Polymorphisms in interstitial lung diseases

Friend or foe?

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Abbreviations

ACCESS	A Case Control Etiologic Study of Sarcoidosis
ADR	adverse drug reaction
Ag	antigen
ANCA	anti-neutrophil cytoplasmic antibody
ANF	antinuclear factor
APC	antigen presenting cell
BAL	bronchoalveolar lavage
BALF	bronchoalvealar lavage fluid
BHL	bilateral hilar lymphadenopathy
BMI	body mass index
BS	buccal swab
BTNL2	butyrophiline-like 2
CARD15	caspase recruitment domain 15
CI	confidence interval
CNS	central nervous system
COX2	cyclooxegenase-2
CR1	complement receptor 1
CT	computed tomography
CV	variation of confidence
CXR	chest X-ray
CYP	cytochrome P450
CYP1A2	cytochrome P450 1A2
CYP2C9	cytochrome P450 2C9
CYP2C19	cytochrome P450 2C19
CYP2D6	cytochrome P450 2D6
CYP3A4	cytochrome P450 3A4
CYP3A5	cytochrome P450 3A5
DAH	diffuse alveolar hemorrhage
DBS	dried blood spot
DI-ILD	drug-induced interstitial lung disease
DLCO	diffusing capacity for carbon monoxide
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
EM	extensive metabolizer
FEV_1	forced expiratory volume in one second
FRET	fluorescence resonance energy transfer
FVC	forced vital capacity
Hb	hemoglobin
HLA	human leukocyte antigen
HRCT	high resolution computed tomography

Ht	hematocrit
HT	heterozygote
IFN-γ	interferon-gamma
IL	interleukin
ILD	interstitial lung disease
IM	intermediate metabolizer
INR	international normalized ratio
IPF	idiopathic pulmonary fibrosis
LD	lactate dehydrogenase
LTA	lymphotoxin alpha
MHC	major histocompatibility complex
MT	mutation
MTHFR	methylenetetrahydrofolate reductase
MTX	methotrexate
MUMC	Maastricht University Medical Centre
NE	norepinephrine
NSIP	non-specific interstitial pneumonia
NVD	N-desmethylvenlafaxine
OR	odds ratio
OVD	O-desmethylvenlafaxine
PCR	polymerase chain reaction
PM	poor metabolizer
RA	rheumatoid arthritis
RFLP	restriction fragment length polymorphism
SD	standard deviation
SNP	single nucleotide polymorphism
SNRI	serotonin-norepinephrine reuptake inhibitor
TDM	therapeutic drug monitoring
TGF-β	transforming growth factor beta
Th1	T-helper cell 1
TLR2	Toll-like receptor 2
TNF-α	tumor necrosis factor alpha
TPMT	thiopurine methyltransferase
UEM	ultra extensive metabolizer
US	United States
UV	ultra violet
VEGF	vascular endothelial growth factor
VKORC1	vitamin K epoxide reductase complex 1
WASOG	World Association of Sarcoidosis and Other Granulomatous diseases
WT	wild type

Chapter 1

General introduction

Introduction

Interstitial lung diseases

Interstitial lung diseases (ILD) are a rapidly growing and increasingly complex component of clinical practice. They represent a group of heterogeneous disorders that diffusely involve the lung parenchyma. The term "interstitial" was originally applied to these disorders because they are associated with thickening of the alveolar septum. The "interstitium" is that part of the alveolar structures bounded by the alveolar epithelial and endothelial basement membranes. The normal alveolar interstitium is composed of connective tissue components (e.g. collagen, elastic fibers, mesenchymal cells), and inflammatory and immune effector cells (monocytes/macrophages and lymphocytes). Generally, ILD involve alveolar epithelial and endothelial cells as well. In addition, although these diseases primarily attack the alveolar structures (inflammation and fibrosis), many also involve airways, arteries and veins.[1,2] ILD can lead to diffuse remodelling and architectural damage to normal lung tissue and progressive loss of lung function.

Although idiopathic pulmonary fibrosis (IPF) and sarcoidosis are the two most common forms of ILD encountered in clinical practice, well over 100 different types of ILD have been identified on the basis of clinical presentation, radiographic findings, and histopathologic examination.[3-5] The patient's age, cigarette-smoking status, and gender will provide useful clues. IPF for example is almost always an adult disorder, typically occurring in patients over 60 years of age. Patients with idiopathic non-specific interstitial pneumonia (NSIP) of the fibrotic variety are usually younger than 60 years of age. Although pulmonary sarcoidosis can manifest in the elderly patient, it is more common in young adults and middle-aged people. Respiratory bronchiolitis associated with ILD (RB-ILD) is seen almost exclusively in cigarette smokers, but it can occur in both men and women of all ages. In contrast, the very rare disorder Lymphangioleiomyomatosis (LAM) occurs exclusively in women of childbearing age.

A detailed occupational history and changes in domestic environment are also essential, as it may lead to identification of a specific inhalation cause or trigger for ILD. At-risk occupations for ILD include miners (pneumoconiosis); sandblasters and granite workers (silicosis); dental workers (dental workers' pneumoconiosis); welders, shipyard workers, pipe fitters, electricians, and mechanics (asbestosis); farm workers, poultry workers, bird fanciers, and bird breeders (hypersensitivity pneumonitis); and workers in aerospace, nuclear, computer, and electronics industries (berylliosis). History of existing, persistent and/or altered environmental 'fibrogenic' factors at home, the workplace, in automobiles or frequently visited facilities/homes or hobbies such as exposure

to birds, moulds, woodworking, use of sauna and hot tubs are often ignored but equally important and may provide a useful clue for specific diagnosis and management of hypersensitivity pneumonitis.[6]
An overview of the different diseases, also known collectively as ILD, is shown in Table 1.1.

Table 1.1 Interstitial Lung Diseases (ILD).

ILD of known causes	**Drug-Induced (DI-ILD)*** Asbestos Beryllium Radiation Fibrosis Silicosis Infection
Idiopathic interstitial pneumonia (IIP)	Idiopathic Pulmonary Fibrosis (IPF) non-IPF IIP: • Familial Pulmonary Fibrosis • Desquamative Interstitial Pneumonia (DIP) • Acute Interstitial Pneumonia (AIP) • Non-specific Interstitial Pneumonia (NSIP) • Cryptogenic Organizing Pneumonia (COP) • Lymphocytic Interstitial Pneumonia (LIP) • Respiratory Bronchiolitis (RB-ILD)
Granulomatous ILD	**Sarcoidosis** Histiocytosis-X Hypersensitivity Pneumonitis (HP)
Other	**Diffuse Alveolar Hemorrhage (DAH)** Connective Tissue Disease-associated ILD (CTD-ILD) Eosinophilic Pneumonia Lymphangioleiomyomatosis (LAM) Pulmonary Alveolar Proteinosis (PAP) Wegener's granulomatosis and other vasculitidies

* the ILD with bold typeface will be addressed in the coming chapters.

Drug-induced interstitial lung disease

Drug-induced interstitial lung disease (DI-ILD) is the most common form of drug-induced respiratory disease. The drugs involved not only include prescribed and over-the-counter-drugs, but also illicit drugs, herbs, alcohol, and dietary ingredients.[7-10] An ever increasing number of drugs can produce or reproduce variegated patterns of naturally-occurring infiltrative lung disease.[7,9,11] Although, only in a limited number of cases drugs unequivocally have been identified as cause, it is important to acknowledge the potential role of medication in the development of drug-induced ILD.[7,12] This is due to the severity of the potentially irreversible damage to the lungs and the improvement that is often easily achieved by stopping administration of drugs.

Rational treatment of drug-toxicities in cases where the mechanism of toxicity is known is common clinical practice. However, often the connection with drug-use and the development of related inflammatory damage or idiosyncratic toxicities is hard to recognize and objectify, especially in those cases using multiple drugs.[13] Furthermore, the list of drugs involved has grown rapidly over the last decades. An example of a drug-induced ILD, in this case caused by nitrofurantoin, is shown in Figure 1.1. For a list of the most important categories of substances which can cause respiratory problems with some examples see Table 1.2, and also www.pneumotox.com for an extensive overview of drugs known to be able to damage the respiratory system.

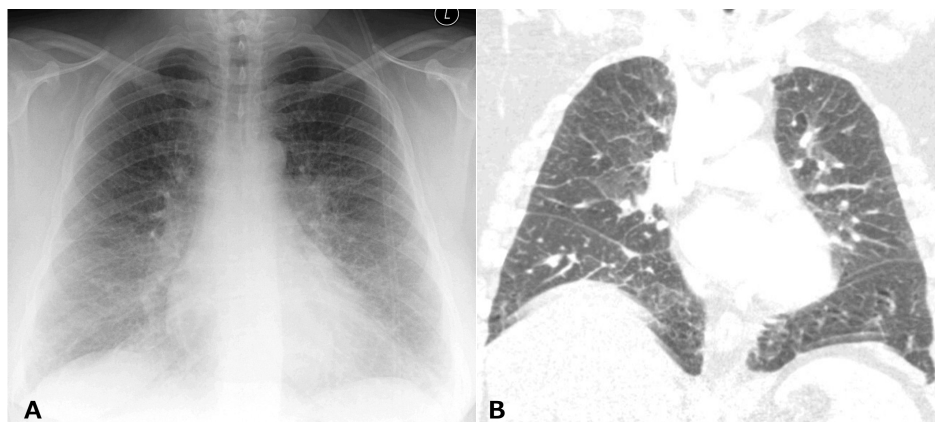

Figure 1.1 Chest X-ray (**A**) showing a diffuse interstitial pattern and a high resolution CT-scan (**B**) demonstrating thickened septa and patchy ground-glass opacities of a patient with drug-induced interstitial lung disease associated with nitrofurantoin.

Table 1.2 Summary of the categories of substances known to cause respiratory problems with some examples of the possible causative agents.

Drugs* (illicit)	Biomolecules	Blood/Stem-cells	Herbs/Dietary supplements	Miscellaneous
Amiodarone	Interferons	Retinoic acid	Ephedra	Talc
Cocaine	Immunoglobulins	Blood products	Comfrey	Silicone
Methotrexate	Anti-thymocyte globuline	Stem-cell transplantation	Germander	Mineral oil
Nitrofurantoin		Blood transfusion	Aristolochic acid	
Methadone				
Oral anticoagulants				

*see appendix for a complete list of drugs and other substances involved, sorted by CYP enzymes.

Diffuse alveolar hemorrhage

Diffuse alveolar hemorrhage (DAH) can be a fulminant and often fatal bleeding complication, but can also occur as an almost sub-clinical condition with a pneumonia-like manifestation. DAH results in accumulation of iron in the lungs and, in turn, iron causes oxidative stress and inflammation. It has been suggested that oxidative damage plays a role in the pathophysiology of various diseases.[14] It is important to prevent or recognize DAH at an early stage to avoid irreversible damage. Particularly, in critically ill patients with unexplained infiltrates, DAH should be considered. DAH events can occur as a result of over-anticoagulation due to coumarin sensitivity, resulting in a relative vitamin K deficiency. It can also occur as a result of the ingestion or inhalation of so-called super-warfarines, (illicit) drugs or toxic fumes.[15-18] An example of a lung with DAH is shown in Figure 1.2 and the histological aspect is illustrated in Figure 1.3A. At present, the diagnosis of DAH is often made by observing an increased percentage of siderophages (iron-positive macrophages, >20%) in bronchoalveolar lavage fluid (BALF), indicated with Perl's staining, as shown in Figure 1.3B.[19]

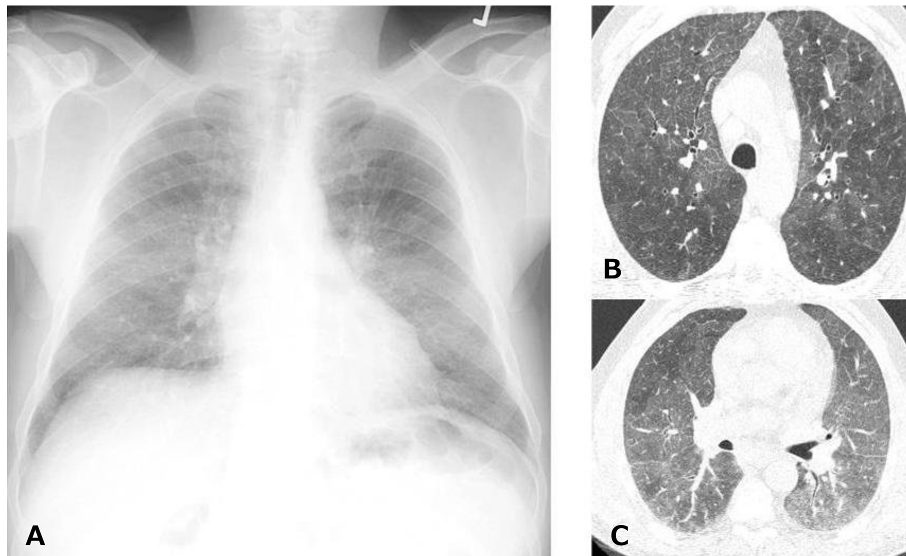

Figure 1.2 Chest X-ray (A) and high resolution CT-scan (B/C) of a patient suffering from diffuse alveolar hemorrhage (DAH) showing widespread ground-glass attenuation.

