected the occurrence of UIP/DAD in every 7 samples (Ambrosini *et al.* 2003). Rice *et al.* investigated the presence of DAD in 15 autopsy samples, and found that DAD was associated with IPF/UIP history in 12 cases, but only one patient had a histopathologically confirmed premortem diagnosis of UIP (Rice *et al.* 2003). In one recent study where 7 IPF patients underwent SLB due to acute exacerbation, a combination of UIP and DAD was present in 5 cases, only UIP was present in one case and DAD alone in another case (Parambil *et al.* 2005). In that study, one patient survived to hospital discharge but the authors did not specify his SLB histology (Parambil *et al.* 2005). Kondoh *et al.* found no hyaline membranes on the 3 OLBs performed during the accelerated phase of IPF and all 3 patients survived (Kondoh *et al.* 1993).

The previous studies consisting of SLB and autopsy samples in a variety of combinations suggest that DAD is frequently associated with UIP, but the systematic comparison between pre- and postmortal lung specimens from the same patient is lacking. It would also be important to obtain more accurate information about the prevalence of UIP and DAD in relation to the course of the disease.

2.1.2 Desquamative interstitial pneumonia

The major histological finding in DIP is the presence of an increased number of alveolar macrophages (Katzenstein & Myers 1998, Travis *et al.* 2002). The uniform histologic pattern is diffuse and the alveoli septas are thickened by inflammatory infiltrates (ATS & ERS 2002). DIP is closely associated with to smoking, i.e. 60–90 % of DIP patients have

a smoking history (Carrington *et al.* 1978, Craig *et al.* 2004). When macrophages are spared in more distal air spaces, the term, respiratory bronchiolitis interstitial lung disease (RB-ILD), is used (Myers *et al.* 1987, Katzenstein & Myers 1998, ATS & ERS 2002). Myer *et al.* suggested that DIP and RB-ILD could be distinct disorders and Craig *et al.* found significantly more interstitial fibrosis and eosinophilic infiltration in DIP than in RB-ILD (Myers *et al.* 1987, Craig *et al.* 2004). Increased number of alveolar macrophages with granules of “smokers pigment” consisting of intracellular yellow to black particles is the predominant histological feature of RB-ILD (Niewoehner *et al.* 1974, King 1993, Travis *et al.* 2002).

The treatments for DIP are cessation of smoking and therapy with corticosteroids (Gaensler *et al.* 1966, Carrington *et al.* 1978). The median survival of 5.6 years is significantly better than that of UIP (Carrington *et al.* 1978). Craig *et al.* found no significant difference in survival between smokers and never smokers in their study of 25 DIP patients (Craig *et al.* 2004).

2.1.3 ILD associated with collagen vascular diseases

Heterogeneous group of collagen vascular diseases (CVD) is associated with different type of ILDs including UIP, DIP, bronchiolitis obliterans organizing pneumonia (BOOP) and NSIP (Lamblin *et al.* 2001, Kim EA *et al.* 2002). The NSIP pattern consists of varying degrees of alveolar wall inflammation and fibrosis (Katzenstein & Fiorelli 1994, Travis *et al.* 2000, Travis *et al.* 2002). Two histologic types of NSIP can be recognized: a cellular pattern with chronic interstitial inflammation including type II pneumocyte hyperplasia and a fibrotic pattern with temporally homogenous fibrosis (Travis *et al.* 2000, ATS & ERS 2002). The uniform type of fibrosis is the difference between NSIP and UIP (Katzenstein & Fiorelli 1994, Nagai *et al.* 1998, Travis *et al.* 2000, ATS & ERS 2002). The major histological type of ILD in scleroderma is NSIP (Bouros *et al.* 2002, Kim DS *et al.* 2002). Surgical lung biopsies of 70 polymyositis-dermatomyositis patients revealed NSIP as the most common ILD also in this CVD (Douglas *et al.* 2001). On the other hand recent studies report that the UIP pattern is prevalent in rheumatoid arthritis associated ILD (Tansey *et al.* 2004, Lee *et al.* 2005).

With respect to of the idiopathic types of the diseases, those patients with NSIP enjoy a better survival than the patients with idiopathic UIP (Bjoraker *et al.* 1998, Nagai *et al.* 1998, Daniil *et al.* 1999, Nicholson *et al.* 2000, Travis *et al.* 2000). The data before the recognition of NSIP revealed that other types of ILDs associated with CVDs had a more favourable prognosis than UIP (Hakala *et al.* 1990, Tazelaar *et al.* 1990). Recent studies have found no difference in the survival between NSIP and UIP associated with CVDs (Bouros *et al.* 2002, Nakamura *et al.* 2003). The prognosis and response to therapy are determined by the histological pattern of ILD and by the underlying CVD.

2.1.4 Sarcoidosis

Sarcoidosis is a systemic disease of unknown etiology affecting the lung and lymphatic systems characterized by non-caseating granulomas, accumulation of T-cells and mononuclear phagocytes at the site of inflammation (Mitchell & Scadding 1974, Thomas & Hunninghake 1987, Dai *et al.* 1998). According to a population based study in the United States, the rates for sarcoidosis were 5.9 per 100 000 person years for men and 6.3 per 100 000 person years for women (Henke *et al.* 1986).

The pathogenesis of sarcoidosis includes granuloma formation beginning with the tissue deposition of poorly soluble antigen material, which is phagocytosed by mononuclear phagocytes and processed into peptide-complexes (Alacron *et al.* 1988). The complexes are then displayed on the surface of antigen-presenting cells for analysis by helper T-lymphocytes (Th) possessing cluster of differentiation 4+ (CD₄⁺)-surface antigen (ATS 1999). Granulomatous inflammation can express both Th 1, such as interferon gamma (IFN γ), and Th 2, such as interleukin (IL) 4, cytokines by activated CD₄⁺ Th-cells (Kunkel *et al.* 1996, Moller 1999). Alveolar macrophages of sarcoidosis patients have been demonstrated to release TNF, which is an important mediator in granulomatous inflammation (Baughman *et al.* 1990). Circulating immune complexes have been detected in sarcoidosis (Hedfors & Norberg 1974, Selroos *et al.* 1980).

IFN γ and IL4 are two important factors involved in the development of fibrosis in sarcoidosis. IFN γ is antifibrotic, i.e. it inhibits the growth of fibroblast and collagen synthesis (Jimenez *et al.* 1984, Clark *et al.* 1989). It has been shown to downregulate TGF α gene expression in bleomycin induced lung fibrosis in animal model (Gurujeyalakshmi & Giri 1995). In contrast to IFN γ , IL4 has a profibrotic effect by stimulating collagen synthesis in fibroblasts (Serpier *et al.* 1997). Fibrotic changes in granulomas are initially expressed at the periphery (Travis *et al.* 2002).

The epithelioid cell granulomas consist of highly differentiated mononuclear phagocytes and lymphocytes (Dail & Hammar 1994, Travis *et al.* 2002). CD₄⁺ lymphocytes are located in the center of the granuloma whereas CD₈⁺ lymphocytes are present in its peripheral regions (Dail & Hammar 1994). T-cells are the most numerous lymphocytes at the site of granuloma formation, but B-cells and plasma cells are also present (Semenzato *et al.* 1984). B-lymphocytes are the source of the immunoglobulins present in sarcoidosis (Hunninghake & Crystal 1981).

The lungs are affected in more than 90 % of sarcoidosis patients with dyspnoea, dry cough and chest pain being the main symptoms (ATS 1999). Radiological findings are classified into five stages from no visible intrathoracic changes up to advanced fibrosis with honeycombing (ATS 1999). There are many extrapulmonary manifestations of sarcoidosis, so the findings and symptoms are related to the organ involved (Roberts *et al.* 2004). Corticosteroids produce relief of the respiratory symptoms, which often reappear after the discontinuation of treatment (Sharma *et al.* 1966, Johns *et al.* 1974, Harkleroad *et al.* 1982). Cytotoxic agents like methotrexate have been used to suppress sarcoidosis achieving a favourable response (Lower & Baughman 1990, Lower & Baughman 1995). The use of systemic therapy in pulmonary manifestations has been limited to patients with severe disease.

2.1.5 Allergic alveolitis

Allergic alveolitis, also known as hypersensitivity pneumonitis (HP) is immunologically induced granulomatous and inflammatory lung disease caused by the inhalation of a variety of environmental agents (Salvaggio & Millhollon 1993, Travis *et al.* 2002). Bird-related HP is induced by exposure to the excrement and protein material from dispersed dust from birds (Perez-Padilla *et al.* 1996). Farmer's lung results from repeated exposure to a high concentration or prolonged exposure to low concentration of inhaled antigens from moldy hay or straw (Erkinjuntti-Pekkarinen *et al.* 1999, Roussel *et al.* 2004). Exposure to aerosols of environmental opportunistic nontuberculous mycobacteria have caused HP in metalworkers doing grinding work operations, attendants in swimming baths and inhabitants of water-damaged buildings (Falkinham 2003). Isocyanates can cause chemically induced HP (Baur 1995).

The disease may be present as an acute form lasting 4 to 8 hours after antigen exposure, with flu-like symptoms, which gradually decline over the next days but recur after the revealed inhalation of the causative agent (Dickie & Franklin 1958, Travis *et al.* 2002). The subacute form develops gradually within weeks or months, with symptoms such as dyspnoea, cough, fatigue and weight loss (Emanuel *et al.* 1964, Travis *et al.* 2002). The chronic form may develop through long-term, low-level antigen exposure or due to recurrent undiagnosed acute or subacute episodes (Yoshizawa *et al.* 1999, Ohtani *et al.* 2003). Chronic HP can be progressive, irreversible and result in lung fibrosis (Pérez-Padilla *et al.* 1993). HP patients with pulmonary fibrosis have diminished survival compared to those without fibrosis (Pérez-Parilla *et al.* 1993, Vourlekis *et al.* 2004).

Both immune-complex mediated and T cell mediated immune responses are involved in HP (Suga *et al.* 1997). The histopathology in acute HP includes neutrophilic infiltration in the alveoli and respiratory bronchioles, sometimes with the pattern of DAD (Travis *et al.* 2002). The histological features of the subacute and chronic forms are a bronchiolocentric interstitial granulomatous pneumonitis with lymphocytes, plasma cells and macrophages (Emanuel *et al.* 1964, Coleman & Colby 1988, Travis *et al.* 2002). Lymphocytic alveolitis is a major finding in BALF (Costabel *et al.* 1984, Yoshizawa *et al.* 1999). Avoidance of organic antigen exposure is the most important factor in the management of HP and corticosteroids are indicated for the treatment of severe acute and progressive forms (Yoshida *et al.* 1989, Kokkarinen *et al.* 1992, Kusaka *et al.* 1993).

2.2 Oxidants and antioxidant-oxidant imbalance in the pathogenesis of interstitial lung diseases

Oxidants are free radicals that scavenge electrons from other molecules (Schraufstatter & Cochrane 1991). Exogenous factors like cigarette smoke, air pollutants and drugs are capable of inducing increased production of oxidants and of activation inflammatory cells including neutrophils, alveolar macrophages and eosinophils to generate free radicals (Kleinerman 1977, Kinnula *et al.* 1995, Rahman *et al.* 1995, Yamazaki *et al.* 1998, Tao *et al.* 2003). ROS such as H_2O_2 , OH^- and O_2^- are powerful oxidants which can be formed in

human lung by mitochondrial electron transport chain, MPO, nicotinamide-adenine dinucleotide phosphate (NADPH) oxidase, eosinophilic peroxidase and xanthine oxidase (Kinnula *et al.* 1995). In addition to ROS, reactive nitrogen species (RNS) can also be formed by the reaction of O_2^- and NO (Beckman & Koppenol 1996). ROS/RNS are toxic by-products of cellular metabolism but they also participate in cell signalling and regulation (Finkel 1998, Rhee *et al.* 1999). The pathways of ROS signalling can lead to oxidative modification of proteins or alterations in the intracellular redox state, where Trx and GSH are important intracellular thiols acting as “redox buffers” (Thannikal & Fanburg 2000).