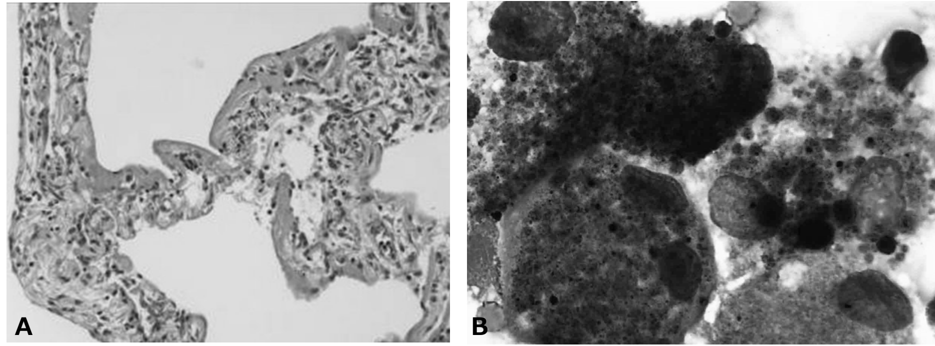

Figure 1.3 Histological appearance (**A**) of diffuse alveolar hemorrhage (DAH). Acute phase of DAH: alveolar walls with interstitial oedema, congested capillaries, and hyaline membranes with regenerating alveolar epithelium underneath. Perls' stained bronchoalveolar lavage (BAL) fluid cells (**B**; dark grey = positive).

Sarcoidosis

Sarcoidosis is a systemic granulomatous disorder of unknown cause, characterized by the presence of noncaseating granuloma, see Figure 1.4 for a schematic presentation of granuloma formation.[20,21]
Sarcoidosis occurs throughout the world, affecting all genders, races and ages. However, several studies demonstrated a peak incidence of the disease occurring in individuals ranging between 25–55 years of age, with a greater presence of men in the under 40-years of age category.[22,23] Epidemiologic studies regarding the incidence, prevalence, and mortality rates of sarcoidosis are difficult because of the highly variable disease presentation[24] and the lack of a known etiology. Regional variabilities due to race and gender, as well as phenotypic variability, further confound epidemiological measurements. Involvement of the lungs or intrathoracic lymph nodes is clinically evident in 90% of the symptomatic patients during their disease and up to 30% show spontaneous remission.[25] In 10-30% of the patients the disease becomes chronic, what can result in significant lung function impairment.[26,27] The diagnosis of sarcoidosis is often one of exclusion of other diseases and involves biopsies and chest X-rays (CXR) or high resolution computed tomography (HRCT) scans. Five stages of the radiographical abnormality were recognized: stage 0 (normal CXR), stage I [bilateral hilar lymphadenopathy (BHL)], stage II (BHL and parenchymal abnormalities), stage III (parenchymal abnormalities without BHL), and stage IV (end stage lung fibrosis), see also Figure 1.5.[28] An acute form of sarcoidosis is the so-called Löfgren's syndrome with distinct skin (Erythema nodosum) and lung (CXR stage I) presentation.

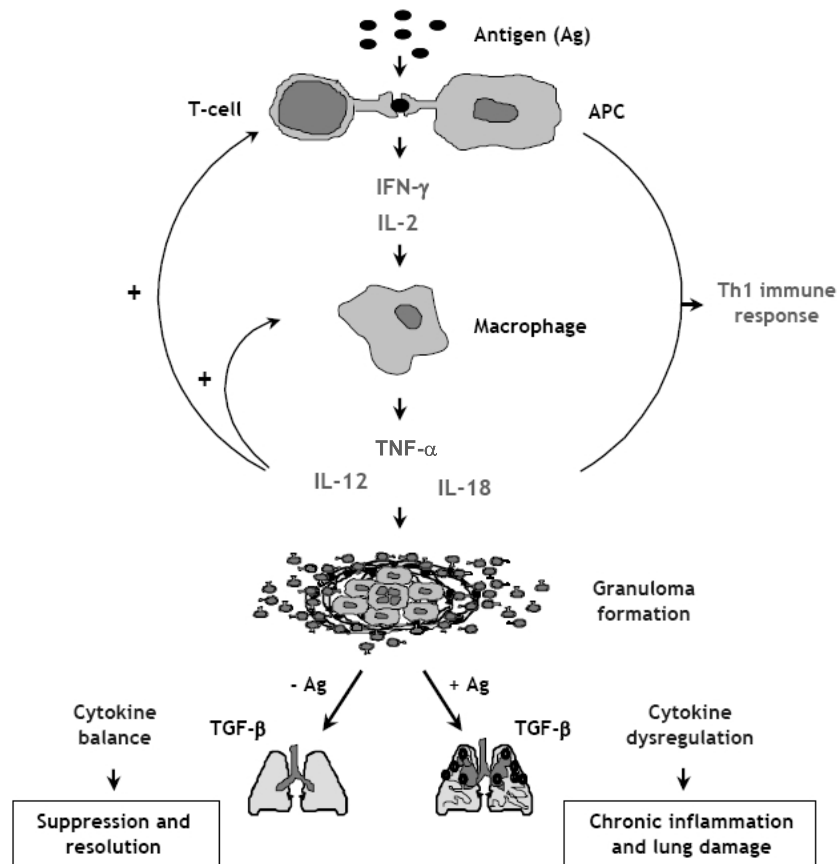

Figure 1.4 A schematic presentation of granuloma formation in sarcoidosis. Ag= antigen; APC= antigen presenting cell; IFN-γ= interferon-gamma; IL= interleukin; Th1= T-helper 1; TNF-α= tumor necrosis factor alpha; TGF-β= transforming growth factor beta (adapted from Moller[29]).
Hypothetical model of the pathogenesis of sarcoidosis. An antigen induces antigen-specific, Th1-mediated granulomatous inflammation with production of Th1 cytokines (IFN-γ, IL-2). Granuloma formation is set in motion by activated macrophages and T-cells along with other effector cells (e.g. fibroblasts) under the regulatory influence of local cytokine production. Removal of the antigen allows TGF-β to downregulate the immune response.

Although the specific trigger of sarcoidosis is still unknown, there appear to be some pre-requisites to develop the condition. The first being predisposition, the second is exposure and the third circumstances. The (genetic) predisposition appears to be not dependent upon a single gene and/or polymorphism, for in

several studies a range of different polymorphisms have been investigated and found to have an influence on the contracting or progression of the disease.[30-36] In Table 1.3 an overview is presented of some of the most common polymorphisms and triggers that have been associated with sarcoidosis, but up until now no single agent or trigger stands out as cause.

Table 1.3 An overview of some of the polymorphisms and triggers that have been associated with sarcoidosis.

HLA	TNF	various	triggers/jobs/exposure
DRB1*03	*TNF-α* G-308A	*BTNL2* G16043A	health care workers
DRB1*1501	*TNF-α* G-238A	*BTNL2* G16071A	firefighters/fumes
DQB1*0602	*TNF-α* C-857T	*CARD15* C2104T	teachers/chalk
		CARD15 A1761G	insecticides/pesticides
		CR1 C5507G	molds/(agricultural) dust/clay
		VEGF C813T	manmade fibers
			bacteria
			metals (e.g. aluminum, zirconium)
			talc

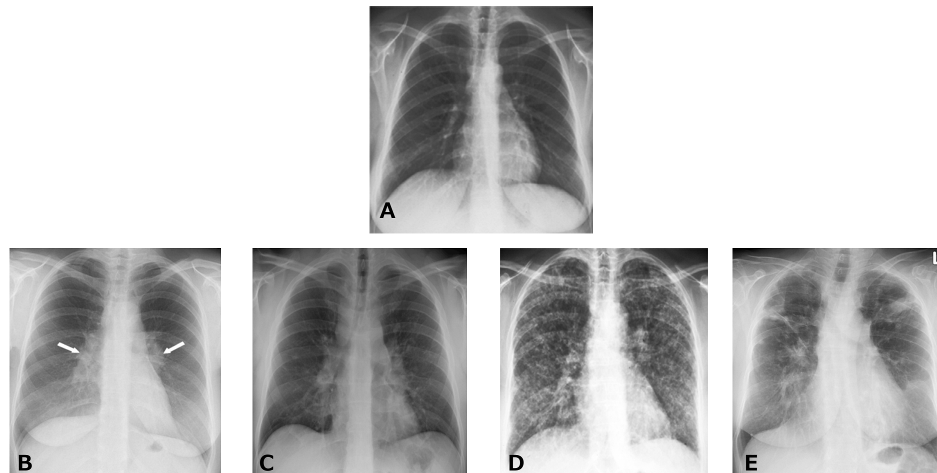

Figure 1.5 Chest X-ray (CXR) stages 0-IV: radiographic appearances of sarcoidosis. (**A**) Stage 0 (5-15%): normal chest radiograph; (**B**) Stage I (45-65%): bilateral hilar lymphadenopathy (arrows), without evidence of interstitial lung disease; (**C**) Stage II (30-40%): both lymphadenopathy and parenchymal abnormalities (nodular and reticulonodular opacities); (**D**) Stage III (10-15%): parenchymal infiltration without hilar lymph node enlargement (reticulonodular infiltrates); (**E**) Stage IV (15-25%): obvious interstitial abnormalities with fibrotic lesions.

Polymorphisms

The human DNA comes with faults (mutations) that can influence the function of a protein or enzyme. Mutations are random mistakes and they are called polymorphisms whenever the DNA mutation occurs in more than one percent of the population. A polymorphism can affect the function of a protein in a positive or negative manner, depending on where it is positioned or what its effect on the length or amino acid build-up of the final protein is. For example, a polymorphism that causes a so-called stop codon in the coding region of the protein will produce a truncated protein that almost always has a severely altered function or no functionality left at all. Whereas a polymorphism in the promoter region of the protein, for instance, can cause a heightened rate of production of the protein involved.

Polymorphisms and, more specific single nucleotide polymorphisms (SNP), can be investigated using different PCR methods or detection techniques (e.g. restriction fragment length polymorphism (RFLP) using restriction enzymes, or real-time PCR assays using fluorescence resonance energy transfer (FRET) or hydrolysis probes). In the studies presented in this thesis SNPs were detected using melting point analysis with fluorescent hybridization probes using the FRET principle. This detection method uses the properties of a donor fluorophore that transfers its energy to an acceptor that has to be in close proximity (no more than three base pairs apart) in order to accept the energy and emit at a different wavelength when returning to its ground state. The FRET principle is visually explained in Figure 1.6 and an example of the melting curve results obtained when running a FRET assay on a LightCycler® are shown in Figure 1.7. In the upper part of Figure 1.7 the lessening of the fluorescence signal as the temperature of the samples was raised is clearly visible. This rise in temperature (the so-called melting) will cause the probes to be melted off their complementary piece of DNA. Moreover, because the distance of the two fluorophores in that situation will become more than the required maximum of three base pairs, FRET is no longer possible and a reduction of signal is apparent (Figure 1.6). As the probes that spans the SNP is complementary, either to the wild type DNA or to the SNP one wishes to examine, more heat is needed to melt it off when the fit is perfect. So when both alleles (heterozygote) are present, one part of the probes will melt off at a lower temperature than the other. When applying the first derivative to these melting curves, the bottom part of Figure 1.7 is produced and the different melting temperatures will be more clearly visible as separate peaks. In the shown example the used probe spanning the SNP was complementary to the wild type, therefore producing a peak for the mutation at a lower temperature.

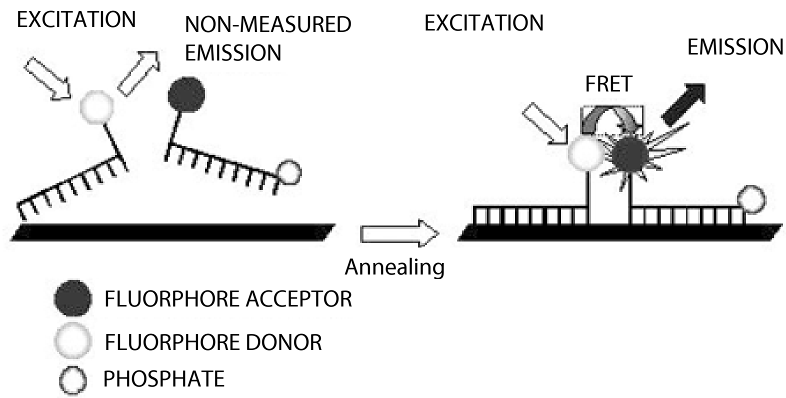

Figure 1.6 Principle of fluorescence resonance energy transfer (FRET) assays.

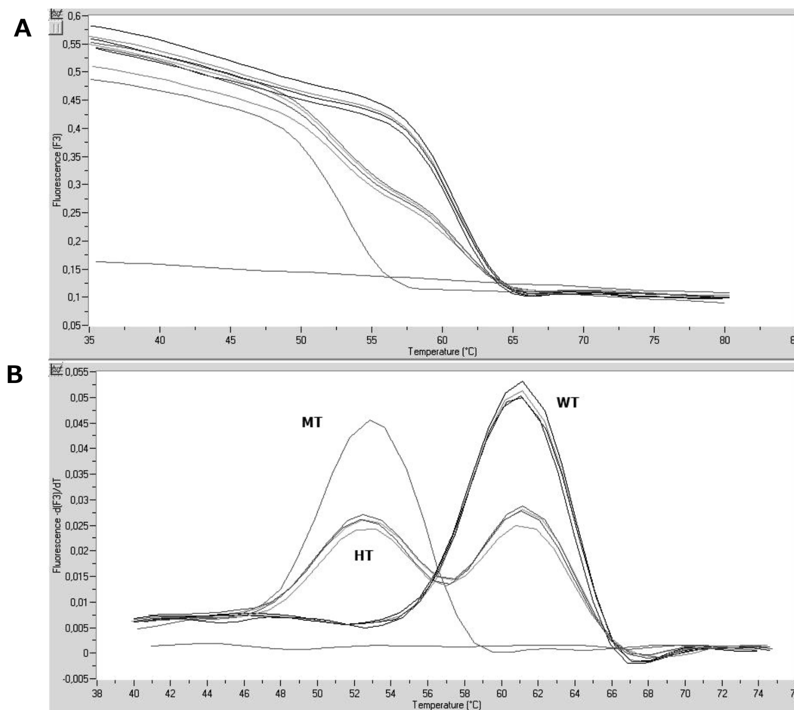

Figure 1.7 Results of a cytochrome 2C19 assay, with melting curves (**A**) and the first derivative (**B**). MT = mutation (*CYP2C19**2/*2), HT = heterozygote (*CYP2C19**1/*2), WT = wildtype (*CYP2C19**1/*1).

Scope and aims of the study

The aims of the studies presented in this thesis were to investigate several polymorphisms and assess their possible role in the cause and course of interstitial lung disease (ILD). ILD, especially drug-induced ILD (DI-ILD), can occur as a cause of drug(s) or drug-drug interactions. The CYP enzyme family plays an important role in the metabolization of all sorts of ingested or inhaled xenobiotic substances. In **Chapter 2** the possible role of cytochrome P450 (CYP) enzymes in DI-ILD is reviewed. **Chapter 3** describes a simple and uncomplicated method to isolate DNA from easy obtainable and patient friendly dried blood spot and/or buccal swab samples prior to real-time polymerase chain reactions (PCR). The aim of **Chapter 4** was to establish whether allelic variation in specific *CYP* polymorphic genes, namely *CYP2D6*, *CYP2C9*, and *CYP2C19*, contributes to variability in drug response and unexpected toxicity. Therefore, a case-control study was conducted. The cases consisted of patients with DI-ILD. Two control groups were used: one group of healthy volunteers and one group of patients with idiopathic pulmonary fibrosis (IPF). In **Chapter 5** the role of *CYP* polymorphisms is further illustrated in the case-report describing the therapeutic failure of venlafaxine in a case lacking CYP2D6 activity. In **Chapter 6** it was hypothesized that in patients treated with coumarins a serious complication i.e. diffuse alveolar hemorrhage (DAH) may be associated with vitamin K epoxide reductase complex 1 (*VKORC1*) and *CYP* (*CYP2C9* and *CYP2C19*) variant alleles. Clinical information of patients using coumarins with at least one episode of DAH was gathered retrospectively during a seven year period. The aim of **Chapter 7** was to evaluate the relationship between the presence of tumor necrosis factor (*TNF*) polymorphisms, human leukocyte antigen (HLA)-DRB1*03 linkage and the prognosis of sarcoidosis. In a retrospective case-control study *TNF-α* G-308A, *TNF-α* G-238A and *LTA* were genotyped in 625 sarcoidosis patients. These patients were classified into patients with persistent disease and patients with non-persistent disease using chest X-ray appearances and lung function parameters after at least two years of follow-up. The aim of **Chapter 8** was to assess the association of BTNL2 G16071A with the course of pulmonary sarcoidosis and verify association with disease predisposition. In addition, the linkage between BTNL2 G16071A and certain HLA-DRB1/DQB1 types was investigated. In a retrospective case-control study BTNL2 G16071A, HLA-DQB1 and DRB1 were typed in 632 sarcoidosis patients. These patients were classified into 304 patients with persistent and 328 patients with non-persistent sarcoidosis using chest X-ray stages after at least two years follow-up. Finally, in **Chapter 9**, a summary and the implications of the findings presented in this thesis are argued and directions for future research are briefly discussed.

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Chapter 2

Relationship between drug-induced interstitial lung diseases and CYP polymorphisms

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Abstract

Background
Interstitial lung disease and especially drug-induced interstitial lung disease can occur as a cause of drug(s) or drug-drug interactions. In this review we summarize the possible role of cytochrome P450 (CYP) enzymes in drug-induced interstitial lung disease.

Recent findings
The CYP enzyme family plays an important role in the metabolization of all sorts of ingested, injected or inhaled xenobiotic substances. Although the liver is considered to be the major metabolization site of CYP enzymes, in recent years more CYP isoforms have been detected in lung tissue. Polymorphisms in these *CYP* genes can influence the metabolic activity of the subsequent enzymes, which in turn may lead to localized (toxic) reactions and tissue damage.

Summary
Drug toxicity can be the consequence of no or very poor enzyme activity, especially if no other metabolic route is available. In case of reduced enzyme activity, dose reduction or prescribing an alternative drug metabolized by a different, unaffected CYP enzyme is recommended to prevent toxic side effects. Therefore, knowing a patient's CYP profile before drug prescription could be a way to prevent drug-induced interstitial lung disease. Moreover, it might be helpful in explaining serious adverse effects from inhaled, injected or ingested xenobiotic substances.

Introduction

The lungs are a target for a variety of xenobiotic and possible toxic substances, because of their large contact surface with both the outside world and circulating blood. Air can deliver (mineral) particles or (toxic) fumes and blood is the main supplier of most drugs, independent of the way of administration. Moreover, they form not only an important first point of contact or barrier, but also can act as a metabolization site for certain substances. Drugs can induce specific respiratory reactions, or the lungs may be affected as part of a generalized response. The most common form of drug-induced respiratory disease is drug-induced interstitial lung disease (DI-ILD). The drugs involved not only include prescribed and over-the-counter-drugs, but also illicit drugs, herbs, alcohol, and dietary ingredients.[1-4] An ever increasing number of drugs can produce or reproduce variegated patterns of naturally occurring infiltrative lung disease, including most forms of interstitial pneumonias, alveolar involvement, and, rarely, vasculitis.[1,3,5] Although, only in a limited number of cases drugs unequivocally have been identified as cause, it is important to acknowledge the potential role of medication in the development of DI-ILD.[1,6] This is due to the severity of the potentially irreversible damage to the lungs and the improvement that is often easily achieved by stopping administration of drugs. Rational treatment of drug-toxicities in cases where the mechanism of toxicity is known is common clinical practice. However, often the connection with drug-use and the development of related inflammatory damage or idiosyncratic toxicities is hard to recognize and objectify, especially in those cases using multiple drugs.[7]
The aim of this review is to discuss drug-induced respiratory reactions and the possible mechanisms involved, focussing on the role of cytochrome P450 (*CYP*) polymorphisms.

Drugs and lungs

The diagnosis of DI-ILD primarily rests on the temporal association of exposure to drug(s), and the development of respiratory symptoms. It is also clinically challenging, especially when trying to find predictors for the possibility that an individual is at risk for developing such a reaction, and moreover, in avoiding re-challenge with the trigger. For an overview of drugs known to be able to damage the respiratory system see www.pneumotox.com. In some cases the evidence that reactions in the lungs are drug-induced is circumstantial.[1] A straightforward interpretation is hampered when the damage is irreversible or when the symptoms aggravate after stopping drug administration. Thus, the

diagnosis of DI-ILD is mainly one of exclusion and requires the meticulous ruling out of all other possible causes.

The variability in drug response among patients is multi-factorial, including extrinsic factors like environmental aspects and also genetic and intrinsic factors that affect the disposition (absorption, distribution, metabolism and excretion) of a certain drug (Table 2.1). The existence of large population differences with small intra-individual variability is consistent with inheritance as determinant of drug response; it is estimated that genetics can account for 20-95% of variability in drug disposition and effects, see also Figure 2.1.[8]

Table 2.1 Factors influencing drug metabolism.

Extrinsic factors	Environment	Smoking Diet Alcohol Inhalation of (toxic) fumes
	Drug use	Prescribed drugs Illicit drugs Over the counter drugs Herbal supplements Concomitant drugs
Intrinsic factors	Demographic	Gender Age Race
	Disease	Disturbed kidney excretion function Diminished liver blood perfusion Changed metabolic function
Genetic factors	Polymorphisms in genes encoding for metabolic enzymes	

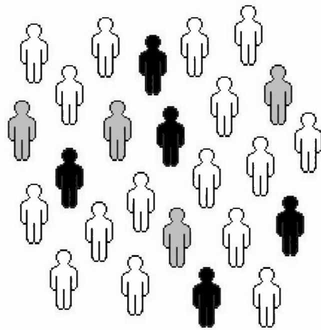

Figure 2.1 Drug response in a population.
Patients with the same diagnosis and treated with the same medication. Most patients react well (white), some of the patients do not respond to the therapy (grey) and some react with adverse reactions or toxicity (black).

Two metabolization routes (phase I and phase II reactions) are responsible for the transformation of the majority of xenobiotic substances, with the purpose of facilitating elimination from the body. Phase I reactions, performed mainly by CYP enzymes, involve hydroxylation, reduction and oxidation while in phase II reactions glucuronidation, sulfation, acetylation or methylation take place. The risk of developing DI-ILD and clinical patterns vary, depending on a variety of host and drug factors. Several different xenobiotic-metabolizing CYP and phase II enzymes are present in the human lung, possibly contributing to in situ activation. Metabolism also affects the biological activity of the drug. Mostly, the biological activity of the parent drug is superior to that of the metabolite, but there are several exceptions. Sometimes CYP metabolism yields very toxic metabolites, e.g. acetaminophen, benzo[a]pyrene or carbamazepine.[9] Occasionally, drugs may cause the formation of reactive oxygen species by uncoupling of the electron transport of the CYP system. These metabolites or reactive oxygen species may damage vital cellular components, such as proteins, lipids or DNA.