Antioxidant enzymes represent the main protective mechanism against free radicals mediated lung injury by detoxifying ROS (Kinnula *et al.* 1995). Antioxidant response element (ARE) mediated expression and coordinated induction of genes encoding detoxifying enzymes are one crucial mechanism involved in the cellular protection against oxidative stress (Rushmore *et al.* 1991). ARE is in turn regulated by nuclear-factor-E2-related factor (NRF2) (Venugopal & Jaiswal 1998). Animal studies suggest that the genes encoding antioxidant enzymes are not inducible in NRF2 knockout animals (Ramos-Gomez *et al.* 2001, Cho *et al.* 2004). In the study of Cho *et al.*, NRF2 knockout mice and wild type mice were exposed to bleomycin to induce pulmonary fibrosis, and the knockout mice expressed severe fibrosis and inflammation in lung histopathology compared to wild type ones suggesting that NRF2 represents a protective factor against pulmonary fibrosis (Cho *et al.* 2004). Furthermore, bleomycin caused significantly higher up regulation of mRNA for Trx and both subunits of γ GCS in wild type mice (Cho *et al.* 2004). Accumulation of neutrophils and increased expression of MMPs were also reported in the knockout mice (Cho *et al.* 2004). Neutrophil oxidants like hypochlorous acid (HOCl), O_2^- and H_2O_2 are able to cleave SH bonds and release complexed zinc, which is required for the activation of MMPs to regulate the formation of extracellular matrix protein (Fliss & Menard 1992, Parks & Mecham 1998).

The antioxidant enzymes include three different SODs, which decompose superoxide radicals to H_2O_2 (Marklund 1984, Kinnula *et al.* 1995). Intracellular copper-zinc SOD (CuZnSOD) is located in bronchial epithelium in healthy lung but also in lung of sarcoidosis patient (Lakari *et al.* 1998). MnSOD is highly expressed in the granulomas of pulmonary sarcoidosis and allergic alveolitis (Lakari *et al.* 1998). In UIP and DIP, alveolar type II epithelial cells and macrophages showed moderate expression of MnSOD, but the immunoreactivity was low or absent in old fibrotic areas in UIP (Lakari *et al.* 2000). Extracellular SOD (ECSOD) protects the cells from extracellularly produced ROS and it is found in the extracellular matrix in UIP (Fattman *et al.* 2003). ECSOD is found in UIP in interstitial mast cells, but as with the expression of two other SODs, there were no immunohistochemical signs of ECSOD expression in the fibrotic areas (Kinnula *et al.* 2006). One of the most important H_2O_2 scavenging enzymes is catalase, which is found in the same areas in UIP and DIP as MnSOD (Simon *et al.* 1989, Lakari *et al.* 2000). The other major H_2O_2 scavenging antioxidant enzyme is glutathione peroxidase (GPx) which utilizes reduced GSH to reduce H_2O_2 and lipid peroxides to their corresponding alcohols (Meister 1988). Extracellular GPx gene expression is upregulated in bronchial epithelial cells as a result of oxidative stress in asthma and in those who have been exposed to an exogenous oxidant such as cigarette smoke (Comhair *et al.* 2000, Comhair *et al.* 2001). In addition to these enzymes already investigated in ILDs, human

lung contains other enzymes with an H_2O_2 consuming capacity, which participate in regulating redox system including Trx, thioredoxin peroxidases (peroxiredoxins) and glutaredoxin (Grx). Trx scavenges ROS by itself or together with Trx-dependent peroxiredoxin (Kang *et al.* 1998a). Koura *et al.* found elevated expression of Trx in the granulomas of pulmonary sarcoidosis (Koura *et al.* 2000). Grx is also a thiol-disulfide oxidoreductase expressed in alveolar macrophages in normal lung, sarcoidosis and UIP, whereas fibrotic lesions in UIP showed no expression (Rhee *et al.* 1999, Peltoniemi *et al.* 2004). The protective antioxidant role of GSH also in lungs is well documented (Rahman & Mac Nee 2000b). The only study on human IPF that has shown some therapeutic favourable effect was done with the precursor of GSH, acetylcysteine. In this study IPF patients who were treated with this thiol compound experienced a lesser deterioration of VC and D_{LCO} at 12 months, although there was no effect on survival (Demedts *et al.* 2005). Figure 2.

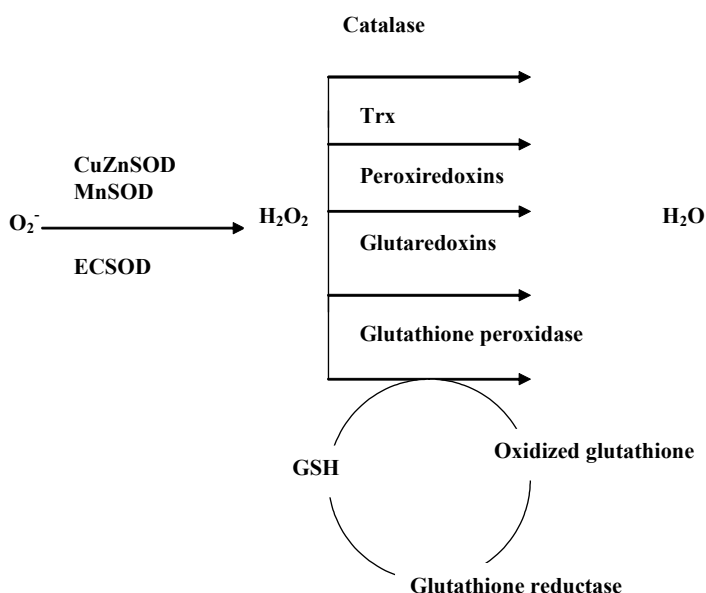


Fig. 2. Major antioxidant pathways of the lung.

In view of the important results emerging from the studies of Cho *et al.* and Demedts *et al.* in conjunction with the fact that GSH is a monothiol and γ GCS as well as Thx are thiol proteins indicate that further investigation of this system in ILDs, including IPF, is needed (Meister & Anderson 1983, Holmgren 1985, Cho *et al.* 2004, Demedts *et al.* 2005).

2.2.1 Thioredoxin system proteins

Mammalian Trx is a small (12 kDa) ubiquitous multifunctional protein containing an oxidation-reduction-active cysteine disulfide sequence: -Cys-Gly-Pro-Cys- (Holmgren *et al.* 1975, Arner & Holmgren 2000). The other members of the thioredoxin system are the major electron donors NADPH and TrxR, which reduce the oxidized thioredoxin (Trx-S₂) (Holmgren 1985). Reduced Trx (Trx-(SH)₂) in turn reduces protein disulfides, generating Trx-S₂ (Holmgren 1985, Arner & Holmgren 2000). Figure 3.

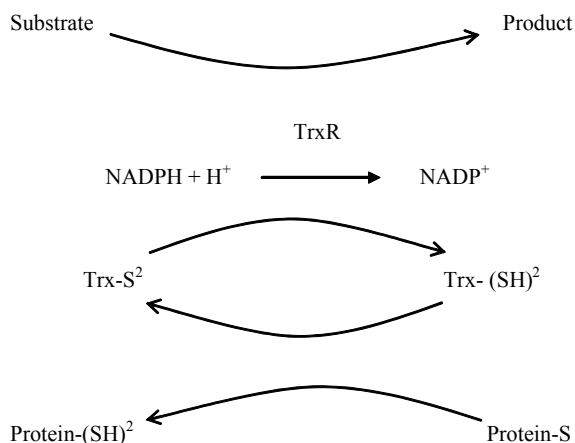


Fig. 3. Thioredoxin system modified from that described by Arner and Holmgren (Holmgren 1985, Arner & Holmgren 2000).

Trx-(SH)₂ functions as a powerful general disulfide reactant, protecting cytosolic proteins via oxidative formation of disulfides and oxidoreductant carrying electrons for catalytic cycles including scavenging ROS (Holmgren *et al.* 1975, Holmgren 1985). Trx functions also as an electron donor for peroxiredoxins, which catalyse the reduction of H₂O₂ (Kang *et al.* 1998a). Trx is a physiological inhibitor of apoptosis signal regulating kinase (ASK) 1, protecting cells against apoptosis (Saitoh *et al.* 1998, Arner & Holmgren 2000). Trx has a central role in thiol redox control of cell functions by modulating the gene transcription of nuclear factor kappa beta (NF β), which controls expression of several inflammatory genes. NF β is a heterodimer consisting of 50 kDa DNA-binding subunit that associates with 65 kDa subunit bound to an inhibitor protein (IB) in the cytoplasm (Baldwin 1996). In response to a variety of stimuli IB dissociates from NF β allowing NF β translocation to nucleus (Bauerle & Baltimore 1988). Trx is responsible for DNA-binding properties of NF β by reducing disulfate bond of the 50 kDa subunit (Matthews *et al.* 1992, Qin *et al.* 1995). Trx exhibits cytokine-like activities such as augmenting the production of TNF, IL6 and IL8 (Schenk *et al.* 1994, Yoshida *et al.* 1999). IL6 is a cytokine capable of stimulating fibroblast growth and production of collagen and it is secreted by fibroblasts in fibrotic lesions (Kondo *et al.* 2001). The overexpression of IL6 evokes interstitial pneumonia in rats (Yoshida *et al.* 1995). IL8 is a monocyte- and

macrophage derived cytokine that can activate macrophages and increased expressions of IL8 have been documented in BALF of IPF patients (Carre *et al.* 1991).

Trx is induced by a variety of stress conditions like exposure to H₂O₂, X-ray irradiation, exposure to ultraviolet (UV) light, ischemic insult and viral infections (Nakamura *et al.* 1997). Diesel exhaust particles (DEP) induce Trx expression in animal models and also in cultured normal human bronchial epithelial cells, exposure to DEP evoked the generation of oxidants and lead to cell necrosis (Matsuo *et al.* 2003, Kaimul *et al.* 2005). ROS are considered to be involved in bleomycin induced lung injury, which has been suppressed by Trx in animal models (Jamieson *et al.* 1987, Tamagawa *et al.* 2000, Hoshino *et al.* 2003).

The first observations of human Trx came from human T cell leukemia virus type 1-transformed T cells (Tagaya *et al.* 1989). Elevated plasma Trx levels have been reported in patients with acute exacerbation of asthma, rheumatoid arthritis, human immunodeficiency virus (HIV) -infection and after a cardiopulmonary bypass operation (Nakamura *et al.* 1996, Nakamura *et al.* 1998, Yoshida *et al.* 1999, Yamada *et al.* 2003). Influenza viruses can cause the global epidemic and therefore the observation that transgenic mice with overexpression of Trx are more resistant to the influenza virus -induced pneumonia is interesting (Nakamura *et al.* 2002).

TrxR is a selenocysteine-containing flavoprotein composed of two identical 57 kDa subunits, with a broad substrate specificity since it can also reduce non-disulfide substrates such as lipid hydroperoxides, selenite and H₂O₂ (Kumar *et al.* 1992, Björnstedt *et al.* 1995, Zhong & Holmgren 2000). TrxR is the only enzyme known to reduce oxidized Trx, and thus it might have regulatory activities on the same functions as Trx (Mustacich & Powis 2000). TrxR can directly remove apoptotic inducers like H₂O₂ or indirectly via anti-apoptotic activity of Trx- (SH)₂ (Zhong & Holmgren 2000, Liu & Min 2002). A positive correlation between the inactivation of TrxR and growth/proliferation inhibition or apoptosis has been found in human lung cell lines (Zhao *et al.* 2005). The activity of TrxR responds to selenium supplementation in several cell lines *in vitro* (Berggren *et al.* 1997, Marcocci *et al.* 1997). The elevated expression of Trx and TrxR messenger ribonucleic acid (mRNA) in newborn baboons, which were exposed to high levels of O₂ point to an important role of TrxR also in protecting the cell against oxidative stress (Kumuda *et al.* 1999). Exposure to cigarette smoke has induced elevated expression of TrxR mRNA in fibroblasts (Gebel & Muller 2001).

Several studies have investigated the overexpression of the Trx/TrxR system in human malignancies (Kahlos *et al.* 2001, Soini *et al.* 2001a). There are few reports about the localization of Trx and TrxR in normal conditions and in non-malignant disorders in humans. In atherosclerotic coronary arteries Trx was expressed in infiltrating macrophages and in nonatherosclerotic arteries it was found in medial smooth muscle cells, taking part in antioxidant protection (Okuda *et al.* 2001). In the endometrium, Trx was found in the ciliated cells of the luminal and glandular epithelium possibly protecting the epithelial cells from the apoptotic actions of the trophoblasts (Stavr us-Evers *et al.* 2002). There are only a few published studies examining the expression of this system in non-malignant lung disorders (Koura *et al.* 2000, Kaarteenaho-Wiik & Kinnula 2004).

2.2.2 Glutathione related redox modulating pathways in human lung

The epithelial lining fluid of the airways contains over 100 fold higher concentrations of GSH compared to plasma, and the levels of GSH are reduced in patients with pulmonary fibrosis and allergic alveolitis (Cantin *et al.* 1987b, Behr *et al.* 1995, Behr *et al.* 2002). GSH is a thiol containing tripeptide synthesized from its constituent amino acids by the sequential action of γ GCS and GSH-synthetase (Meister & Andersson 1983). Figure 4.