CYP enzymes

The most common DNA variations in the human genome are called single nucleotide polymorphisms (SNPs). The presence of certain SNPs can result in a less functional enzyme and a subsequently changed, i.e. slower, metabolization for certain drugs. SNPs in the *CYP* genes are one of the key factors known to cause variation in drug response between individuals.[2,3,7-11] In recent years more CYP enzymes were detected within lung tissue. Local metabolization, or rather the lack of metabolization in some cases, might explain adverse reactions and subsequent tissue damage in the lungs.[2,10,12-14]
With the widespread possibility to determine the genetic profile of the CYP enzymes, their metabolic capacity can be determined. Patients can be divided into ultra-extensive (UM), extensive (EM), intermediate (IM) or poor metabolizers (PM).[15]
The most important CYP enzymes for drug metabolism are CYP1A2, CYP2C9, CYP2C19, CYP2D6, CYP3A4, and CYP3A5. This review will focus on clinical relevance and prevalence of *CYP* polymorphisms that are also important in relation to the lungs (Table 2.2).
The completion of the sequence of the human genome revealed the presence of 115 human *CYP* genes: 57 active and 58 pseudo-genes.[16] The CYP enzymes are a superfamily of hemoproteins found in a wide range of different organs and tissues.[9,17-21]

Table 2.2 CYP distribution and metabolic activity in the liver and presence in lung tissue.

CYP	% Distribution	% Metabolic activity	Presence in the lung
3A	30	55	+++
2C	20	10	++
1A2	13	2	+
2E1	7	1.5	+++
2A6	4	1.5	+++
2D6	2	30	++

CYP proteins are conveniently arranged into families and subfamilies on the basis of percentage amino acid sequence identity.[22] Figure 2.2 illustrates an example of CYP enzyme nomenclature, see also www.cypalleles.ki.se for an overview of human CYP allele nomenclature. The CYP isoenzymes in families 1-3 are responsible for 70-80% of all phase I-dependent metabolism of clinically used drugs and also participate in the metabolism of a large number of xenobiotic substances.[23-25]

Substances (medicines or other compounds) that are metabolized by CYP enzymes are called substrates. Inhibitors can reduce the action of a cytochrome, while so-called inducers can enhance the metabolism of a specific CYP enzyme.

The metabolic capacity of the different CYP enzymes is also defined as low affinity/high capacity or high affinity/low capacity. The CYP2D6 enzyme is, like CYP2C9 and CYP2C19, defined as a high affinity/low capacity, which implies that these CYP enzymes prefer to metabolize specific substrates at a low concentration. As the concentration of a medicine increases, the metabolism can possibly spill over to CYP3A4 and CYP1A2, which are low affinity/high capacity enzymes.

Several CYP enzymes, important for metabolism in the liver, but also in the lungs, will be discussed in detail.

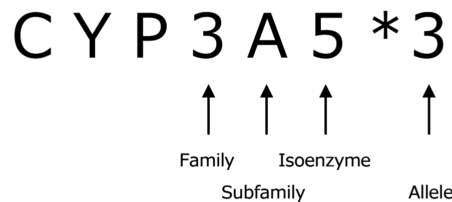

Figure 2.2 An example of the nomenclature of CYP enzymes.
The fully functional allele is indicated with *1, whereas the variant alleles are indicated with higher allele numbers and result in an aberrant functioning enzyme.

CYP1A2

Until recently, CYP1A2 was assumed to be present exclusively in the liver, but with more sensitive techniques available it has been detected in other tissues, including the lungs.[9,12,13] Sixteen variant alleles, not counting the 20 isotypes of the *1 allele, have been identified to date. The metabolic activity of CYP1A2 consists primarily of hydroxylating and demethylating compounds through oxidative metabolism. Substrates for CYP1A2 metabolism are, e.g. caffeine[26], theophylline, and naproxen.[27] Used in combination with an inhibitor, grapefruit juice for example, serum levels of substrates may increase, with toxicity and adverse drug reactions (ADRs) as a possible result.[28] Induction of the CYP1A2 metabolism can be achieved by cruciferous vegetables such as Brussels sprouts, broccoli or cabbage, and charbroiled foods (burned meats). Another important inducer of CYP1A2 is tobacco smoke.[29]
CYP1A2 is a low affinity/high capacity enzyme in contrast to CYP2D6, CYP2C9, and CYP2C19 in the metabolism of many drugs.[30] Gender differences have been found in the Chinese population, with men having more CYP1A2 activity compared with women.[31]

CYP2C9

Of the CYP2C family, CYP2C9 is considered to be the most important isoform, being the largest contributor and responsible for the metabolic clearance of up to 15% of all drugs (among which a host of clinically important drugs such as NSAIDs, oral anticoagulants, and angiotensin II blockers) undergoing phase I metabolism.[32] Most of the CYP2C9 activity in terms of drug metabolism takes place in the liver, but it is found in various other tissues.[33] To date, 34 variant alleles of the CYP2C9 enzymes have been identified and Lee et al.[34] determined that two of these *CYP2C9* variant alleles, *2 (430T) and *3 (1075C), were found in 35% of the Caucasians. These *CYP2C9* variant alleles are present much less frequently in African-Americans and Asians (about 2% and 5%, respectively).[35] Patients with *CYP2C9**2 or *CYP2C9**3 variant alleles require lower doses of drugs metabolized by CYP2C9, because of the reduced activity of these common variants.[36]
The only clearly recognized inducer of CYP2C9 is rifampicin. Moreover, in the case of concomitant rifampicin use, serum levels of drugs metabolized by CYP2C9 have been shown to reduce, by induction. In the case of e.g. warfarin use, this can lead to not enough anticoagulation and in turn could cause thrombotic events.[37]

CYP2C19

CYP2C19 is found in many tissues, but predominantly in the liver were it accounts together with CYP2C9, for approximately 20% of the total CYP activity. Until recently, 26 different variant alleles for CYP2C19 were identified.[38] The prevalence of CYP2C19 enzyme polymorphisms differs significantly between ethnic groups. For example, the PM phenotype occurs in 2-6% of Caucasians, 10-20% of Africans, and in 15-30% of Asians.[39,40] Variant alleles of *CYP2C19* lead to reduced or no enzyme function. Determining the metabolizer phenotype may also help in the case of treatment with drugs that rely on CYP2C19. Rifampicin has been identified as an inducer of both CYP2C19 and CYP2C9.[41] Other drugs (anti-convulsants and steroids) that typically induce other CYP enzymes may also induce CYP2C19, but to a lesser extent than CYP2C9 and CYP3A4.[42]

CYP2D6

Although CYP2D6 represents only 1-2% of the liver CYP isoenzymes by weight, it takes care of some 30% of its metabolic activity (Table 2.2).[43,44] Next to its important role in the liver's drug metabolism, CYP2D6 is also found in many other tissues, including the lungs.[45,46] For many drugs, especially psychotropic drugs, CYP2D6 is considered a high affinity/low capacity enzyme, which implies that CYP2D6 will preferentially metabolize drugs at lower concentrations.[47]
Until recently, 75 different variant alleles for *CYP2D6* were identified. There are ethnic differences in the distribution of EMs, PMs, and UMs. PMs are present in approximately 5-14% of Caucasians.[48,49] Bradford[50] indicated that Asians, Pacific Islanders, Africans and African Americans have a higher percentage of reduced-function or non-functional CYP2D6 (40-50%) than do Caucasians (26%). UMs carry a duplication of a fully functional *CYP2D6* allele which results in higher CYP2D6 enzyme levels. Due to these higher CYP2D6 enzyme levels, UMs require a higher daily dose to obtain a therapeutic drug blood level. UMs are generally rare representing 1-3% of the Caucasian population.[51]
CYP2D6 is the main metabolic enzyme for a whole range of (psychotropic) drugs and the presence of a less functional enzyme can have serious treatment consequences or lead to severe ADRs.[52,53]

CASE: A 43-year-old female presented with a non-productive cough and dyspnoea. The chest X-ray (CXR) and high resolution CT-scan (Figure 2.3A) showed coarse reticular opacities indicative of ILD. She used metoprolol for her hypertension, flecainide as an antiarrhythmic, and fenfluramide for obesity. Bronchoalveolar lavage fluid (BALF) showed an increased number of cells, predominantly lymphocytes, and the presence of plasma cells and foamy alveolar macrophages (Figure 2.3B), indicative of hypersensitivity pneumonitis or DI-ILD. Lung biopsy specimens demonstrated non-specific interstitial pneumonia (NSIP) of the cellular type. The patient's clinical condition deteriorated and artificial respiration was required for 6 weeks. She was treated with prednisone and fenfluramine was stopped. Hereafter, the clinical condition improved spectacularly and the CXR abnormalities resolved completely. Four years later, the patient's initial complaints returned. Again, the CXR showed the earlier reported reticular opacities and BALF analysis revealed the aforementioned signs of DI-ILD. To date, dexfenfluramine was started six weeks prior to this admission. The first clinical deterioration was not recognized as DI-ILD related to fenfluramine. The second deterioration appeared after starting dexfenfluramine as adjuvant therapy for her obesity. At that time, a role for (dex)fenfluramine was assumed in the development of pneumonitis. In addition genotyping revealed a *CYP2D6**1/*3 heterozygote variant. She used metoprolol and flecainide on a regular base. When another drug metabolized by the CYP2D6 system was added she deteriorated. Therefore, the use of various drugs metabolized by the same affected enzyme should be avoided, for this may result in significant accumulation of these drug(s), leading to toxic serum levels and severe side effects. Avoiding those drugs or dose reduction appeared to be beneficial in this patient and protected her from similar adverse effects until now.

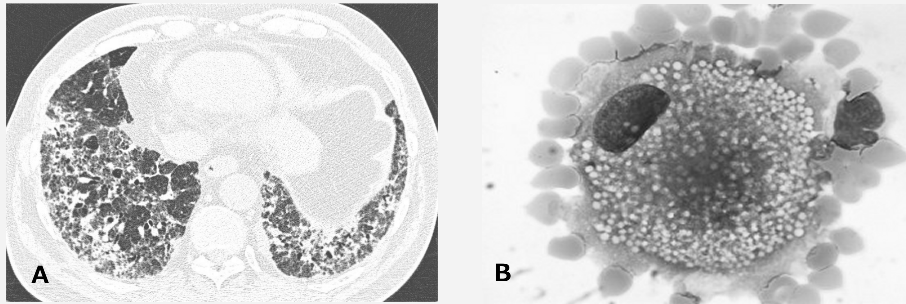

Figure 2.3 (**A**) High resolution CT-scan; (**B**) Foamy alveolar macrophage.

Table 2.2 CYP distribution and metabolic activity in the liver and presence in lung tissue.

CYP	% Distribution	% Metabolic activity	Presence in the lung
3A	30	55	+++
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2E1	7	1.5	+++
2A6	4	1.5	+++
2D6	2	30	++

CYP3A4

To date, 20 *CYP3A4* allelic variants have been identified. CYP3A4 is considered to be one of the most contributing CYP enzymes in all phase I drug metabolizations. In the liver the CYP3A enzymes make up about 30% of all CYPs present, but take care of about 55% of the metabolism. CYP3A4, together with CYP3A5, are considered to be the major forms present. Whenever the concentration of a substrate increases beyond the capacity of the main metabolizing CYP enzyme, the metabolism can spill over to CYP3A4, which is a low affinity/high capacity enzyme.
The most common variant in the 5'-untranslated region is *CYP3A4**1B, which is strongly associated with increased CYP3A5 expression in a study by Wojnowski et al.[54] and also associated with enhanced CYP3A4 expression.[55] Allelic frequencies range from 0% in Asians to 4-10% in Caucasians and 48-80% in African-Americans.[56] Inducers of CYP3A4 are e.g. rifampicine, carbamazepine, phenobarbital, and St. John's Wort. Grapefruit juice, amiodarone and verapramil are some of the possible inhibitors of CYP3A4.[4]

CYP3A5

CYP3A5, together with CYP3A4, is one of the most contributing CYP enzymes, but not so much in the Caucasian population. For the homozygous *CYP3A5**3/*3 variant, which is a less functional enzyme, is the more prevalent genotype in the Caucasian population. Moreover, in Caucasians the frequency of the *CYP3A5**3 allele is about 90-95% and of the functional *CYP3A5**1 allele only about 10%.[57,58] In Asians the *CYP3A5**3 allelic frequency is about 75% and about 35% in African-Americans.[58] In all CYPs the term 'wild type' stands for the most prevalent and fully functional (*1/*1) enzyme, CYP3A5 being the exception to the rule. As a consequence, an individual who possesses one or two *CYP3A5**1 alleles, needs a higher (two to threefold) maintenance dose of medicines metabolized by CYP3A5, such as i.e. the immune modulator tacrolimus.[59]
Until recently, 11 different *CYP3A5* allelic variants have been found. Furthermore, contrary to the situation in the liver, in the lungs CYP3A5 is the main CYP3A form expressed.[20]

Discussion

Many prescribed drugs are effective in only 25-60% of the patients (Figure 2.1).[60] Therefore, it is also important to determine co-factors in drug metabolism, as depicted in Table 2.1. A disadvantage of drug development is the fact that most drugs are tested in a standardized population, which rules

out severe toxicity and will not always predict drug interaction(s). Also, in a lot of trials multiple drug prescription or intake is not taken into account. Moreover, in many cases genetic metabolic differences, like the presence of one or multiple polymorphisms in CYP enzymes, can make it difficult to predict therapeutic drug reactions.

Although in most cases the clinical consequences may be minor, the impact can be enormous for patients receiving medicines with a narrow therapeutic index, due to either, sub-therapeutic drug levels, or (severe) ADRs, or increased mortality rates. For the latter this was established in a recent study about tamoxifen use and *CYP2D6* polymorphisms.[52] An example of *CYP* polymorphisms and subsequent lung involvement is shown in another recent study, in which the link between *CYP2C9* and *VKORC1* SNPs, the prescription of oral anticoagulants and the occurrence of diffuse alveolar hemorrhage (DAH) was established.[61] Patients with *CYP2C9**2 and/or *CYP2C9**3 variant alleles require up to 61% lower maintenance doses of warfarin because of the reduced enzyme activity of these common variants.[36] A common serious ADR in these patients, therefore, can be over-anticoagulation, resulting in (life threatening) bleeding complications, such as DAH.

Furthermore, in a case-control study it was found that 91.5% of patients with DI-ILD had at least one of the studied *CYP2D6*, *CYP2C9* or *CYP2C19* variant alleles compared with 70.5% ($p<0.001$) of healthy volunteers. DI-ILD appeared to be associated with the presence of at least one variant *CYP* allele.[2]

These and other studies support the potential usefulness of personalized medicine by genotyping aiming to improve efficacy, tolerability and drug safety.[25,62]

Different patient categories should be tested for CYP polymorphisms: elderly patients with many drugs for different diseases, patients using drugs with a small therapeutic range and patients with unexplained side effects. Although the genotypic profile does not always predict the phenotypic expression, the interaction profile between different drugs can be estimated by computer models. Starting with a lower dose or using a medicine that is metabolized by another enzyme or route is often a way to prevent ADRs and reduce interactions. In addition, the best way to check the effect is to measure serum levels of the drug and its metabolites, the so called therapeutic drug monitoring (TDM). However, therapeutic serum levels of many drugs are not available or expensive to ascertain. In organ transplantation medicine, TDM together with CYP genotyping is already daily practice and can be cost effective, because of the high costs and the small therapeutic range of the immunosuppressive medication used.[59,63]

Nevertheless, genotyping should be considered to identify patients that might be at risk of severe toxic responses to environmental, pharmacological, herbal remedies and/or nutritional stimuli, in order to guide appropriate individual

dosage(s).[25] Nowadays, DNA material for genotyping can be easily obtained and patients do not even have to go to hospital for sample drawing.[64] Both clinical and genetic risk stratification (pharmacogenomics) may lead to more accurate prevention of drug-induced damage in the future.[11,25] However, further research is needed to explore the clinical relevance.

Conclusion

An ideal situation would be the introduction of a genetic medical passport for each patient to achieve a system in which therapeutic drug monitoring will be standard clinical practice. In this manner the incidence of ADRs and related medical consumption will decrease, which in the end will lead to a better pharmacotherapy for patients and reduced healthcare costs. To achieve this, a multi-disciplinary approach will be necessary to individualize pharmacotherapy on the basis of the pharmcogenetic profile.

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Chapter 3

Pharmacogenetic testing after a simple DNA isolation method

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Adaptation from:

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Genotyping with a dried blood spot method: a useful technique for application in Pharmacogenetics*. Clin Chim Acta*. 2008;388:189–191

and

P Wijnen, M Drent, M van Dieijen-Visser, O Bekers

Pharmacogenetic testing after a simple DNA isolation method on buccal swab samples*. Pharmacogenomics.* 2009;10:983–987

Abstract

Background

Several commercial DNA isolation kits are available for extracting genomic DNA from whole blood samples, but these procedures require quite some hands-on time and are rather expensive. An alternative technique could be dried blood spot (DBS) sampling, with which DNA isolation is faster, cheaper and logistics are easier. We have developed a non-commercial DBS method and examined whether the quality and quantity of DNA, isolated from DBS and noninvasively obtained buccal swab (BS) samples, was satisfactory.

Study design

DNA isolation from EDTA blood samples and blood spots on filter paper were compared after DNA isolation with a column method and two different DBS methods. BS samples were obtained by rubbing a sterile, dry cotton swab against the inside of the subject's cheek. In addition, the quantity of the obtained DNA was measured and melting curve analyses of five cytochrome P450 and three ATP-binding cassette polymorphisms were performed with real-time PCR FRET assays to establish the quality of the obtained DNA from both the DBS and BS samples.

Results

In all cases the genotype results corresponded completely. Moreover, the derivative melting curves of the DNA samples obtained from the capillary blood and BS were comparable and highly reproducible. The mean DNA concentrations measured were 16.0 ng/µl (12.6-19.4 ng/µl) and 70.2 ng/µl (57.3-83.1 ng/µl), respectively, for DBS and BS samples ($p<0.001$).

Conclusion

The DBS DNA isolation appears to be a useful alternative for the commercially available DNA isolation kits and an extremely useful method to discriminate between genotypes. This expands the possibilities of this quick and easy DNA isolation procedure. In particular, the noninvasive BS sampling method appeared to be a good alternative to invasive sampling methods.

Introduction

Pharmacogenetics, the study of the role of inheritance in the individual variation in drug response, is still a growing field and it is gaining more importance in the treatment and investigation of the cause of certain diseases, symptoms and adverse drug reactions (ADR). The promise of pharmacogenetics lies in its potential to identify the right drug and dose for each patient. Even though individual differences in drug response can result from the effects of age, sex, disease or drug interactions, genetic factors also influence both the efficacy of a drug and the likelihood of an adverse reaction occurring.[1-3] There is an increasing number of examples in which pharmacogenetic studies have indicated that a genetic test prior to treatment may be useful either for setting the individual dose or making a decision to use a particular drug.[4-6] Therefore, genetic testing of individuals, specific patient groups and possibly their family members or genotyping prior to prescribing certain drugs is becoming more important. The materials used for genotyping are mostly ethylene diamine tetra-acetic acid (EDTA) whole blood obtained by venous sampling and tissue samples. However, both require an invasive sampling method and for a number of subjects, this can be a reason to refrain from signing up to an investigation or trial.

Several commercial DNA isolation kits are available for extracting genomic DNA from EDTA whole blood samples. To obtain DNA from whole blood, commercially available DNA isolation procedures, require approximately one hour hands-on time of a technician and are rather expensive (approximately €4 per isolation). Simplification of the standard DNA isolation procedures by the dried blood spot method (DBS) might be advantageous. DBS sampling has already become common practice for newborns. Over the past decade many applications have been reported for both qualitative and quantitative screening of metabolic disorders.[7] Possible advantages of genotyping with DBS are: 1. The patient does not have to leave home and no phlebotomist is necessary. 2. Genotyping results are known when the patient visits the clinician and the clinician can take these results into account when he prescribes (other) drugs. 3. Transport is easy and there is a decrease in transport costs because only an envelope with the DBS of the patient is required. 4. Lower isolation costs and faster handling: DNA isolation with DBS is rapid, simple and no expensive DNA isolation kits are necessary.

Previously, the usefulness of DBS genotyping has been described.[8,9] However, a non-commercial method for DNA isolation with DBS in association with pharmacogenetics has not yet been reported in literature. In the present study a new non-commercial DBS method was developed for isolating DNA from capillary blood and this method is validated versus a standard commercial

available DNA isolation kit and also compared to an already existing extraction method for DBS described by Fischer et al.[10]
The aim of this study was also to test if the same DNA isolation protocol as applied to DBS could be used for isolating DNA from noninvasive buccal swab (BS) samples. Subsequently, the DNA quality and quantity of the BS samples were compared with the obtained DBS samples. Real-time PCR melting curve analyses were performed and DNA yields were measured using UV spectrometry. Additional storage tests were performed in order to ascertain whether delays in sending the sample to the laboratory or rather time from acquiring the BS sample to isolation, had an influence on the final result. Moreover, establishing the turnaround time and storage conditions required to avoid problems allows storage recommendations to be provided for the patient to follow.

Materials and methods

Samples and materials

EDTA whole blood samples and blood spots on filter paper made from the same samples were obtained from 106 Chinese renal transplant recipients and isolated by using 200 μl EDTA whole blood with a column method QIAamp blood mini kit, (Qiagen, Leusden, The Netherlands) according to manufacturer's instructions and the DBS method developed by our lab. Additionally, DNA was isolated from 10 samples obtained from healthy volunteers using the column method for EDTA blood and simultaneously obtained finger prick blood was isolated using the DBS method described in this study and the one described by Fischer et al.
Furthermore, another 100 DBS samples were collected by finger prick from healthy volunteers to examine our DBS method in clinical practice. When using the DBS method capillary blood can be obtained by a finger prick by the patients themselves. The drop of blood is spotted on filter paper (Whatman® Schleicher and Schuell, code 903 (2992), Whatman®, Dassel, Germany).
Buccal swabs were obtained by thoroughly rubbing a sterile, dry cotton stick (Copan plain swab sterile plastic applicator rayon tipped, ref 155C, Copan, Brescia, Italy) twice up and down against the inside of the individual's cheek on the one side of the mouth. One DBS and one BS sample were collected at the same time from 25 healthy volunteers, resulting in a total of 25 DBS and 25 BS samples to examine the two sampling methods that could be used in clinical practice. In addition, the samples were isolated according to our DBS protocol.
After obtaining 12 BS samples per individual from a total of five healthy volunteers for a storage test, four swabs from each subject were kept at room temperature, four were put in a refrigerator, and four in a freezer. The BS

samples kept at room temperature were isolated at day 1, 3, 5, and 7 after sampling. The samples kept at 4–8°C were isolated at 1, 2, 3, and 4 weeks and the BS samples stored at -20°C were isolated at 1, 2, 3, and 4 months after sampling.
The DNA concentrations of the samples were measured on the NanoDrop® ND-1000 UV Spectrophotometer (Witec AG, Littau, Switzerland).
Written informed consent for participation in this study was obtained from all subjects.

DNA isolation

EDTA whole blood samples, capillary DBS and BS samples were collected. In the laboratory, after drying for ½ h a 3 mm paper disk was cut out from the blood spot sample with a puncher (Harris Uni-core™) or the tip was cut off the cotton stick. In addition, the 3 mm bloodstained paper disk or the cotton tip was placed into a cup, 500 µl sterilized Milli-Q water (Millipore B.V., Amsterdam, The Netherlands) was added and vortexed three-times for 5 s each. The water was pipetted off. After adding 200 µl 10% Chelex® 100 solution (Bio-Rad Laboratories, CA, USA), the cup was placed in a water bath at 95°C for 30 min. Finally, this DNA solution was pipetted into a new cup and the DNA was ready for use.
With the method described by Fischer et al.[10] the 3 mm disk is placed in an Eppendorf™ cup and washed twice with 1 ml of phosphated-buffered saline-0.1% Tween during 10 min with shaking. After transferring the disk to a 2 ml screw-cap cup and adding 200 µl 5% Chelex-100 solution (Sigma-Aldrich Chemie B.V., Zwijndrecht, The Netherlands), the cup is placed in a water bath at 60°C for 30 min, followed by 30 min in a water bath boiling at 100°C.