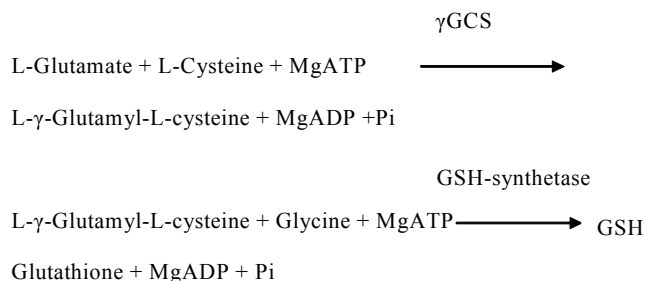


Fig. 4. The synthesis of glutathione as described by Meister & Andersson (Meister & Andersson 1983).

Intracellular GSH is oxidized to glutathione disulfide (GSSG), which is reduced back to GSH by GSSG reductase at the expense of NADPH (Meister & Andersson 1983). GSH reaction pathways are linked to the reactions of other thiol containing proteins in scavenging of H_2O_2 and thus is involved in the regulation of the redox balance of the cells (Kinnula *et al.* 2004).

2.2.2.1 Gamma-glutamylcysteine synthetase

γ GCS is the rate-limiting enzyme catalysing the generation of GSH, which is feedback inhibited by GSH (Meister & Andersson 1983, Franklin *et al.* 2002). Two subunits form γ GCS: these are the catalytically active heavy subunit (γ GCS_h \approx 73 kDa) which contains all of the substrate binding sites, and the light subunit (γ GCS_l \approx 28 kDa) that modulates the affinity of the heavy subunit for substrates and inhibitors, this is presumably being mediated by a redox-sensitive disulfide bond between the two subunits (Huang *et al.* 1993, Griffith 1999).

The regulation of γ GCS is complex. Oxidants, antioxidants and agents related to inflammatory processes modulate the intracellular redox state influencing the activity of key transcription factors such as NF κ B and activator protein-1 (AP-1) (Griffith 1999, Rahman & MacNee 2000b). Overexpression of γ GCS inhibits NF κ B activation and gene expression *in vitro* (Manna *et al.* 1998). AP-1 is involved in the transcriptional up-regulation of γ GCS_h gene under oxidative stress including cigarette smoke (Rahman *et al.* 1996b, Rahman *et al.* 1998). NFR2 regulates also γ GCS and in bleomycin induced lung

injury, the expression of both subunits was significantly reduced in NFR2 knockout mice leading to pulmonary fibrosis (Cho *et al.* 2004). The activation of both NF and AP-1 by oxidative stress was inhibited by NAC *in vitro* (Schreck *et al.* 1991, Collart *et al.* 1995). Furthermore, *in vitro* studies indicate that the γ GSH content in A549 cells is affected also by culture conditions even without exposure to oxidants (Post *et al.* 1983).

TNF α modulates chronic inflammatory changes also by inducing antioxidant levels in mammalian cells (Männel *et al.* 1980, Wong & Goeddel 1988). *In vitro* studies, oxidative stress imposed on A549 cells and on pulmonary mucoepidermoidal carcinoma cells by H₂O₂, menadione or TNF α , increased GSH, γ GCS protein as well as the γ GCS α mRNA and the changes occurred at 6-24 hours after exposure (Rahman *et al.* 1996a, Rahman *et al.* 1999, Ray *et al.* 2002). Conversely, treatment with the anti-inflammatory agent, dexamethasone, decreased the levels of γ GCS and γ GCS α mRNA (Rahman *et al.* 1999). TGF β ₁ is a chemoattractant for fibroblasts being the main mediator of fibrogenesis in lung (Raghow *et al.* 1987, Khalil *et al.* 1991). TGF β ₁ causes depletion of GSH by down regulation of γ GCS, at least partly, via decreased expression of γ GCS α mRNA (Arsalene *et al.* 1997, Jardine *et al.* 2002).

As with the Trx system, the levels of γ GCS are highly elevated in lung and pleural malignancies such as mesothelioma and non-small cell carcinoma (Soini *et al.* 2001a, Soini *et al.* 2001b, Järvinen *et al.* 2002, Kinnula *et al.* 2004). Increased immunoreactivities for both subunits expressed in bronchiolar epithelium and alveolar macrophages of nonsmokers compared to those of smokers with or without chronic obstructive pulmonary disease (COPD) (Harju *et al.* 2002). In human lung, γ GCS can be detected at the 17th gestational week (Kaarteenaho-Wiik & Kinnula 2004). No investigations of the cell specific expressions of both subunits of γ GCS in ILDs are available.

3 Aims of the study

The aims of this study were to evaluate the safety of SLB, the histopathological features and prognostic factors of UIP, the most common idiopathic interstitial pneumonia. Another goal of the study was to investigate the role of the major thiol containing redox-regulated proteins of human lung in selected ILDs, including UIP.

The specific aims of the study were:

1. to evaluate the morbidity and short-term mortality of biopsied patients with UIP (I)
2. to assess the number of FF in premortem lung samples and to correlate this parameter with survival, and to evaluate the histopathological findings at autopsy and to associate them with the course of IPF/UIP (II)
3. to determine the expression and distribution of the thioredoxin system enzymes such as thioredoxin and thioredoxin reductase, in IPF/UIP and selected ILDs (III)
4. to investigate the expression and distribution of the rate-limiting enzyme in GSH synthesis, gamma-glutamylcysteine synthetase, in IPF/UIP and selected ILDs (IV).

4 Materials and methods

4.1 Clinical information (I, II, III, IV)

The clinical data was collected from Oulu University Hospital, Päivärinne Hospital, Oulaskangas Hospital, Central Hospital of Länsi-Pohja and Lapland Central Hospital. The first patient data was from September 1973 and the end points of these studies were the last visit before January 1st 2003 or the patient's death. The clinical data of the 76 patients in studies I and II included age at the time of the biopsy, sex, smoking status, exposure to asbestos, other diseases, medication in general and for IPF/UIP, need for home oxygen therapy, date of biopsy, type of biopsy, major postoperative complications, date and causes of death. The delay between the first visit and biopsy was calculated as well as the survival time after the biopsy. The associated symptoms such as exertional dyspnoea and cough were noted. Pulmonary function tests included preoperative spirometry and D_{LCO} . The radiology involved standard chest radiographs and HRCT.

The III study included 68 patients and the IV study 66 patients and their clinical data was obtained from the patient records of the University Hospital and Päivärinne Hospital including age at the time of biopsy, sex, pulmonary function tests and smoking habits. The end point of these studies was April 1999, when the last lung biopsy sample was gathered.

4.2 Human tissue specimens (I, II)

76 lung samples with typical UIP histopathology taken between January 1973 and January 2003 were gathered from the files of the Department of Pathology, Oulu University Hospital. OLB were performed from January 1973 to December 1992, after that time VATS was mainly used. The subjects fulfilled the UIP diagnosis based on the ATS/ERS criteria (ATS & ERS 2002). Biopsies were taken from different parts of the left or right lung. The biopsy material was fixed in 10 % formalin under vacuum in order to expand the tissue and to remove air bubbles or perfused by injecting the fixative, using a

small syringe, into the bronchioles as described by Dail and Hammar (Dail & Hammar 1994). The specimens were then dehydrated and embedded in paraffin.

In the second study where the number of FF in the lung biopsy sample were evaluated in relation to the course of IPF/UIP, 11 autopsy samples from the same patients were added to the material (II). Lung tissue samples were taken from all five lobes in all but one case, in that instance samples only from right lung lobes were taken.

4.3 Human tissue specimens (III, IV)

Histopathologically typical cases of interstitial lung diseases were retrieved from the files of the Department of Pathology, Oulu University Hospital, by re-evaluating lung biopsies taken either by open or thoracoscopic method between September 1973 and April 1999. The diagnoses were based on light-microscopic evaluations using the histological criteria of Katzenstein (Katzenstein 1997). 60 patients were included into study III and 62 into study IV representing UIP (n=15 (III, IV)), DIP (n=9 (III) and n=10 (IV)), sarcoidosis (n=18 (III) and n=19 (IV)), allergic alveolitis (n=8 (III, IV) and ILD-CVD (n=10 (III, IV)) whose biopsies were obtained from patients with rheumatoid arthritis (n=3 (III) and n=2 (IV)), scleroderma n=3 (III, IV)), polymyositis (n=2 (III) and n=3 (IV)), systemic lupus erythematosus (SLE) (n=1 (III, IV)) and mixed connective tissue disease (n=1 (III, IV)). Six controls were available with uninvolved peripheral lung tissue. These were obtained from normal, healthy-looking lung of six patients operated on for carcinoid tumour of the lung. The biopsy material was handled as described in chapter 4.2.

4.4 Lung biopsies (I, II, III, IV)

Lung biopsies were performed under general anaesthesia. The site for the biopsy was selected on the basis of chest radiographic or computed tomography findings.

In OLB, the most common approach used was small anterior or lateral thoracotomy. The biopsy was usually obtained with an autosuture device (TA 30-60-90 or GIA) from the representative lung parenchyma, frequently the edge of the lobe was suitable. Chest tube and pleural suction or a Heimlich-valve was usually used for one to three days.

VATS was always performed with double lumen endotracheal intubation with single lung ventilation. The patient was placed in the full lateral position. Three access sites and 5.5 mm ports were used. The thoracoscope was introduced through the seventh or eighth interspace on the midaxillary line and the placement of the latter two additional ports was determined. An endostapler was used to perform the wedge resection. Chest tube insertion was accomplished and the other two sites were closed.

In studies I and II, the biopsies were taken from the right lower lobe in 32 cases, right middle lobe in 20 cases, left lower lobe in 13 cases, lingula in 8 cases and right upper lobe in 3 cases. Only one specimen was taken in 75 cases, and the mean size of biopsy was 13.7 cm². The size of the sample was large enough for the diagnosis of UIP in all cases.

4.5 Bronchoalveolar lavage (III)

Fiberoptic bronchoscopy for sampling of BALF was performed as recommended by the European Task Group on BAL (Kleeh & Hutter 1990) for 16 patients (UIP n=3, sarcoidosis n=5, allergic alveolitis n=4, control n=4). The control group consisted of nonsmoking patients, who had been investigated for minor respiratory symptoms of unknown etiology. The cell number and distribution as well as the chemical inflammatory parameters of BALF of the control subjects were normal. Lavage was carried out in the lingual or the right middle lobe. Aliquots of 20 ml of sterile saline of 37° C (for a total of 200 ml) were installed into the segment to be lavaged. The fluid recovered from the first aliquot was excluded from the study. The effluent was aspirated manually using a syringe. Cells were harvested from the BALF by centrifugation at 400xg for 15 min, fixed in 10 % formalin, embedded in 2 % agar, and after solidification, further embedded in paraffin prior to evaluation.

4.6 Cell culture and exposures (IV)

A549 cells (American Type Culture Collection, Rockville, MD, USA) were grown in monolayer in Ham's Nutrient mixture F-12 with L-glutamine supplied with 15% fetal bovine serum (FBS), 100 U/ml penicillin and 100µg/ml streptomycin (all from Gibco BLR, Life Technologies, Paisley, UK). The cells were exposed to TGFβ₁ (2 ng/ml) or TNFα (50 ng/ml) for 24, 48 and 72 hours and the expressions of γGCSH and γGCSI were determined by Western blot analyses where the cell pellets were melted with the electrophoresis sample buffer, and boiled for 5 min at 95 °C. TGFβ₁ and TNFα were selected for the challenge test since they are known to express in IPF (Piquet *et al.* 1993, Khalil *et al.* 1996). Furthermore they promote infiltration of inflammatory cells and proliferation of fibroblasts (Broekelmann *et al.* 1991, Zhang *et al.* 1993) The protein concentration of samples was measured using the Bio-Rad protein assay (Bio-Rad, Hercules, CA) and 50 µg of cell protein per lane was applied to a 12 % sodium dodecyl sulfate polyacrylamide gel. The gel was electrophoresed for 1.5 h (90V), and then transferred (45 min, 100 V) onto Hybond ECL nitrocellulose membrane (Amersham, Buckinghamshire, UK) in Mini Protean II Cell (Bio-Rad).

The blotted membranes were incubated with antibodies to recombinant MnSOD (a gift from Prof JD Crapo, Jewish Medical and Research Center, Denver, CO, USA) (dilution 1:10 000), γGCSH or γGCSI peptides (dilutions 1:10 000 and 1:5000, respectively) or -actin (Sigma Aldrich, St Louis, MO, USA) (dilution 1:5000) followed by treatment with horseradish peroxidase-conjugated anti-rabbit or anti-mouse secondary antibodies (both from Amersham).

The antibodies directed against the γGCSH or the γGCSI peptides were from a rabbit and were a gift from D.T. Kavanagh, University of Washington, Seattle, WA, USA.

The regulation of well characterized antioxidant enzyme, MnSOD was used as a positive control in both situations, since its regulation in human lung has been intensively investigated and documented to express strongly in the granulomas of pulmonary

sarcoidosis and allergic alveolitis, but the expression is weak in old fibrotic areas of the lung (Kinnula *et al.* 1995, Lakari *et al.* 1998).

4.7 Histopathological evaluation (I, III, IV)

Lung biopsy samples with typical UIP histopathology were re-evaluated independently by two pathologists (Riitta Kaarteenaho-Wiik, Paavo Pääkkö) with a light microscope based on the criteria presented by Dail & Hammar and Katzenstein (Dail & Hammar 1994, Katzenstein 1997). The same criteria were used when DIP, sarcoidosis and allergic alveolitis were re-evaluated in studies III and IV (Dail & Hammar 1994, Katzenstein 1997).

4.8 Histopathological evaluation (II)

The histopathology of UIP was re-evaluated as previously described (Chapter 4.7). Sections 4 µm thick lung tissue samples were stained with haematoxylin/eosin (HE) - and alcian blue periodic acid schiff (AB-PAS) -methods. The total numbers of FF were analysed independently by two pathologists (Riitta Kaarteenaho-Wiik, Paavo Pääkkö). The surface area of lung tissue in one slide of each case was defined by image-analysis (MC ID – M4 for Windows 3.0 Rev 1.1, Imaging Research Inc., Brock University, St Catharines, Ontario, Canada) and the numbers of FF were divided into two subgroups (50 or less FF/ 1 cm² or more than 50 FF/ 1 cm²) (See statistical analyses).

Autopsy was carried out in 11 UIP cases and the lung samples were re-analysed by HE-staining. The histological features including UIP, squamous-type metaplastic alveolar epithelium, accumulation of intra-alveolar neutrophils and hyaline membranes were classified (RK-W, PP) independently as either negative (-) or positive (+). The quantifying of DAD in the lung specimen referred to the extent of lung tissue involvement as follows: no evidence of DAD (-), focal patches of DAD (+) or widely spread DAD (++)

4.9 Immunohistochemistry and evaluation of immunoreactivity (III, IV)

Four micrometers thick sections were cut from representative paraffin blocks. The sections were first deparaffinized in xylene and rehydrated in descending ethanol series. In order to enhance the immunoreactivity, the sections were incubated in 10 mM citrate buffer (pH 6.0), boiled in a microwave oven for 2 min at 850 W, and then for 8 min in 350 W. Endogenous peroxidase activity was eliminated by incubation in 0.1% hydrogen peroxide in absolute methanol for 10 min. After incubation at +4°C overnight with the

affinity purified goat antihuman Trx antibody at a dilution of 1:2000 (American Diagnostica, Greenwich, CT), a biotinylated secondary anti-goat antibody was applied (dilution 1:400) followed by the avidin-biotin-peroxidase complex (both from Dakopatts, Glostrup, Denmark)

The antibody to TrxR was the gammaglobulin fraction of a polyclonal rabbit antibody directed against rat cytosolic thioredoxin reductase and was a gift from A. Holmgren, Karolinska Institutet, Stockholm, Sweden. The antibodies directed against the γ GCSH and γ GCSI were from rabbit and were a gift from D.T. Kavanagh, University of Washington, Seattle, WA, USA. The dilutions used were all 1:1000.

Replacement of the primary antibody by phosphate buffered saline (PBS) at pH 7.2 and rabbit primary antibody isotype control (Zymed Laboratories, Inc, San Francisco, CA) was used as a negative control. The colour was developed using aminoethylcarbazole (AEC), and the sections were lightly counterstained with hematoxylin and mounted with Eukitt (Kindler, Freiburg, Germany).

The scoring of the cytoplasmic staining was evaluated semiquantitatively by two pathologists independently from one tissue section (roughly 1 cm²) in alveolar, metaplastic alveolar, bronchial and bronchiolar epithelium, macrophages, fibroblast foci and old fibrotic lesions. The results were classified into four groups: 0, no immunostaining; +, weak positive staining; ++, moderate staining and +++, strong staining.

4.10 Immunoelectron microscopy (III, IV)

Fresh lung tissues of sarcoidosis and UIP patients were obtained from surgical operations and small pieces were fixed in 4 % paraformaldehyde in 0.1 M phosphate buffer with 2.5 % sucrose, pH 7.4, for 2 hours, immersed in 2.3 M sucrose and frozen in liquid nitrogen. Thin cryosections were cut with a Leica Ultracut UCT microtome (Leica Microsystems, Vienna, Austria). For immunolabelling, the sections were first incubated in 0.05 M glycine in PBS and then in 5 % bovine serum albumin (BSA) with 0.1 % cold water fish skin gelatin (Aurion, Wageningen, The Netherlands). Antibodies and gold conjugate were diluted in 0.1 % BSA-C (Aurion) in PBS. All washings were performed in 0.1 % BSA-C in PBS. The sections were then incubated with antibodies to Trx, TrxR, γ GCSH γ GCSH which were the same as previously described (Chapter 4.6 and 4.9) for 60 min followed by protein A-gold complex (size 10 nm) for 30 min, made according to Slot (Slot & Geuze 1985). The controls were prepared by carrying out the labelling procedure without the primary antibody. The sections were embedded in methylcellulose and examined with a Philips CM 100 transmission electron microscope (FEI Company, Eindhoven, the Netherlands). Images were captured by a CCD camera equipped with TCL-EM-menu version 3 from Tietz Video and Image Processing Systems SmbH (Gaunting, Germany).

4.11 Statistical analyses (I, II, III, IV)

The statistical analyses were performed with the SPSS for Windows software (SPSS for Windows, Rel. 11.5.1 2002.Chicago). The significance of the associations was determined using Fisher's exact probability test which is designed for small sample groups. P-values less than 0.05 were considered statistically significant.

In the studies I and II the median survival was used, whenever possible comparing the survival between different groups, but if this could not be calculated, then the mean survival was used. Cumulative survivals were estimated using the Kaplan-Meier method, the log-rank test was used to compare survival of different groups.

In the studies II, III and IV, the inter-observer reliability between two pathologists was investigated using Cohen's kappa statistics (Cohen 1960, Landis & Koch 1977). Since no linear correlation between the number of FF and survival was observed, receiver operating characteristics (ROC) -curve was used to find the cut-off value (i.e. the number of FF) on which basis the material was divided into two subsets (Armitage 2002).

In studies III and IV, the correlations between the groups were determined by Spearman correlation test.

4.12 Ethical considerations (I, II, III, IV)

The instructions of the National advisory board on research ethics were followed in all the studies included into this thesis. The personal data of the patients has not been used, instead serial numbers were used both in collecting and analysing the material. The personal identification registry is kept in the separate archive. The study material consisting of the lung biopsy specimens and BALF samples as well as the clinical patient information and test results were initially attained for diagnostic purposes only. The study protocols were approved by the Ethical committee of the Oulu University Hospital.

5 Results

5.1 Safety of the thoracoscopic and open lung biopsies (I)

5.1.1 *Clinical findings and symptoms (I, II)*

The study population consisted of 42 (55.3 %) men and 34 (44.7 %) women, with a mean age at the time of biopsy of 56.7 years (range, 21 to 77). The mean observation time after the first visit due to symptoms was 89.2 months (range, 2 to 336). A total of 42 patients (55.3 %) had open lung biopsy and 34 (44.7 %) underwent the thoracoscopic procedure. The mean observation period after the lung biopsy was 69 months (range, 1 to 296). There were 17 (22.4 %) current and 23 (30.3 %) former smokers with average 22.6 pack years, whose median survival was 60 months (95% confidence interval (CI) 51 to 69) considering those patients who died during the study period. Compared to the survival of never smokers, 49 months (95% CI 0 to 99), this 11 months difference was not statistically significant. The main symptoms were exertional dyspnoea in 40 patients (52.6 %) and non-productive cough in 26 cases (34.2 %). The physical examination revealed inspiratory crackle in 55 patients (72.4 %). Finger clubbing was seen in 24 cases (31.6 %) and 15 patients (36.6 %) who died of UIP exhibited this symptom. A family history of IPF like disease was found in 5 cases (6.6 %).

Bilateral reticular opacities (34 cases, 44.7 %) and fibrosis (50 cases, 65.8 %) were the main findings in the chest radiograph. Since the first biopsies were taken in 1973 and HRCT only came into general use during the 1990s, the preoperative HRCT was available in only 38 cases (50 %). The predominant pattern of HRCT was fibrosis in 32 cases (84.2 %) including 20 cases (52.6 %) of honeycombing, whereas ground glass opacities were observed in 11 cases (28.9 %).

Pulmonary function tests including forced vital capacity (FVC), forced expiratory volume (FEV₁) and D_{LCO} were available in 73 cases (96 %). Those patients having D_{LCO} 50 % or more of predicted value experienced a median survival of 85 months (95 % CI 56 to 114, p=0.009), while the group with less than 50 % of the predicted value lived only 49 months (95 % CI 37 to 61, p = 0.009).

5.1.2 Lung biopsy and survival (I)

By the end of the study, 51 patients (67.1 %) were dead. UIP was the main cause of death in 43 cases (56.6 %) including only 2 patients with CVD. Four patients (5.3 %) died of lung carcinoma (squamous cell carcinoma n = 2, small cell carcinoma n = 2), they all had idiopathic type of UIP. One patient died of ventricular bleeding due to an ulcer and 3 patients died of acute myocardial infarction. Table 2. The median survival after the first visit to hospital due to symptoms of the whole group was 91 months (95 % CI 62-120) and after lung biopsy, 59 months (95 % CI 44-74).

Table 2. Characteristics of the patients with idiopathic UIP and UIP with connective tissue disease.

Parameter	Idiopathic UIP	UIP with connective tissue disease*	Total
	Number (%)	Number (%)	Number (%)
Number of patients / %	64 (84.2 %)	12 (15.8 %)	76 (100 %)
Male	38 / 76 (50 %)	4 / 76 (5.3 %)	42 (55.3 %)
Female	26 / 76 (34.1 %)	8 / 76 (10.6 %)	34 (44.7 %)
Median age at the time of biopsy, Years (range)	58.9 (37-77)	45.3 (21-74)	56.7 (21-77)
Open lung biopsy	34	8	42
Thoracoscopic biopsy	30	4	34
Previous /Active smoker	35 / 64 (54.7 %)	5 / 12 (41.7 %)	40 / 76 (52.6 %)
Pack Years	23.9	13	22.6
Median survival after diagnosis (Months (95% CI))	69 (47-91)	232** (182-281)	91(62-120)
Median survival after biopsy (Months (95 % CI))	49(40-58)	223**(167-280)	59(44-74)
Dead	47 / 64 (73.4 %)	4 / 12 (33.3 %)	51 / 76 (67.1%)
Death due to UIP	41 / 64 (64.3 %)	2 / 12 (16.7 %)	43 / 76 (56.6 %)

UIP = usual interstitial pneumonia, CI = confidence interval, * rheumatoid arthritis (n = 4), scleroderma (n = 4), dermatomyositis (n = 3), mixed connective tissue disease (n = 1), ** mean survival was used since over half of the patients (4/6) were alive at the end of the study, median survival was used for those patients who were dead by the end of the study

The biopsies in the 1970s (10 cases) and 1980s (23 cases) were all OLBs. After the 1990s, there were 9 open and 34 VATS lung biopsies. There was a 20 month difference in the median survival depending on the type of biopsy, in the open thoracotomy group it was 51 months (95 % CI 36 to 66) and in the thoracoscopic group 71 months (95 % CI 43 to 99). The median survival after the lung biopsy did not change significantly over these three decades. Eight patients died in the 1970s having median survival of 47 months (95 % CI 3-91), the corresponding numbers from the 1980s were 19 patients / 60 months (95 % CI 17-105) and from the 1990s 23 patients / 63 months (95 % CI 42-84). After the year 2000, only one patient has died with 10 months survival after the lung biopsy. Thirty-five patients (46 %) who had an open thoracotomy and 16 of the patients (21.1 %) who underwent thoracoscopic procedure were dead by the end of the study.

The histopathological verification of UIP led to a change in the treatment in 58 cases (76.8 %) with no statistical effect on survival. The 58 patients with altered treatment had a median survival 61 months (95% CI 37-85), those 9 patients (11.8 %) who underwent no change in their medication had a median survival of 85 months (95% CI 38-132). Nine patients who received no treatment had a median survival of 49 months (95% CI 26-72). Corticosteroids were supplemented in 45 cases (59.2 %) of these 16 cases received additional treatment with azathioprine, whereas cyclophosphamide was combined in 4 cases. Two patients underwent lung transplantation because of UIP.