Genotyping

A total of five previously described cytochrome P450 (*CYP*) and three ATP-binding cassette (*ABCB1*) polymorphisms were performed in this study, using real-time PCR fluorescence resonance energy transfer (FRET) assays (TIB MOLBIOL, Berlin, Germany), namely: *CYP3A4-V* A-392G, *CYP3A5**3 A6986G, *CYP2C9**2 C430T, *CYP2C9**3 A1075C, *CYP2C19**2 G681A, *CYP2C19**3 G636A, *CYP2D6**3 2549delA, *CYP2D6**4 G1846A, *ABCB1* C1236T, *ABCB1* G2677T/A, and *ABCB1* C3435T.[11-15] The single nucleotide polymorphisms (SNPs) were genotyped by examining the melting curves from the aforementioned FRET assays using the LightCycler® (Roche Diagnostics, Basel, Switzerland).
For comparison an EDTA sample, isolated on the MagNA Pure Compact (Roche Diagnostics), was genotyped within the same run as the DBS and BS samples. Furthermore, a heterozygote plasmid sample supplied with the primer/probes sets of each assay and a negative water control were analysed within each run.

Statistical analysis

The paired t-test was used to compare the two sampling methods.
Melting curves were generated by the LightCycler® and its software/integration program calculates melting temperature(s) for each detected peak.
Statistical analyses were performed with SPSS 15.0 (SPSS. Inc., IL, USA) for Windows. A p-value of less than 0.05 (two sided) was considered to indicate statistical significance.

Results

After analysing the melting curves obtained by real-time PCR FRET assays for the different DNA isolation methods, the results obtained with the DNA extracted with the column method were in complete concordance with the results of the DNA isolated with our DBS method. Additionally, the DNA isolation with the QIAamp blood mini kit and the two DBS methods performed for 10 healthy volunteers were also completely in concordance. Genotyping with the DNA isolated with our DBS method for the 100 healthy volunteers gave also satisfactory melting curves.
Although analysis of the column and previously described DBS DNA isolation methods were performed for five different real-time PCR FRET assays, an overview of the results is only given for the *CYP3A5* A6986G polymorphism. Melting of a sample homozygous for the *CYP3A5* 6986G allele produced a melting peak at approximately 57.1°C±0.1 (mean±2 SD) and 57.3°C±0.2, and the *CYP3A5* 6986A allele gave a melting peak at approximately 62.3°C±0.2 and 62.5°C±0.5, for respectively the QIAamp blood mini kit and our DBS DNA isolation method. Heterozygous samples contained both type of targets and thus generated both peaks. In addition, for heterozygous samples (n=40), the difference between the two melting temperatures (5.2°C±0.1) had a coefficient of variation (CV) of respectively 0.64% and 1.24%.
Hence, the derivative melting curves of the commercial DNA isolation method and our DBS method were highly reproducible and can therefore be used perfectly for discrimination between the *CYP3A5**1 and *CYP3A5**3 allele, which is illustrated in Figure 3.1. The results of the other real-time PCR FRET assays were similar to the results presented for the *CYP3A5* A6986G polymorphism.
An issue that may be of importance when the DBS method is used for DNA isolation is a possible contamination risk. Because it is likely that the filter paper used will be contaminated before and/or after the blood spot is actually on the filter paper. Therefore, we examined whether the DBS DNA isolation procedure is robust enough to discriminate the blood spot of the patient from the contamination caused by rubbing vigorously over respectively the dry filter paper, the dry filter paper before adding a blood spot and the dried blood spot

on the filter paper. In the cases with the blood sample only the genotypes of the patients were seen and in the case of the no blood blank disk, no signal was found, which implies that there is no disturbing influence when the filter paper is contaminated by hands before or after adding the blood spot on the filter paper.

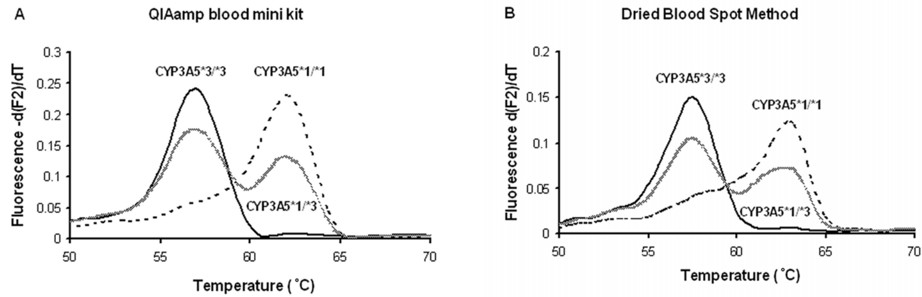

Figure 3.1 Examples of melting curves from DNA isolated with the QIAamp blood mini kit from EDTA whole blood, and the dried blood spot method from capillary blood.
Genotyping of the A6986G polymorphism of the *CYP3A5* gene with allele specific fluorescent probes by derivative melting curve plots using DNA samples isolated with the QIAamp blood mini kit (**A**) and with our dried blood spot DNA isolation method (**B**).
The -dF2/dT derivative melting curves are read in channel 2 for the detection of the *CYP3A5* fragment. The derivative melting curves is plotted for a similar sample homozygous for the 6986G (*CYP3A5**3/*3) allele (Tm 57°C); a similar heterozygous sample (Tm 57°C (*CYP3A5**3) and 62°C (*CYP3A5**1)) and a similar sample homozygous for the 6986A (*CYP3A5**1/*1) allele (Tm 62°C).

The DNA concentrations of the DBS and BS samples resulted in mean concentrations of 16.0 ng/µl (12.6-19.4 ng/µl) and 70.2 ng/µl (57.3-83.1 ng/µl), respectively (p<0.001) obtained using our isolation method. As a comparison, the samples isolated using the dried blood spot method previously described by Fischer *et al*.[10] resulted in a mean concentration of 17.5 ng/µl (12.8-22.2 ng/µl) for these DBS samples (Figure 3.2).
The derivative melting curves of the DNA samples obtained from capillary blood and BS samples were comparable and highly reproducible for all SNPs studied. As a representative example, the melting results of the *CYP2C9**2 C430T SNP are hereby presented below. Melting of a sample homozygous for the 430T allele produced a melting peak at approximately 50.6°C±0.4 and 50.7°C±0.4 for a BS and a DBS sample, respectively. In a sample homozygous for the 430C allele a melting peak was obtained at approximately 59.5°C±1.2 and 59.6°C±1.3, respectively, for a BS and a DBS sample. Heterozygous samples contained both type of targets and therefore generated both melting peaks, with a mean±2 SD difference of 9.6°C±0.4 and 9.5°C±0.7 for the BS and DBS

samples, respectively. An overview of all the melting temperature ranges for the SNPs investigated, stratified per sample type, are listed in Table 3.1.
Genotyping confirmed the results demonstrating the same amount of peaks at the same melting temperatures and concurrent results were obtained for the DBS and BS sample types per subject for the *CYP3A4-V*, *CYP3A5*, *CYP2C9*, *CYP2C19* and *CYP2D6* SNPs that were investigated (Table 3.1). An EDTA sample, isolated with the QIAamp blood mini kit, was also analyzed in the same run to illustrate and compare the height and position of the melting curves of the different sample types. A representative example of a FRET melting curve analyses is shown for the *CYP2C9**2 assay in Figure 3.2.
Furthermore, we investigated the stability of the BS samples obtained using a plain cotton swab under different storage conditions. When kept at room temperature, the performed FRET assays gave only a weak signal that was difficult to interpret, or no signal at all, after isolating the swabs after a 7-day period. In contrast, when isolating the BS samples at day five all the samples still performed well. Storage at 4-8°C extended that period to one month, whereas storing the BS samples at -20°C gave good results up until at least four months.

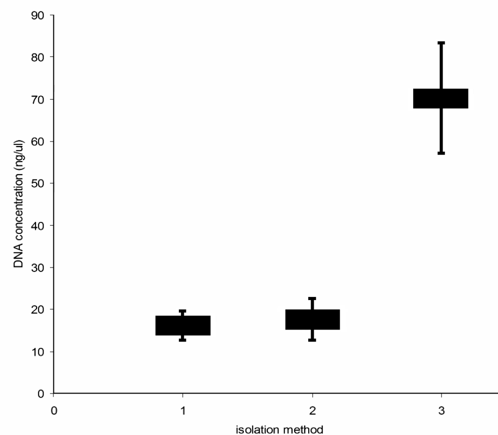

Figure 3.2 Mean DNA quantities, isolated with two different DBS DNA isolation protocols, using capillary blood DBS and BS samples.
1: DBS sample isolated using our method
2: DBS sample isolated using the method described by Fischer et al.[10]
3: BS sample isolated using our method
BS: Buccal Swab; DBS: Dried Blood Spot.

Table 3.1 Overview of melting temperatures ± 2 standard deviations per sample type for the CYP450 SNPs examined and isolated with our dried blood spot method.

CYP450	SNP	Tm	BS (°C)	DBS (°C)	Expected Tm (°C)
CYP2D6	*3 2549delA	Tm1	NA	NA	52.1 ± 0.4
		Tm2	58.8 ± 0.6	58.8 ± 0.6	59.4 ± 0.2
	*4 G1846A	Tm1	57.7 ± 1.2	58.0 ± 1.9	58.2 ± 0.2
		Tm2	65.2 ± 1.5	66.9 ± 1.8	66.4 ± 0.8
CYP2C9	*2 C430T	Tm1	50.6 ± 0.4	50.7 ± 0.4	50.5 ± 0.2
		Tm2	59.5 ± 1.2	59.6 ± 1.3	60.4 ± 1.0
	*3 A1075C	Tm1	50.1 ± 0.2	50.2 ± 0.2	50.2 ± 0.3
		Tm2	NA	NA	59.4 ± 0.1
CYP2C19	*2 G681A	Tm1	49.3 ± 1.0	49.0 ± 1.0	49.3 ± 0.1
		Tm2	55.4 ± 1.2	55.2 ± 0.8	56.1 ± 0.1
	*3 G636A	Tm1	54.0 ± 0.6	54.2 ± 0.2	54.1 ± 0.2
		Tm2	NA	NA	61.4 ± 0.2
CYP3A4-V	A-392G	Tm1	NA	NA	50.1 ± 0.4
		Tm2	58.5 ± 0.8	58.6 ± 0.3	58.8 ± 0.4
CYP3A5	*3 A6986G	Tm1	57.6 ± 0.2	57.5 ± 0.2	57.4 ± 0.2
		Tm2	NA	NA	62.6 ± 0.2

BS: Buccal Swab sample; CYP450: Cytochrome P450; DBS: Dried Blood Spot sample; Expected Tm: Positive control, a plasmid sample; NA: Not available; Tm: Melting temperature.

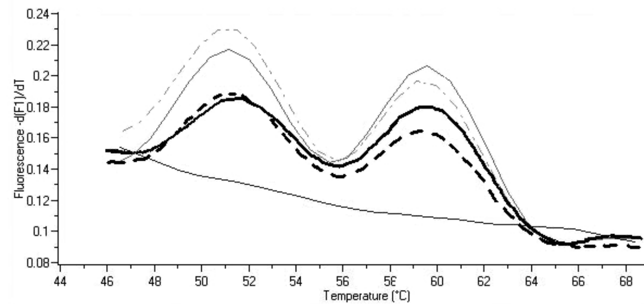

Figure 3.3 An example of completely concurrent *CYP2C9**2 melting curves of an EDTA blood sample, a DBS sample and a BS sample.
Positive control — • — — •
EDTA sample ———— (grey)
DBS sample — — — (black)
BS sample ———— (black)
Negative control ————
The positive control is a heterozygote plasmid sample, supplied with the primer/probes of each assay. The negative control is sterile water (no peaks).
BS: Buccal Swab; DBS: Dried Blood Spot; EDTA: Ethylenediaminetetraacetic acid.