The age of the patients at the time of the biopsy was a significant factor, with a correlation to the median survival after biopsy (Spearman ~ -0.228 , $p = 0.048$). The patients who were 50 years of age or younger had 211 months median survival (95 % CI 109 to 313, $p = 0.006$) compared to 51 months (95 % CI 42 to 60, $p = 0.006$) of the patients who were older than 50 years.

Four (5.3 %) patients died within one month after the biopsy. All these patients underwent OLB due to atypical symptoms or radiological findings of IPF, and the histological features of UIP were found in all four cases. In addition to UIP, the organizing stage of diffuse alveolar damage (DAD) was observed at autopsy in all cases, one patient had also pneumonia in his autopsy samples. None of these individuals had connective tissue disease and only one had received corticosteroids prior to SLB and none of the four needed mechanical ventilation preoperatively.

The connective tissue disease patients with better survival were younger and smoked less than the idiopathic group (Table 2). The median age at the time of lung biopsy was 45.3 years for the patients with connective tissue disease compared to 58.9 years of idiopathic group. In the connective tissue disease group the median survival after diagnosis and biopsy was longer than the survival of patients with the idiopathic type UIP, 69 vs. 232 months and 49 vs. 223 months, respectively. Only 2 out of 12 patients (16.7 %) with connective tissue disease had died of UIP by the end of the study whereas 41 out of 64 patients (64.3 %) had passed away due to UIP in the idiopathic group. Table 2.

5.2 Fibroblast foci, diffuse alveolar damage and exacerbation of idiopathic pulmonary fibrosis/usual interstitial pneumonia (II)

5.2.1 Clinical features (II)

The clinical findings and symptoms are described in chapter 5.1.2, since the study material was same.

Seven patients (9.2 %) fulfilled all the criteria of accelerated deterioration of IPF, and in two cases one criteria was missing, i.e. the PaO₂ value from the last visit was not available in one case, and in another case there were problems in assessing the chest x-ray and excluding infection (Akira *et al.* 1997). Six males and 3 females were included in this subgroup. Their mean age at the time of biopsy was 54.6 years (range, 21 to 69) and at the time of death 60.1 years (range, 45 to 80). The 2 years frequency of acute exacerbation

before death was 2 cases out of 76, i.e. 2.6 %, when only those patients who fulfilled the criteria of Akira *et al.* were included (Akira *et al.* 1997). The total survival after the first visit to hospital due to symptoms was 96 months (~ 8 years) (range, 10 to 247) and survival after biopsy 75 months (~ 6.3 years) (range, 6 to 245). Acute exacerbation before death was found in 20.9 % of the IPF/UIP related deaths.

5.2.2 Histopathologic findings of lung biopsies and their correlation with survival (II)

FF were observed in all cases and the average size of analysed material was 1.64 cm². The number of FF varied from 2 to 327 / 1 cm² and the average number of FF / 1 cm² was 76 in patients with idiopathic UIP and 50 / 1 cm² in the patients with connective tissue disease. The numbers of the idiopathic UIP patients with 1-50 and over 50 FF / 1 cm² were 28 and 36, respectively, and the numbers of those UIP patients with connective tissue disease were 6 and 6, respectively. As expected, patients with lower numbers of FF had better prognosis. Using Kaplan-Meier method the patients having 50 or less FF / 1 cm² (n=34) had median survival of 89 months (95% CI 38 to 140, p=0.0358) when that of the patients with more than 50 FF / 1 cm² (n=42) was 49 months (95% CI 36 to 62, p=0.0358) (Table 3). The 223 months (95% CI 167 to 280, p= 0.0002) survival after biopsy of UIP-patients with connective tissue disease was significantly longer compared to 49 months (95 % CI 40 to 58, p=0.0002) of idiopathic group. The interobserver repeatability between the scoring results of the pathologists was moderate by using Cohen's kappa statistics (Kappa coefficient = 0.568).

There was a significant negative correlation between D_{LCO} and the number of FF (Spearman \sim -0.302, p= 0.009) showing that the patients with diminished D_{LCO} had more often higher number of FF in their lung biopsy specimens.

According to our unpublished finding the median survival after biopsy of never smoker was 116 months (95 % CI 23 to 209) having 50 or less FF / 1 cm² and 40 months (95 % CI 20 to 60) when the number of FF / 1 cm² was over 50, the numbers of former and active smokers were 60 months (95 % CI 55 to 65) and 56 months (95 % CI 36 to 76) respectively. The correlations between the number of FF / 1 cm² and the subgroups of the patients classified by smoking habits were not statistically significant

5.2.3 Histopathologic findings of autopsy samples (II)

Histopathologic findings of autopsy material revealed DAD with hyaline membranes in 10 out of 11 cases. However, the extent of DAD varied from case to case. In 3 cases DAD was observed in focal patches while in 7 samples it was observed widely throughout the whole lung tissue. In 6 cases DAD was so strong that it nearly obscured the typical histological features of UIP. The post-mortem analysis revealed neutrophil infiltrates in 9 cases. Squamous-type metaplastic alveolar epithelium could be detected in 10/11 cases.

In 6 patients there were clinical sings of pneumonia, like chest x-ray findings or elevated CRP levels, but histopathological analyses of their autopsy samples revealed typical histopathological features of pneumonia in only 2 cases. On the other hand 2 other patients with typical pneumonia in their autopsy lung samples did not have any changes in pre-mortem chest x-ray findings or elevated CRP levels. 3 patients had clinical sings of acute exacerbation before death (Akira *et al.* 1997). Table 3.

Table 3. Histopathological findings of autopsy samples and the occurrence of acute exacerbation of IPF/UIP before death of the 11 patients with UIP.

Case number	DAD	Intra-alveolar neutrophils	Metaplastic squamous epithelium	Pneumonia at autopsy	Acute exacerbation before death
1.	++	No	Yes	No	No
2.	+	Yes	Yes	No	Yes
3.	+	Yes	Yes	Yes	No
4.	++	Yes	Yes	No	Yes
5.	++	Yes	Yes	No	No
6.	-	Yes	Yes	Yes	No
7.	++	Yes	No	Yes	No
8.	++	Yes	Yes	No	No
9.	++	Yes	Yes	No	No
10.	+	No	Yes	No	Yes
11	++	Yes	Yes	Yes	No

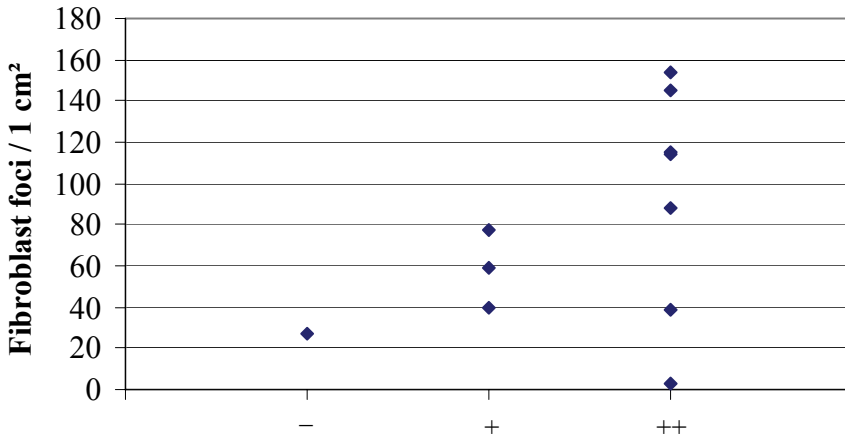
DAD = diffuse alveolar damage

- = no evidence of DAD, + = focal patches of DAD

++ = widely spread DAD

5.2.4 The associations between FF, DAD and acute exacerbations before death (II)

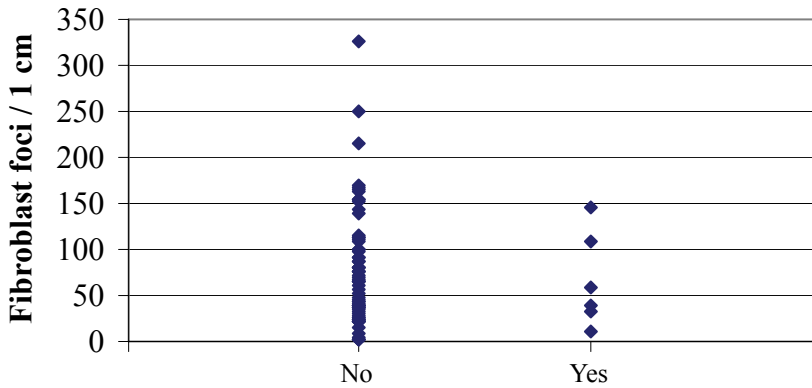
When the existence of DAD was categorized and correlated with FF, there appeared to be no significant association between them. Generally DAD could be found both from those with low and high numbers of FF (Figure 5).



Degree of DAD

- = no evidence of DAD, + = focal patches of DAD, ++ = widely spread DAD, DAD = diffuse alveolar damage
 No significant association was found between the number of FF and accelerated phase of the disease (Figure 6).

Fig. 5. The association between the number of fibroblast foci / 1 cm² and diffuse alveolar damage IPF/UIP.



Accelerated phase

Fig. 6. The association between the number of fibroblast foci / 1 cm² and accelerated phase of IPF.

There was no correlation between the occurrence of the accelerated exacerbation of IPF/UIP and the extent of DAD, since 6 out of 11 autopsied patients had widely spread DAD in their autopsy samples without clinical signs of acute exacerbation before death (Table 4).

Table 4. The numbers of IPF/UIP patients classified by the occurrence of acute exacerbation and the extent of DAD.

Degree of DAD	Accelerated exacerbation of IPF	No accelerated exacerbation of IPF
Widely spread DAD	1	6
Focal patches of DAD	2	1
No evidence of DAD	0	1

5.3 Expression of the Thioredoxin system in interstitial lung diseases (III)

5.3.1 Clinical features (III, IV)

The cases selected in the studies III and IV were similar including patients with UIP, DIP, interstitial lung disease associated with collagen vascular disease, sarcoidosis and allergic alveolitis. The mean age was 50 years (range 21 to 74 years), including 28 males (41.2 % (III), 42.4 % (IV)) in both studies and 40 (58.8 %) females in the III study and 38 (57.6 %) females in the IV study. In pulmonary function tests the mean FEV₁ % was 78.2 % of the predicted value, FVC % was 66.8 % of the predicted value and D_{LCO} was 70.8 % of predicted value in the III study, and the numbers were 77 %, 82 %, and 70 %, in the study IV, respectively. There were 23 active smokers in both studies.

5.3.2 Localization for Trx and TrxR by immunohistochemistry (III)

5.3.2.1 Trx and TrxR in normal lung (III)

Normal peripheral lung showed weak or moderate Trx and TrxR immunoreactivity in the epithelium of bronchus and bronchiolus, and in alveolar macrophages. Normal alveolar epithelium, consisting of type I and II pneumocytes, and lung fibroblasts were negative by immunohistochemistry (Table 5). The staining was mainly cytoplasmic, but some bronchial epithelial cells showed also nuclear immunoreactivity.

5.3.2.2 Trx and TrxR in UIP, DIP and ILD-CVD (III)

In UIP, metaplastic alveolar epithelium showed mainly moderate to strong expression for Trx and TrxR, this was observed in all three types i.e. alveolar cuboidal type II, bronchiolar and squamous type metaplastic epithelium (Table 5). The immunostainings were both cytoplasmic and nuclear, and especially strong in the nuclei of the bronchiolar-

type metaplastic and squamous-type metaplastic epithelium. Alveolar macrophages showed significantly higher immunostaining for Trx ($p = 0.03$) and TrxR ($p = 0.04$) than controls. The immunoreactivity was also increased in the epithelium of bronchus and bronchiolus both in the cytoplasmic and nuclear compartments. The epithelium of the fibrotic areas showed expression for Trx and TrxR, but fibroblasts and myofibroblasts were negative in the active fibroblastic foci and in the scar-like old fibrotic areas. Table 5.

In DIP, the strongest immunohistochemical expressions for both Trx ($p = 0.002$) and TrxR ($p = 0.019$) were in the alveolar macrophages compared to controls. The expression in the metaplastic alveolar epithelium was moderate to strong for Trx and weak for TrxR. As with UIP, the positivity was both cytoplasmic and nuclear, the latter being again detectable especially in the metaplastic alveolar epithelium. Table 5.

In interstitial lung diseases-collagen vascular diseases (ILD-CVD) the immunohistochemical reaction for Trx and TrxR appeared to be stronger when compared to the normal lung, but lower than in UIP and DIP. The cytoplasmic and nuclear compartments of the metaplastic alveolar epithelium showed occasionally moderate to strong expressions for both enzymes. Table 5.