Discussion

A large difference was found for labour time and costs between the QIAamp blood mini kit and our DBS method: it took approximately one hour and €4.00 per blood sample to isolate DNA from EDTA whole blood with the QIAmp mini blood kit while DNA isolation with the DBS method takes about 45 min hands-on time and less than €2.00 per capillary blood sample. Moreover, there was also a significant difference when comparing the two DBS methods, not so much in costs, but in hands-on and total time. The method described by Fischer et al.[10] takes about one hour more before DNA is obtained. Furthermore a Tween/phosphate-buffered saline solution is necessary for washing the DBS. For DBS samples, there was virtually no difference in DNA yield between the isolation method previously described by Fischer et al.[10] and the one described in this study. Moreover, an advantage of our method is that the use of chemicals is minimal, except for a 10% Chelex solution, and it has an incubation time of only 30 min.
Although the amount of blood required for this DBS DNA isolation is less, an invasive finger prick is still required to obtain capillary blood. Another restriction of this DBS method can be the sometimes relatively low amount of DNA obtained from the blood spots.
Combined with the noninvasive BS as a sampling method, this provides a patient friendly and easy way of obtaining DNA samples that can be used for genotyping. Especially when conducting a large trial including family members, the BSs can be sent by post. Furthermore, attending the hospital in order to have a blood sample drawn would no longer be necessary. The sending by post and not having to travel to a hospital are advantages that also apply to DBS samples. However, when using that sampling method there is still the need to draw blood. The noninvasive nature of a BS sample combined with the other benefits implicate that there are even fewer drawbacks and people will be more motivated to participate.
We used plain sterile swabs that are readily available and cheap (approximately €0.10 each) to investigate if buccal-cell collection and subsequent isolation would work. Isolation with our method only takes 30 min in a water bath, and adding preparation and pipetting time, the procedure is completed well within an hour and with minimal chemical use and cost. On the other hand, commercially available saliva collection methods (up to approximately €18.00 per collection vial) currently being used in most community-based studies require an additional kit to isolate DNA, and all at considerably higher costs. The obtained BS samples, when using plain sterile cotton swabs, should be isolated within five days from the time of sampling to prevent loss of signal when performing melting curve analyses. The fact that we did not get a signal in the cases that were a week old was a strong indication that amplification of

the desired fragment was no longer possible and DNA degradation was present. Whenever isolation within five days time is not possible, the BS samples can be stored for up to a month at 4-8°C or for up to at least four months at -20°C prior to isolation. Therefore, amending the instructions to the patient to include: “Store in the refrigerator when you are not able to send it off by mail the same day”, is an important addition that will improve the final result.
After isolation, the ready to use DNA solution (approximately 150 µl per isolation), when pipetted off the Chelex and transferred into a new vial, can be stored at 4-8°C or at -20°C for more than one year.

Conclusion

The DNA isolation method described appeared to be extremely useful for the different sampling procedures (capillary blood and BS). Furthermore, it is able to isolate DNA with less hands-on time, is less invasive for the patient, and saves transport expenses and DNA isolation costs. Comparison of our DBS method to another non-commercially available DBS method resulted in similar DNA yields, less use of chemicals and it was less time consuming. All these advantages make our DSB method very useful in clinical practice.
Moreover, it appeared to be a useful alternative for commercially available DNA isolation kits. The DNA yields of BS samples were considerably higher compared with the yields of DBS samples. For DNA isolated from BS samples could be used perfectly to distinguish genotypes or polymorphisms. This expands the possibilities of this quick and easy DNA isolation procedure. Especially, the noninvasive BS sampling method appeared to be a good and patient friendly-alternative to invasive sampling methods.

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Chapter 4

Role of cytochrome P450 polymorphisms in the development of pulmonary drug toxicity

A case-control study in the Netherlands

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Abstract

Background
Drug-induced pulmonary toxicity is a serious and expanding problem with often unknown aetiology. Many drugs are metabolized by cytochrome P450 (CYP) enzymes.
To establish whether allelic variation in CYP polymorphic genes contributes to variability in drug response and unexpected toxicity.

Study design
A case-control study was conducted. The cases consisted of patients with drug-induced interstitial lung disease (DI-ILD; n=59). Two control groups were used: one group of healthy volunteers (n=173) and one group of patients with idiopathic pulmonary fibrosis (IPF; n=110).

Results
Of the patients with DI-ILD 91.5% (54/59) had at least one of the studied variant genes compared with 70.5% (122/173, $p<0.001$) of the healthy volunteers and 69.1% (76/110, $p<0.001$) of the IPF patients. The percentage of individuals with one or more variant CYP genes was higher in DI-ILD group. Odds ratios were significantly increased and ranged from 3.25 to 40.8, indicating a significant association between the development of DI-ILD and the presence of one or more variant CYP genes.

Conclusion
DI-ILD appeared to be associated with the presence of at least one variant CYP allele. This study supports the potential usefulness of personalized medicine by genotyping aiming to improve efficacy, tolerability and drug safety.

Background

One may view the lung as bathed in two dissimilar environments: inhaled air and circulating blood. Both can carry noxious substances that may inflict damage to the lung. Air can deliver noxious particles and blood is the main supplier of most drugs, independent of the route of administration, and adverse respiratory reactions may follow by the most unlikely routes. Drugs can induce specific respiratory reactions, or the lungs may be affected as part of a generalized response. The most common form of drug-induced respiratory disease is diffuse interstitial lung disease (ILD). The drugs involved not only comprise prescribed and over-the-counter-drugs, but also illicit drugs, herbs, alcohol and ingredients of the diet. The list of compounds involved has grown rapidly over the last decades.[1,2] Although drugs have been unequivocally identified as the cause in only a limited number of cases, it is important to acknowledge the potential role of medication in the development of ILD. This is due to the severity of the potentially irreversible damage to the lungs and the improvement that is often easily brought about by stopping administration of the drug involved.

The diagnosis of drug-induced ILD (DI-ILD) primarily rests on the link between drug intake and the subsequent development of respiratory symptoms. In some cases, the evidence that reactions in the lung are drug-induced is circumstantial. A straightforward interpretation is hampered when the damage is irreversible or when the symptoms are aggravated after stopping drug administration. Rechallenging the patient with the drug involved is frequently unethical and dangerous. Sensitive and specific tests are lacking. The key to diagnosis is a high clinical acuity.[2] All medication needs to be recorded and reviewed meticulously.

The molecular mechanism involved in the development of DI-ILD is still enigmatic. Inflammation initiated by drugs, drug metabolites or drug-induced free radical generation processes has been implicated.[3] There is also emerging evidence for a role of genetic factors in the development of ILD such as differences in gene expression profiles between pulmonary fibrosis (IPF) and hypersensitivity pneumonitis.[4,5]

Cytochrome P450 (CYP) single nucleotide polymorphisms (SNPs) are one of the key factors known to cause a variation in drug response between individuals.[3,6-9] CYP is a super family of microsomal enzymes that metabolize various endogenous compounds and xenobiotics, including most drugs. Through CYP metabolism the chemical structure of drugs is changed, in general facilitating elimination of the drug from the body.[3,7]

The risk for development of DI-ILD and clinical patterns vary depending on a variety of host and drug factors.[2] Several different phase I xenobiotic-metabolizing CYP and phase II enzymes (i.e. conjugation enzymes including

several transferases) are present in the human lung, possibly contributing to *in situ* activation.[10-14] Metabolism also affects the biological activity of the drug. The biological activity of the parent drug is usually superior to that of the metabolite, but there are several exceptions. Sometimes CYP metabolism yields very toxic metabolites, for example, with paracetamol (acetaminophen), benzo[a]pyrene and carbamazepine.[14] Occasionally, drugs may cause the formation of reactive oxygen species by uncoupling of the electron transport of the CYP system.[15] These metabolites or reactive oxygen species may damage vital cellular components, such as proteins, lipids or DNA.[16] This might be the cause of clinically relevant drug-induced pulmonary toxicity.[12,17,18]

Recently, we reported two cases of patients with interstitial pneumonia who developed cardiac failure following treatment with venlafaxine.[19] In both cases, a strong relationship between the development of the patients' illness and the initiation of venlafaxine treatment was identified. Furthermore, members of the CYP family are involved in the metabolism of venlafaxine. Therefore, we hypothesized that genetically or environmentally induced inter-individual differences in the expression of pulmonary biotransformation enzymes such as CYP may form the basis for, or contribute to, the risk of DI-ILD.[2,10,13,14]

The aim of this study was to establish whether variation in *CYP* genes contributes to variability in individual drug response and toxicity. Therefore, the presence of the most clinically relevant variants of *CYP* genes (*CYP2D6*, *CYP2C9* and *CYP2C19* variants) was compared in a population of patients with DI-ILD with the presence of these variant genes in a population of patients with IPF and one of healthy volunteers.

Materials and methods

Setting and study population

The study was conducted as a case-control study. Between 2002 and 2006, 575 bronchoalveolar lavages (BAL) were performed on patients referred to the department of Respiratory Medicine of the Maastricht University Medical Centre (MUMC), Maastricht, The Netherlands, suspected of a non-infectious or infectious disorder. Out of these 575 cases, 51.1% appeared to have an infectious disorder, 17.8% had diffuse alveolar haemorrhage and 31.1% an ILD. Of this latter group, 59 (10.2%) met the criteria of DI-ILD. All of the 59 patients were Caucasian, used multiple drugs for various indications and did not have a history of any pulmonary disorder. The clinical presentation of drug-induced pulmonary toxicity varied. Besides pulmonary symptoms, some patients (n=7; 12%) showed signs of other toxicity such as skin, cardiac and gastrointestinal involvement. The diagnosis DI-ILD was established by clinical presentation including dyspnoea and hypoxia, diffuse interstitial features on

chest X-ray and a high-resolution CT scan as well as a BAL fluid (BALF) profile compatible with DI-ILD, excluding an infectious cause.[2,20,21] A lung biopsy was performed in 20% (n=12) of the patients. Clinical records were reviewed carefully and present and past drug use was documented. After reviewing the drug use of all DI-ILD patients, the most important groups appeared to be antihypertensive medication, β-adrenergic receptor antagonists, anti-arrythmic agents, antidepressants and anticoagulants. Every DI-ILD patient used a combination of at least two or more of these drugs. Withdrawal of the suspected causative drug(s) led to a favorable outcome in 75% of the patients, including an improvement of the respiratory symptoms, lung function test results, especially the diffusing capacity, and the chest X-ray abnormalities. In 25% of cases the damage was irreversible. Subjects were genotyped retrospectively for *CYP* polymorphisms and the drug(s) which are metabolized by the CYP enzymes that show genetic polymorphisms were identified.

The first control group consisted of 173 healthy Caucasian volunteers, recruited for method validation, who did not use medication nor had any relevant medical history, especially no history of pulmonary complaints.[22] All healthy volunteers were hospital employees. This healthy volunteer control group was also used to establish the distribution of allele variants in the general population.

The second control group consisted of 110 Caucasian patients with IPF, known at our out-patient clinic and collected during the timeframe of this study, who also used medication for various indications. This group was selected as a control group, for it was expected that drug use did not play a substantial role in the pathogenesis of this disease. The diagnosis IPF was based on consistent clinical features, radiographic findings and BALF analysis results. According to the international consensus a biopsy was obtained from 50% of the IPF group, which confirmed the diagnosis histologically as being usual interstitial pneumonia.[23] Clinical records were reviewed carefully and present and past drug use was documented. All IPF patients used one or more drug(s). For the DI-ILD group as well as for the IPF group it was checked whether the drugs used were metabolized by polymorphic CYP enzymes.
The study was performed in accordance with the Declaration of Helsinki and its amendments. The protocol was approved by the local Medical Ethics Board of the MUMC. Written informed consent for participation in this study was obtained from all subjects.

Genotyping

DNA was obtained from all subjects by using venous EDTA anti-coagulated blood and isolating with a High Pure PCR Template Preparation Kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions.