5.3.2.3 Trx and TrxR in granulomatous diseases (III)

In sarcoidosis alveolar macrophages showed mainly moderate expression for Trx and TrxR. Most granulomas showed moderate or strong expression for Trx, and weak to moderate expression for TrxR. Bronchial epithelium was mainly weak for Trx and weak or moderate for TrxR. Table 5.

In allergic alveolitis, alveolar macrophages showed mainly weak or moderate stainings, granulomas had occasionally immunoreactivity for Trx, the staining for TrxR was weak or absent. The expressions in bronchial epithelium varied between negative to moderate, while normal alveolar epithelium showed no immunoreactivity. Table 5.

Table 5. The cytoplasmic and nuclear immunostainings for Trx and TrxR in controls, UIP, DIP, CVD-ILD and granulomatous diseases of the lung.

Disease		Alveolar Epithelium	Metaplastic Alveolar Epithelium	Bronchiolar Epithelium	Macrophages	Nuclear Staining	Granulomas
		Trx/TrxR	Trx/TrxR	Trx/TrxR	Trx/TrxR	Trx/TrxR	Trx/TrxR
Controls	0	6/6	0/0	0/3	1/3	3/4	0/0
N = 6	+	0/0	0/0	3/3	5/3	2/2	0/0
	++	0/0	0/0	3/0	0/0	1/0	0/0
	+++	0/0	0/0	0/0	0/0	0/0	0/0
UIP N = 15	0	2*/0	2/1	1/6	1*/0*	4/5	0/0
	+	7/5	6/3	4/3	5/6	3/1	0/0
	++	5/4	4/4	7/3	5/5	8/7	0/0
DIP N = 10	0	1*/1*	1/1	5/6	0*/1*	0/1	0/0
	+	3/4	2/4	1/0	1/2	8/4	0/0
	++	3/2	2/3	2/2	1/5	1/3	0/0
ILD-CVD N = 10	0	2/2	4/1	1/1	7/1	0/1	0/0
	+	3/5	3/5	2/6	2/5	4/5	0/0
	++	3/3	3/3	4/2	4/5	4/1	0/0
Sarcoidosis N = 18	0	2/2	2/0	4/1	4/0	2/4	0/0
	+	18/17	0/0	6/3	4/0*	4/7	2/2
	++	0/1	0/0	11/10	11/13	14/11	3/9
Allergic alveolitis N = 8	0	0/0	0/0	1/5	3/5	0/0	8/7
	+	8/8	0/0	3/1	1/0*	1/5	4/6
	++	0/0	0/0	3/5	4/7	7/3	1/2
	+++	0/0	0/0	2/2	3/1	0/0	2/0
		0/0	0/0	0/0	0/0	0/0	1/0

0 = no positive staining; + = <30 % of cells showing positivity, weak; ++ = 30-60 % of cells showing positivity, moderate; +++ = >60 % of cells showing positivity, intense, * p < 0,05 compared to the expression of the investigated enzyme in the corresponding cell type from normal looking, healthy lung of the patients with no interstitial lung disease

The interobserver reability of the assessment of 2 independent investigators varied from moderate to almost perfect (Kappa coefficient 0.46-0.94).

5.3.3 Immunohistochemical analyses of BAL samples (III)

The BALF samples of healthy controls, patients with UIP, sarcoidosis and allergic alveolitis confirmed the expression of Trx and TrxR in alveolar macrophages and showed no expression for Trx or TrxR in the lymphocytes.

5.3.4 Ultrastructural distribution of Trx and TrxR in UIP (III)

Trx and TrxR were expressed in alveolar macrophages, metaplastic type II pneumocytes and bronchial epithelial cells. The staining was diffuse in the cytoplasm and occasionally in the nuclear compartment and along the plasma membranes. Bronchial epithelial cells showed prominent expression in the lateral cell protrusions. Trx and TrxR could also be detected in the apical areas of the ciliary epithelial cells. No labelling was seen in fibroblasts.

5.4 Cell-specific regulation of gamma-glutamylcysteine synthetase in human interstitial lung diseases (IV)

5.4.1 Localization of γ GCS_h and γ GCS_l by immunohistochemistry (IV)

5.4.1.1 γ GCS_h and γ GCS_l in normal lung (IV)

Both subunits of γ GCS were localized to bronchiolar epithelium, the intensity varied from weak to strong for γ GCS_h and for γ GCS_l was mainly weak. Alveolar macrophages were weakly positive for both subunits. Normal alveolar epithelium showed no immunohistochemical expression for either subunit. Table 6.

5.4.1.2 γ GCS_l and γ GCS_h in UIP, DIP and ILD-CVD (IV)

In UIP, the strongest expressions of both subunits were observed in bronchiolar epithelium (Table 6). The metaplastic alveolar epithelium, which was found only in the diseased lung, showed moderate to strong reactivity for both subunits, diffuse expression of both subunits could also be detected in fibrotic areas, but fibroblasts and myofibroblasts were negative in the active fibroblast foci and in the scar like fibrotic areas. In DIP the most intense expression for both γ GCS subunits was seen in alveolar macrophages, while bronchiolar epithelium and metaplastic alveolar epithelium showed mainly weak immunoreactivity. Table 6.

The alveolar epithelium in DIP showed increased intensities for both subunits compared to the control lung ($p = 0.007$ for γ GCS_h and $p = 0.001$ for γ GCS_l). In ILD-CVD the bronchiolar epithelium and metaplastic alveolar epithelium were variably positive for both subunits but once again fibroblasts, fibromyxoid lesions and the fibrotic areas were negative. Table 6.

5.4.1.3 γ GCS_h and γ GCS_l in granulomatous diseases (IV)

In sarcoidosis, the bronchiolar epithelium showed moderate to strong expression for γ GCS_h and weak to moderate expression for γ GCS_l in most cases. In the majority of the cases, alveolar macrophages were weakly to moderately positive for both subunits. Granulomas were from moderately to strongly positive for γ GCS_h and weakly positive for γ GCS_l. Table 6.

In allergic alveolitis the granulomas are in general less conspicuous and not so well developed as in sarcoidosis, the immunoreactivity of both γ GCS subunits was mainly weak or negative except for a few cases with strong γ GCS_h expression. Bronchiolar epithelium and alveolar macrophages showed weak to moderate expression. Table 6.

Table 6. Immunoreactivities for γ GCS heavy (h) and light (l) subunits in the lung.

Disease		Alveolar	Metaplastic	Bronchiolar	Alveolar	Granulo-
		Epithelium	Alveolar Epithelium	Epithelium	Macrophages	mas
		h/l	h/l	h/l	h/l	h/l
Controls N = 6	0	6/6	0/0	0/0	6/6	0/0
	+	0/0	0/0	2/5	0/0	0/0
	++	0/0	0/0	2/1	0/0	0/0
	+++	0/0	0/0	2/0	0/0	0/0
UIP N = 15	0	0/0	0/0	1/1	7/11	0/0
	+	7/9	4/6	0/3	8/4	0/0
	++	6/5	8/7	3/7	0/0	0/0
	+++	2/1	3/2	11/4	0/0	0/0
DIP N = 10	0	2/1	2/1	3/1	0/0	0/0
	+	8/9	8/7	1/5	8/8	0/0
	++	0/0	0/2	2/3	0/2	0/0
	+++	0/0	0/0	4/1	2/1	0/0
ILD-CVD N = 10	0	1/1	2/1	1/0	1/0	0/0
	+	6/6	5/5	5/4	7/5	0/0
	++	2/1	2/1	1/3	1/4	0/0
	+++	1/2	1/3	3/3	1/1	0/0
Sarcoidosis N = 19	0	17/12	0/0	1/1	2/2	0/1
	+	1/4	0/0	5/9	11/12	5/15
	++	0/2	0/0	7/9	6/5	5/3
	+++	1/1	0/0	6/0	0/0	9/0
Allergic alveolitis N = 8	0	7/7	0/0	2/2	1/0	4/5
	+	0/1	0/0	3/5	4/7	1/3
	++	1/0	0/0	3/1	3/1	1/0
	+++	0/0	0/0	0/0	0/0	2/0

0 = no positive immunostaining; + = < 30 % of cells showing positivity, weakly positive; ++ = 30-60 % of cells showing positivity; moderately positive; +++ = > 60 % of cells showing positivity, strongly positive

The interobserver reability of the assessment of 2 independent investigators varied from fair to almost perfect (Kappa coefficient 0.33-1.0) (Cohen 1960, Landis & Koch 1977).

5.4.2 Ultrastructural localization for γ GCSI and γ GCSH in UIP and sarcoidosis (IV)

Immunolabeling for both subunits of γ GCS was found in the metaplastic type II pneumocytes in UIP and macrophages in both diseases. Labeling of γ GCSH was diffuse in the cytoplasm, whereas γ GCSI appeared to concentrate to the near proximity of the plasma membrane.

5.4.3 In vitro experiments with A549 cells (IV)

To get more accurate insight to the regulation of γ GCS in lung cells, A549 cells were exposed to $\text{TNF}\alpha$ and $\text{TGF}\beta_1$, since these cytokines are important pathogenic mediators of lung fibrosis (Agostini & Gurreri 2006). $\text{TNF}\alpha$ caused 131 % and 113 % increase in MnSOD (a positive control) after 24 h and 48 h. The corresponding values for γ GCSH were 23 % (24 h) and 0 % (48 h) and for γ GCSI 19 % (24 h) and 16 % (48 h), respectively. $\text{TGF}\beta_1$ caused 19 % (24 h) and 20 % (48 h) decrease in the expression of MnSOD, and 8 % and 47 % decrease after 24 h and 48 h for γ GCSH, the corresponding percentages for γ GCSI being 6 % and 9 %.

6 Discussion

6.1 Methodological aspects (I, II, III, IV)

Considering the fact that UIP is a rare disease our material consisting of 76 UIP patients who fulfilled the criteria of UIP collected over three decades is sufficient for comparing the results with previous studies were Utz *et al.* included 68 UIP patients and Riha *et al.* 59 UIP patients (Utz *et al.* 2001, Riha *et al.* 2002). We focused only on those patients with histopathologically confirmed UIP instead of including all kind of ILDs in the study (Gaensler & Carrington 1980, Rena *et al.* 1999, Zegdi *et al.* 1998, Ayed & Raghunathan 2000, Yamaguchi *et al.* 2004, Lettieri *et al.* 2005). Also the fact that we evaluated only the patients whose diagnosis was attained by SLB leaving out the diagnoses made by TLB helps drawing reliable conclusions from our material (Hunninghake *et al.* 2001). The assessment of the patient's preoperative constitution is important issue in evaluating the impact of SLB on mortality. In the retrospective re-evaluation of the short-term mortality after SLB none of our patients fulfilled the criteria of acute exacerbation of IPF before lung biopsy in contrast to Utz *et al.* study where 4 cases of 10 IPF patients were biopsied because of accelerated decline in respiratory status (Utz *et al.* 2001).

The evaluation of predicting histopathological factors was carried out advisely by focusing on a single hallmark of UIP, that is FF, to achieve simple and repeatable method instead of deterring several factors resulting in derivation of different scores (Cherniack *et al.* 1991, King *et al.* 2001a). The concern about the sufficiency of the lung biopsy sample for diagnosis must always notice, so the counting of the actual number of FF from the whole available sample makes the diagnosis reliable comparing to those results made from few standard areas (Nicholson *et al.* 2002b). Previous studies concerning the assessment of lung pathology in IPF have included four different stainings of biopsy samples, whereas only one staining was used in our study making our method simple and useful (Cherniack *et al.* 1991, Hyde *et al.* 1992, King *et al.* 2001a).

The possibility of comparing 11 lung biopsy specimens taken during lifetime and autopsy samples from the same patients is unique, since previous studies have investigated pre- and post-mortem cases in variable ratios. Ambrosini *et al.* had 5 IPF cases where 3 of them underwent SLB, autopsy was carried out in all 4 cases when

patient died (Ambrosini *et al.* 2003). Parambil *et al.* had 7 SLBs and 2 autopsy samples from the same patients (Parambil *et al.* 2005). Rice *et al.* had 12 autopsy cases with a single premortem SLB from one patients including into the study group (Rice *et al.* 2003).

Using human tissue samples, which were lung biopsies taken for diagnostic purposes, we could evaluate the remaining tissue structure and true localization of the enzymes studied, this would have not been possible with tissue homogenates. The expressions of Trx, TrxR and both subunits of γ GCS have not been previously studied systematically in non-malignant lung diseases. In fact Koura *et al.* focused in describing the expression of Trx in granulomas and lymph nodes in sarcoidosis (Koura *et al.* 2000).

The heavy subunit of γ GCS has been studied more, since it is considered to be functionally more imposing than the light one. However both subunits are induced by oxidant, so it is important to describe the observation concerning γ GCSl too. To obtain better understanding the regulation of γ GCS, lung cells were exposed to cytokine, TNF α , and growth factor TGF β ₁, which are important factors especially in inflammatory and fibrotic disorders of the lung.

6.2 Survival and safety of SLB in usual interstitial pneumonia (I)

Our study material shares the characteristics of IPF patients previously described, the patients have passed their middle-ages, clinical features like clubbing and inspiratory crackle were common and chest x-ray findings were in line with former studies (Jonsthon *et al.* 1997, ATS & ERS 2002). Median survival after lung biopsy in our study for idiopathic UIP was 69 months, this equals well with another available composition were postoperative median survival was 78 months (Riha *et al.* 2002). The improved health care during three decades had no significant impact on survival after lung biopsy probably due to unresolved clinical tasks in treating IPF/UIP.

Age at the time of presentation is significantly related to the survival (Tukiainen *et al.* 1983, King *et al.* 2001a). This study indicates that the age at the time of the biopsy is also a significant predictive factor for survival. IPF is an independent risk for lung cancer, and there were 4 (5.3 %) lung cancers in our study, which is also in line with earlier reported risk for lung cancer (4.4 %) in pulmonary fibrosis (Hubbard *et al.* 2000).

According to our study UIP with CVD had better survival than idiopathic type of UIP. The reason for this could partly be due to the fact that the patients belonging to CVD subgroup were younger and they had better pulmonary function results, the both being independent prognostic factors for survival (Tukiainen *et al.* 1983, King *et al.* 2001b, Latsi *et al.* 2003). Since no histologic differences were found between UIP and UIP associated with CVD according to one previous study, where also NSIP cases were excluded, this interesting observation needs to study further (Nagao *et al.* 2001). On the contrary Kocheril *et al.* reported that ILDs connected with CVD compared to IIPs appeared to be associated with a worse prognosis when adjusted to age in study population without histological verification The recent study where CVD associated with ILDs were compared to IIPs without histological verification (Kocheril *et al.* 2005). The opposite results of these studies indicate the relevance of the histopathological findings in predicting response to therapy and prognosis also in patients with CVD.

The short-term postoperative mortality of IPF/UIP patients in our study was 5.3 %, which was substantially lower than that of Utz *et al.* 21.7 % (Utz *et al.* 2001). In that particular study, however, seven out of ten dead IPF/UIP patients were biopsied during the accelerated disease progression or sub-acute worsening of disease (Utz *et al.* 2001). The four patients in our study who died within 30 days after the biopsy exhibited a histological pattern of DAD in their autopsies, even though they did not fulfil the criteria of acute exacerbation (Akira *et al.* 1997). No DAD had been observed in their pre-mortem lung samples. On the other hand, Utz *et al.* found DAD on UIP in 2 cases of idiopathic IPF, who died within 30 days after SLB, one of them was biopsied because of an accelerated decline in respiratory status (Utz *et al.* 2001). Our finding is consistent with previous studies, where the histopathological finding of the pre-mortem sample may differ from that obtained at autopsy after disease acceleration (Katzenstein & Myers 1998, Rice *et al.* 2003). In another recently published study, the accelerated exacerbation of IPF developed immediately after SLB in 2 cases of 11, and the authors concluded that the procedure could aggravate the rapid worsening of respiratory conditions in IPF (Kim *et al.* 2006). Whether SLB is justified at the time when the patient is showing a rapid deterioration of IPF when additional indications like exclusion of associated malignancy or infection are lacking needs to be considered critically if the major criteria of IPF fulfil otherwise.

The mortality of 5.3 % (4 cases of 76) within 30 days after the procedure in our study includes only patients who underwent OLB compared to the study of Utz *et al.* where the corresponding frequency was 12 % (7 cases of 60) concerning the patients subjected to thoracotomy and biopsy (Utz *et al.* 2001). The writers did not find any differences in surgical techniques that would account for the short-term survival, but detailed information about the SLB methods used in those 7 patients who were biopsied because of accelerated disease progression was not published (Utz *et al.* 2001). In the present study those patients who died within one month after the operation had undergone thoracotomy suggesting that the thoracoscopic lung biopsy is a safer method for diagnosing UIP.

IPF/UIP is a fatal disease with low prevalence. The prognosis has remained unchanged despite the latest diagnostic methods like HRCT. The requirement of the diagnostic accuracy indicate increasing utility for surgical lung biopsy, since in the 1990s the clinical diagnosis proved to be correct in two thirds of the suspected IPF cases and after the millennium the exact diagnosis of IPF was achieved in little over half of the cases (Raghu *et al.* 1999, Hunninghake *et al.* 2001, Peckham *et al.* 2004). This could partly stem from more detailed classification of IIPs (Liebow 1974, ATS & ERS 2002).

The promising therapeutical approaches for IPF indicate the earlier histological verification of IPF/UIP to exclude it from other ILDs with better prognosis. This study documents the safety of VATS in diagnosing IPF and found no short-term mortality. Considering the insufficient accuracy of ATS/ERS criteria for clinical diagnosis of IPF in routine pulmonary practice, it needs to be re-evaluated if these criteria are acceptable alternatives for SLB. In addition to that this procedure provides lung samples which can subsequently be used for scientific investigations and which may clarify the pathogenesis of UIP.

6.3 Fibroblast foci, diffuse alveolar damage and exacerbation of idiopathic pulmonary fibrosis/usual interstitial pneumonia (II)

The present study was done by analysing the number of FF in interstitial tissue and in intra-alveolar spaces from the whole morphometrically defined tissue samples using AB-PAS-staining, in contrast to earlier studies where semiquantitative grading and several stainings have been used (Cherniack *et al.* 1991, Cheniack *et al.* 1995, King *et al.* 2001a, Nicholson *et al.* 2002b). As expected, a subclass having a lower number of FF / 1 cm² enjoyed better survival, which is in line with previous studies (King *et al.* 2001a, Nicholson *et al.* 2002b). Nicholson *et al.* scored the number of FF from one to three standard areas creating an overall index and found a correlation between the increase in the extent and mortality (Nicholson *et al.* 2002b). Our results are in line with the study of Kaarteenaho-Wiik *et al.* who found increased tenascin-C expression especially under metaplastic bronchiolar type epithelium, which was associated with shortened survival (Kaarteenaho-Wiik *et al.* 1996). These two studies suggest that it might be possible to find distinct histological features of UIP that reliably predict survival.

We focused on FF, which is a single fairly well demarcated key feature of pulmonary fibrosis at sites of active fibrosis using AB-PAS-staining since this is better at detecting loose fibrotic tissue of FF with proteoglycans and glycoproteins than HE -methodology (Anderson *et al.* 1991). The simplicity of our method makes it repeatable and reliable.

Earlier studies have shown that there is no critical degree of abnormality of pulmonary function values that predicts diminished survival, although D_{LCO} is the single value that reflects the severity of IPF also over longer observation periods (Turner-Warwick *et al.* 1980, Tukiainen *et al.* 1983, Schwartz *et al.* 1994, Xaubet *et al.* 1998, Latsi *et al.* 2003). This is the first study revealing the negative correlation between the D_{LCO} of predicted value and the number of FF / 1 cm². Our results are in line with the study of King *et al.* who found a significant relationship between the fibrosis factor including FF and a scoring system comprising clinical, radiological and physiological determinants including D_{LCO} (King *et al.* 2001b). Nicholson *et al.* reported also the association between increasing FF score done assessed semiquantitatively and decline of D_{LCO} (Nicholson *et al.* 2002b). Cherniack *et al.* found no significant correlation between the scoring results including FF and physiological parameters like D_{LCO} (Cherniack *et al.* 1995). These discrepancies may be due to differences in the analytical approach and scoring of the histological alterations, so our use of a simple and repeatable method is justified.

Cigarette smoking is a risk factor for IPF, and 52.6 % of our patients were current or ex-smokers, but their median survival after lung biopsy was better than never smokers (Baumgartner *et al.* 1997). Our results considering the extended survival of active smokers is in line with the study of King *et al.*, where they found longer survival time from the initial visit associated with lower scoring results of granulation/young connective tissue in semiquantitative grading of lung biopsy samples of IPF patients who were active smokers (King *et al.* 2001b). This histopathological finding could reflect increased inflammation secondary to accumulation of macrophages as a result of smoking (King *et al.* 2001b). In a multivariate analysis adjusting for smoking among other factors, a positive smoking history was a protective parameter in predicting survival (Flaherty *et al.* 2002). Previously the degree of fibrotic changes including FF has not shown any

correlation to smoking in agreement with our results (Cherniack *et al.* 1995, King *et al.* 2001a). Since one published study has documented altered histopathology of IPF/UIP according to smoking habits this discrepancy needs to be clarified in further investigations (King *et al.* 2001b).

There were 9 cases of acute exacerbation of IPF/UIP among those 43 patients who had UIP as the main cause of death in our study. The percentage found here of acute exacerbations (20.9 %) is less than 47 % reported by Martinez *et al.* (Martinez *et al.* 2005). Substantial differences between these two studies can be identified. All our patients were biopsy-proven UIP cases whereas only 58 % of the study population underwent SLB in Martinez *et al.* study (Martinez *et al.* 2005). The observation time was 247 months in our study compared to 19 months in the referred study (Martinez *et al.* 2005). We did not include in our study any patient showing clinical signs of pneumonia or cor pulmonale. If we scrutinize the cases in the Martinez *et al.* study and include only those patients who seem to fulfil the criteria of acute exacerbation according to Akira *et al.*, we can identify 6 patients, which is 18.8 % of the IPF-related deaths in the study and rather similar to our results (Akira *et al.* 1997, Martinez *et al.* 2005). The 2 year frequency of acute exacerbation of IPF before death in our study was 2.6 %, which is clearly lower than that reported by Kim *et al.* study (18 %) evaluated by the criteria of Akira *et al.* where the mortality rate was 81.8 % compared to ours where all patients with acute exacerbation of IPF/UIP were dead by the end of the study (Akira *et al.* 1997, Kim *et al.* 2006). These results underline the importance of reporting the acute exacerbations of IPF/UIP if one wishes to evaluate the lifelong history of acute deteriorations in these patients.

This is the first study where the number of FF in premortem lung samples was compared systematically with the histological findings at autopsy and acute exacerbations of IPF/UIP before death. Previous studies investigating the pre- and post-mortem histopathological changes of IPF patients have been done mainly during the acute deterioration of the disease, so the finding of DAD also in premortem SLB is not surprising (Saydain *et al.* 2002, Ambrosini *et al.* 2003, Rice *et al.* 2003, Parambil *et al.* 2005). In our study, there were no signs of DAD in the premortem SLB samples in agreement with Kim *et al.* results with respect to these SLB histopathological findings prior to the acute exacerbation of IPF (Kim *et al.* 2006). Furthermore the DAD in the autopsy samples could be so extensive that the correct diagnosis of the underlying UIP might have been overlooked if no premortem pulmonary biopsies had been available. There are no reports about the histopathological features in the stable phase of IPF/UIP and their correlation to the acute deterioration of the disease, thus our findings for a lack of any correlation between the number of FF in premortem sample and DAD at autopsy or appearance of acute exacerbation of IPF/UIP before death has not previously described.

The finding of an accumulation of intra-alveolar neutrophils in most (9/11) autopsied cases compared to histopathological verification of pneumonia in only 4 cases is interesting. There are very few reports, which have examined the expression of neutrophils in lung tissue of IPF/UIP patients, but Rice *et al.* has described honeycomb changes containing aggregates of neutrophils in acute exacerbation of IPF (Rice *et al.* 2003). In our autopsied patients only 2 out of those 3 who fulfilled the criteria of acute exacerbation of IPF had intra-alveolar neutrophils in their lung samples (Akira *et al.*

1997). In view of the complex role of neutrophils in the pathogenesis of IPF the interrelationship between inflammatory cells and the course of IPF must be studied further.

This study is evidence of the practicality of the AB-PAS -method in quantifying the number of FF from lung samples of UIP and illustrates the interrelationship between the detected number of FF and survival. Furthermore DAD is a common and frequently intense feature in the autopsy sample of UIP patient without acute exacerbation before death, but this cannot be predicted by counting FF from the premortem SLB samples.

6.4 The expression of the thioredoxin system in interstitial lung diseases (III)

As oxidant/antioxidant imbalance is a potential feature in ILDs, this study investigated two pathways that had not been earlier described in parenchymal diseases. Theoretically these two enzymes, thioredoxin and gamma-glutamylcysteinesynthetase, are of major importance in the regulation of cellular redox state. Both enzymes have thiol groups in their active centers or in the reactions in which they participate, e.g., GSH metabolism. Both enzymes are regulated by NRF2 and the inhibition of NRF2 has recently been demonstrated to be associated with pulmonary fibrosis (Kim *et al.* 2003, Cho *et al.* 2004, Sakurai *et al.* 2005).

Trx was generally weakly expressed in bronchial epithelium and alveolar macrophages as also found earlier (Soini *et al.* 2001a, Soini *et al.* 2003). The finding of Trx and TrxR in normal bronchial epithelium is not surprising, since it is the first barrier against the exogenous oxidant stress.

The levels of expression of both Trx and TrxR in the metaplastic alveolar epithelium of UIP were positive to strong. This finding is very similar to the previous findings in specimens from premalignant and malignant lung lesions with increased proliferation and apoptosis (Soini *et al.* 2001a, Soini *et al.* 2003). Our findings suggest that Trx might have a role also in the pathogenesis of IPF. One potential mechanism could be via mitogen activated proteins (MAP) kinases which are enzymes linking cell-surface receptors to intracellular regulatory targets, and their activation is known to occur in lung tissue of IPF patients e.g. in epithelial cells and fibroblasts (Yoshida *et al.* 2002). One *in vitro* study has indicated that ASK1, which has Trx as a physiological inhibitor, participates in the signalling cascade activating the MAP kinases leading to apoptosis (Ichijo *et al.* 1997). The absence of both Trx and TrxR in old fibrotic areas is in line with the studies showing low or absent expression of other antioxidant enzymes such as MnSOD and hemoxygenase 1 (HO-1) in old fibrotic lesions of UIP (Lakari *et al.* 1998, Lakari *et al.* 2001). TGF β ₁ is present in the lung of IPF patients and it is capable of inhibiting epithelial cell proliferation and in that way can regulate the extent of fibrosis and influence remodelling in IPF (Khalil *et al.* 2001). TGF β ₁ downregulates HO-1 in rat lung as well as decreases gene and protein expression of TrxR *in vitro* (Pellacani *et al.* 1998, Zhao *et al.* 2004). The possibility that this down-regulation is one mechanism affecting

the natural defence by Trx/TrxR in fibrotic lung disorders contributing to the progression of the disease needs to be studied further.

CVDs with confirmed UIP histopathology were included in this study. The expressions of both Trx and TrxR were elevated in the same locations as in UIP, but the intensities were weaker. This could partly reflect from less active fibrogenesis, since we found less FF / 1 cm² in the UIP patients with CVD than in the idiopathic group in the previous study (II).

The increased expressions of both Trx and TrxR in metaplastic alveolar epithelium and macrophages emphasize the importance of these enzymes also in protection against oxidant stress in IPF. This is in agreement with the study of Kuwano *et al.*, who investigated mitochondria, the most important cellular source of ROS, and found elevated mitochondrial mass in hyperplastic epithelial cells in UIP (Kuwano *et al.* 2003). In previous studies where nitrotyrosine and nitric oxide synthases (NOS) were used as parameters of oxidant stress in IPF elevated expressions were detected also in alveolar epithelium and macrophages (Saleh *et al.* 1997, Lakari *et al.* 2002).

Trx has been studied in sarcoidosis, where it appeared in and was produced by granulomas (Koura *et al.* 2000). Our study confirms this finding, we found expression of Trx and TrxR in several macrophage associated diseases not only sarcoidosis, but also allergic alveolitis and especially DIP, where we detected the highest intensity of both enzymes in alveolar macrophages suggesting also increased oxidant resistance of alveolar macrophages *in vivo*. In ILDs, alveolar macrophages express high levels of other antioxidant enzymes such as MnSOD and HO-1, in addition to the fact Trx can induce both MnSOD and HO-1 (Das *et al.* 1997, Lakari *et al.* 1998, Wiesel *et al.* 2000, Lakari *et al.* 2001).

The ultrastructural localization of Trx and TrxR was mainly cytoplasmic. This is in agreement with a previous study where Trx was localized by indirect immunofluorescence predominantly in the cytoplasm of isolated HeLa cells (Hirota *et al.* 1997). TrxR was detected also along the plasma membrane, which has been reported previously for melanoma cells (Söderberg *et al.* 2000). The reason for occasional detection in nuclear compartment might be due to the antibody used, which though directed towards the cytosolic TrxR, has been shown to detect also TrxR from the isolated mitochondrial fraction of cultured lung cells (Soini *et al.* 2001a). On the other hand, the occasional mitochondrial location could mean that TrxR might be involved in the protection of aerobic cells against oxidant stress (Soini *et al.* 2001a). The verification of the ultrastructural localizations of both enzymes in nonmalignant cells is also new additional information.

Trx and TrxR are redox active, cytoprotective proteins that act in synergy, but they also may have role in proliferative and regenerative changes occurring in lung disorders. In our study, the endothelial lining and macrophages expressed increased immunoreactivity emphasizing the importance of Trx and TrxR in the primary antioxidant defence in the human lung. The highest expressions of both enzymes were in alveolar macrophages in DIP and metaplastic epithelium in UIP, but fibrotic areas in UIP were mainly negative, thus Trx and TrxR could act as markers of inflammation and of ongoing cell regeneration.

6.5 Cell specific regulation of gamma-glutamylcysteine synthetase in human interstitial lung diseases (IV)

There are no previous systematic studies about the expression of GCS in human parenchymal lung diseases. According to our study normal lung expresses significant immunoreactivity for both subunits in bronchiolar epithelium, alveolar macrophages were stained weakly, and there was no staining in alveolar epithelium in agreement with the results of Harju *et al.* (Harju *et al.* 2002). In developing human lung, both subunits have been also detected in bronchial epithelium (Kaarteenaho-Wiik & Kinnula 2004). Previous findings have pointed to the importance of γ GCS as a first-line defence also in normal lung against oxidants, preserving an optimal level of GSH in the airways with the present results being in full agreement.

The involvement of alveolar macrophages in antioxidant protection of lung was demonstrated also in this study, since the expression of both γ GCS_h and γ GCS_l was elevated in DIP, sarcoidosis and allergic alveolitis. An *in vitro* study revealed that activated macrophages produce ROS in response to endogenous and exogenous oxidative stress (Rochelle *et al.* 1998). This leads to a depletion of GSH, resulting in increased expressions of γ GCS both subunits.

In UIP and ILD-CVD with UIP histopathology, the strongest expressions of both subunits were observed in bronchiolar epithelium especially in the metaplastic epithelium. Previous studies have also documented the strongest expression of γ GCS_h in metaplastic alveolar epithelium where it was attributed to malignant transformation of lung epithelial cells (Harju *et al.* 2002, Soini *et al.* 2003). The elevated expressions in UIP, might be related to apoptosis and proliferation, since in UIP the alveolar type II cells have an important role in re-epithelialization and they are the main site of synthesis of TGF β ₁ and TNF α , which also promote apoptosis in epithelial and alveolar epithelial cells (Khalil *et al.* 1991, Kapanaci *et al.* 1995, Wang *et al.* 2000, Hagimoto *et al.* 2002).

The expressions of both GCS subunits were negative in new and old fibrotic lesions. Previously the fibroblasts in COPD have been demonstrated to exhibit no immunoreactivity for γ GCS (Harju *et al.* 2002). This may be partly due to down-regulation of γ GCS caused by TGF β ₁ (Arsalene *et al.* 1997). This was confirmed also in the *in vitro* part of our study. The immunoreactivities in UIP highlight the importance of oxidative stress in the pathogenesis of the disease, since ROS enhance the release of TGF β ₁ *in vitro* studies (Belloq *et al.* 1999). In addition, the decreased GSH levels in IPF may have direct consequences for fibroblasts, since low GSH levels in BALF of IPF patients may facilitate fibroblast proliferation (Cantin *et al.* 1990).

The studies of γ GCS regulation have focused on the heavy, catalytic subunit, which contains all the substrate binding sites, but the light, regulatory, subunit modulates the affinity of the heavy subunits for substrates and inhibitors, so we examined the regulation of both heavy and light subunits exposed to TGF β ₁ and TNF α (Huang *et al.* 1993, Morales *et al.* 1997, Griffith 1999, Kondo *et al.* 1999, Järvinen *et al.* 2000, Jardine *et al.* 2002). A549 cells were chosen since they are known to express moderate levels of γ GCS_h (Järvinen *et al.* 2000). TGF β ₁ decreased in the activity of catalytic γ GCS_h, which is in agreement with the study of Arsalene *et al.* (Arsalene *et al.* 1997). The activity of heavy subunit is modulated by γ GCS_l, and there was a minor decrease in the immunoreactivity

of light subunit by TGF β ₁, which has not been described previously. The reason for this is unclear, but Jardine *et al.* proposed a mechanism of $\alpha\gamma$ GCS_h gene down-regulation where a negative gene modulator was recruited into the active AP-1 complex in response to TGF β ₁ in epithelial cells (Jardine *et al.* 2002). The human γ GCS_h gene contains AP-1 binding sites, but also the γ GCS_l gene has AP-1 binding sites (Moinova & Mulchany 1998, Griffith 1999). According to our study, TNF produced only a modest increase in the immunoreactivities of both γ GCS subunits, this being in line with a previous study where TNF α produced a significant elevation of γ GCS_h and mRNA (Rahman *et al.* 1999). There are several reasons for the variability of intensities including cultural conditions, availability of GSH needed for cell activation and Haddad *et al.* documented that TNF α is a redox-sensitive cytokine itself (Post *et al.* 1983, Poot *et al.* 1995, Ray *et al.* 1999 Haddad *et al.* 2002). Another sequence of AP-1 might be a key factor also in the up-regulation of γ GCS subunits in TNF α treated cells (Rahman *et al.* 1999).

This study provides novel information about the cell specific expression of both subunits of γ GCS, which were simultaneously expressed in the alveolar macrophages as an adaptive response to oxidative stress in UIP and to a lesser degree in DIP and CVD-ILD, and in metaplastic epithelium induced by cell proliferation. The old fibrotic areas in UIP were mainly negative, possibly due to downregulation by TGF β ₁, which was confirmed also in the *in vitro* part of our study.

7 Conclusions

This study provides new information on the histopathology of the most common interstitial pneumonia, IPF/UIP, and the clinical course of the disease as well as data on the safety of histopathological verification by lung biopsy. The analysis of the expression of selected antioxidant enzymes related to thiol metabolism and maintenance of cellular redox state in ILDs is important, since they may play a central role in the progression of these diseases. The following conclusions can be drawn.

1. When non-invasive interventions cannot confirm the diagnosis of IPF, VATS is a safe method for diagnosing IPF/UIP, in the early and stable stages of the disease.
2. In IPF/UIP counting the number of FF represents a repeatable and reliable method for predicting the survival, but the number of FF cannot predict the course of the disease, including acute exacerbation before death of IPF/UIP.
3. Trx and TrxR are important thiol containing redox regulated proteins that are expressed in bronchial and alveolar epithelium and macrophages, emphasizing their role in the antioxidant defence of the human lung. The elevated expression of Trx and TrxR in the metaplastic epithelium and the absence of both enzymes in fibrotic areas point to the possibility of Trx and TrxR serving as markers of ongoing cell regeneration.
4. The expression of γ GCS, the rate-limiting enzyme in GSH synthesis in metaplastic alveolar epithelium and macrophages emphasizes its role in antioxidant defence in ILDs. Both subunits of γ GCS were absent in the fibrotic areas of the lung, probably due to downregulation by TGF. The overall decline of this major defensive enzyme in human lung may further contribute to the progression of these diseases, by enhancing oxidant injury.

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Original publications

This thesis is based on the following publications, which are referred to in the text by their Roman numerals:

- I Tiitto L, Heiskanen U, Bloigu R, Pääkkö P, Kinnula V & Kaarteenaho-Wiik R (2005) Thoracoscopic lung biopsy is a safe procedure in diagnosing usual interstitial pneumonia. *Chest* 128: 2375–2380.
- II Tiitto L, Bloigu R, Heiskanen U, Pääkkö P, Kinnula V & Kaarteenaho-Wiik R (2006) Fibroblast foci, diffuse alveolar damage and exacerbation of idiopathic pulmonary fibrosis / usual interstitial pneumonia. *Thorax* 12: Epub ahead of print.
- III Tiitto L, Kaarteenaho-Wiik R, Sormunen R, Holmgren A, Pääkkö P, Soini Y & Kinnula V (2003) Expression of the thioredoxin system in interstitial lung disease. *Journal of Pathology* 201: 363–370.
- IV Tiitto L, Peltoniemi M, Kaarteenaho-Wiik R, Soini Y, Pääkkö P, Sormunen R & Kinnula V (2004) Cell specific regulation of gammaglutamylcysteine synthetase in human interstitial lung diseases. *Human Pathology* 35: 832–839.

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