In this study, genotyping was carried out for the following *CYP* allelic variants: *CYP2C9**2 C430T, *CYP2C9**3 A1075C, *CYP2C19**2 G681A, *CYP2C19**3 G636A, *CYP2D6**3 2549delA, and *CYP2D6**4 G1846A. For genotyping the *CYP2C9**1,*2,*3 and *CYP2C19**1,*2,*3 SNPs, real-time PCR Fluorescence Resonance Energy Transfer (FRET) assays were performed using the *CYP2C9* and *CYP2C19* Mutation Detection Kits (Roche Diagnostics, Mannheim, Germany). The *CYP2D6**1,*3 and *4 SNPs were genotyped using FRET assays (TIB MOLBIOL, Berlin, Germany) as described by Stamer et al.[24] and Müller et al.[25] on the LightCycler® (Roche Diagnostics, Mannheim, Germany)

Statistical analysis

Statistical analyses were performed with SPSS 15.0 (SPSS. Inc., Chicago, IL, USA) for Windows. In order to evaluate the association between the presence of pulmonary disease and the presence of CYP polymorphisms, odds ratios (ORs) with corresponding 95% confidence intervals (CI) were calculated. Actual allele distributions were compared against the expected frequencies calculated, using the Hardy–Weinberg equilibrium. Deviations from Hardy–Weinberg equilibrium were analysed using the Chi-square test. A p-value of <0.05 (two sided) was considered to indicate statistical significance. A Bonferroni correction, to adjust for multiple comparisons, was applied were it was appropriate ($p<0.01$, indicating statistical significance).

Results

A summary of the characteristics of the DI-ILD, IPF and healthy volunteer population is shown in Table 4.1.

The subjects in the healthy volunteers group were younger than the subjects in the other two populations and used no medication. The age difference had no influence on the distribution of genetically variant genes, however, it does indicate that (multiple) drug use increases with age. The IPF control patients did use (multiple) drugs, but to a lesser extent than the DI-ILD patients. Table 4.1 also shows that of the patients in the ILD group, substantial larger percentages were receiving drugs that are metabolized by a polymorphic CYP enzyme system. For example, of the 43 patients who received a drug that

should be metabolized by CYP2D6, 73% had a genetic polymorphism that would be likely to influence their ability to metabolize drugs efficiently.

Table 4.1 Baseline characteristics of the drug-induced interstitial lung disease (DI-ILD), idiopathic pulmonary fibrosis (IPF) and healthy volunteers (HV) populations.

Characteristic	DI-ILD	IPF	HV
Subjects (no.)	59	110	173
Male/female (no.)	28/31	62/48	78/95
Age (mean/range (y))	65.4/21-87	63.3/27-89	38.5/19-59
Percentages of above populations taking one or more drugs metabolized by CYP polymorphic enzymes:			
CYP2D6	73	18	0
CYP2C9	52	19	0
CYP2C19	88	17	0

CYP = cytochrome P450.

The percentages of individuals having one of the studied individual polymorphisms (*CYP2D6*, *CYP2C9* and *CYP2C19*) or combinations in DI-ILD, healthy volunteer and IPF control patient groups, respectively, are shown in Figure 4.1.

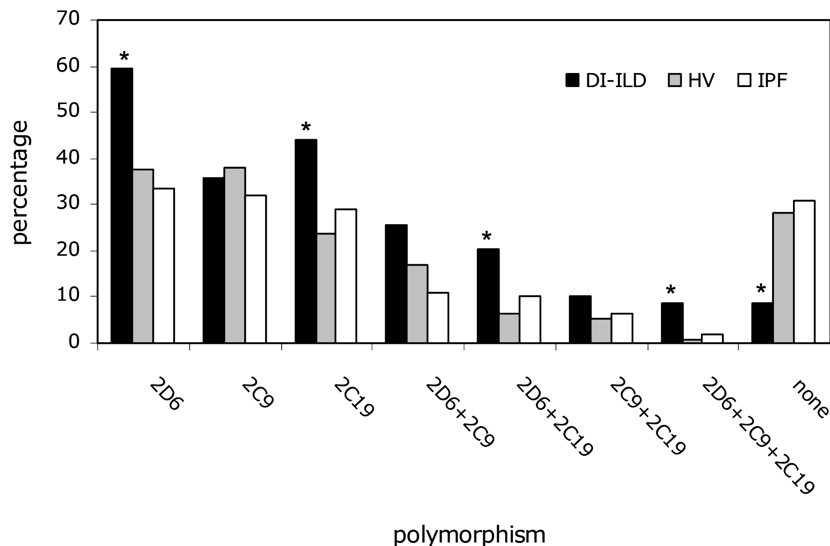

Figure 4.1 Percentage of individual and combinations of cytochrome P450 (CYP) polymorphic genes in individuals with drug-induced interstitial lung disease (DI-ILD, n=59), idiopathic pulmonary fibrosis (IPF, n=110) and healthy volunteer (HV, n=173) groups. * $p<0.05$.

The genotype distribution for the three groups in this study showed that all results were generally consistent with the Hardy-Weinberg equilibrium (Table 4.2, upper panel, expected values not shown).[26-29] One exception was the *CYP2D6* polymorphism of the healthy volunteer group, in which a small but significant deviation from the Hardy-Weinberg equilibrium was observed ($p<0.01$). Fewer heterozygotes (45 actually present in the healthy volunteer group versus 56 expected, calculated using the Hardy-Weinberg equilibrium) and more homozygotes (13 versus 7) were found. However, the allele frequencies in both control groups (healthy volunteers and IPF patients) were comparable with those in the reference populations from the literature (Table 4.2, lower panel).[26,27,29]

Table 4.3 presents the results from the analyses that compared the proportions of persons with one ore more variant genes that express the polymorphic CYP enzymes between DI-ILD patients and both control groups. The proportion of persons without any allelic variants was only 8.5% among DI-ILD cases versus 30.9% and 29.5% in IPF patients and healthy volunteers. Furthermore, it appeared that there was a higher prevalence of combinations of variant genes within the DI-ILD patients compared with the IPF patients and healthy volunteers (Table 4.3, upper panel). Subjects without any *CYP* variant genes within the DI-ILD group and healthy volunteer group were used as reference group for the calculation of ORs associated with the presence of one or more of the studied genetic polymorphisms. Strong and significant associations were found comparing the prevalence of *CYP2D6* and *CYP2C19* genetic variants in DI-ILD patients with those of the healthy volunteers. The ORs calculated were 5.80 (95% CI: 2.17-15.4, $p<0.001$) for *CYP2D6*, 3.25 (95% CI: 1.18-8.86, $p=0.026$) for *CYP2C9* and 6.47 (95% CI: 2.35-17.7, $p<0.001$) for *CYP2C19*, respectively (Table 4.3). For the combined genotypes with variant *CYP* alleles, significant ORs were found of 4.91, 14.0, 6.80 and 40.8 (Table 4.3).
Similar results were found comparing the prevalence of variant *CYP* genes in DI-ILD patients with the IPF control patients. These analyses, as shown in Table 4.3, also resulted in significant associations between the development of DI-ILD and the presence of *CYP* genetic variants.
The percentage of DI-ILD patients having a *CYP* variant gene and receiving one or more drug(s) metabolized by a polymorphic CYP enzyme was 87% (47/54), compared with only 16% (12/76) of the IPF patients.
Table 4.4 lists the (suspected) causative drugs, the CYP enzymes involved in the metabolism of the mentioned drugs, the number of DI-ILD patients involved (with and without variant *CYP* genes) and also shows which of the variant forms of *CYP* genes were found. The last column lists the number of literature references mentioned on www.pneumotox.com, concerning the stated causative drug in relation to lung disease.

Table 4.2 dwars.

Table 4.3 dwars

Table 4.4 List of potentially causative drugs and the cytochrome P450 (*CYP*) genes and variant genotypes expressing enzymes involved in their metabolism.

Causative drug	*CYP* gene[a]	Allelic variants present	Drug+ variant gene[b]	No variant gene+ inhibitor[c]	Literature[d]
Acenocoumarol	2C9	*1/*2 *2/*3	5	7	4
	2C19	*1/*2 *2/*2	8	2	4
Acetaminophen	2D6	*1/*4	2	2	11
	2C9	*1/*2 *2/*3	2	1	11
Amiodarone	2D6	*1/*4 *1/*3 *3/*4 *4/*4	8	7	276
	2C19	*1/*2	6	9	276
Amitriptyline	2D6	*1/*4	2		4
	2C19	*1/*2	1		4
Captopril	2D6	*3/*4	1	1	27
Carbamazepine	2C9			1	53
Carvedilol	2D6	*1/*3	1	1	2
Cotrimoxazol	2C9			1	11
Cyclophosphamid	2C19	*1/*2	1		65
Diazepam	2C19	*1/*2	1		
Diclofenac	2C9	*3/*3	1	1	3
	2C19	*1/*2	1		3
Fenfluramine	2D6	*1/*3	1		39
Flecainide	2D6	*1/*3	1		10
Fluoxetine	2D6	*1/*4 *1/*3	2	1	8
	2C19	*1/*2	2		8
Haloperidol	2D6			1	2
Ibuprofen	2C9	*1/*2	1	1	6
	2C19	*1/*2	1		6
Methotrexate				2	117
Metoprolol	2D6	*1/*4 *4/*4 *1/*3	10	1	2
Montelukast	2C9	*1/*2	1		6
Morphine	2D6	*1/*4	1		16
Naproxen	2C9	*1/*2	1	1	14
Omeprazol	2C19	*1/*2	4	5	1
Pantoprazol	2C19	*1/*2 *2/*2	5	2	
Paroxetine	2D6	*1/*4	2	2	2
Propranolol	2D6	*1/*4 *3/*4	1	1	11
Ranitidine	2D6	*1/*4	2	1	
	2C19	*1/*2	2	1	
Risperidone	2D6	*1/*4	1		1
Tamsulosin	2D6	*1/*3 *3/*4	3	1	
Temazepam	2C19	*1/*2 *2/*2	4		
Tramadol	2D6	*1/*4	1		2
Valsartan	2C9	*1/*3 *1/*2 *2/*3	3	1	3
Venlafaxine	2D6	*1/*4	2		5
	2C19	*1/*2	1		5

[a] = Gene expressing CYP enzyme involved in the metabolism of the drug.
[b] = Total number of patients with a variant *CYP* gene receiving the drug.
[c] = Total number of patients receiving the drug, without a variant *CYP* gene, but with the drug being associated with an inhibition of the CYP enzyme involved in the metabolism of the drug.
[d] = Total number of articles at www.pneumotox.com referring to the mentioned drug.

Discussion

To the best of our knowledge, this is the first study indicating that DI-ILD may be attributable to a reduced metabolic capacity by CYP enzymes. According to our results, there seems to be an association between having one or more *CYP* genetic variants that may lead to reduced metabolism, receiving drugs that are metabolized inadequately by the affected system and the development of DI-ILD. Looking at the ORs, ranging from 3.25 to 40.8, patients with variant CYP enzymes appear to be at a substantially greater risk of developing a DI-ILD when prescribed multiple drugs. These findings strengthened our presumption that inadequate drug metabolism predispose an individual for the development of DI-ILD. In 91.5% of the studied DI-ILD patients at least one *CYP* variant gene was present, whereas this percentage was about 70% in the control populations. In the DI-ILD group the prevalence of having more than one *CYP* variant gene was also substantially higher. The results support the potential usefulness of CYP genotyping in selecting appropriate drugs or dosages of drugs and avoiding subsequent serious adverse effects.

CYP isoenzymes have been detected in animal as well as in human lung tissues.[30] It is generally agreed that the CYP super family of enzymes forms the first step in the inactivation and elimination of numerous drugs by oxidation and reduction. Considering the fact that bio-activation by CYP enzymes plays an important role in human drug toxicity, polymorphisms in the CYP450 enzyme system may result in large inter-individual variations in the metabolism and toxicity of xenobiotics.

Given the ever-increasing number of patients, especially seriously ill and/or elderly, who take more than one drug, also often metabolized by the same CYP enzyme, the inherent problems of drug-induced toxicity are alarming.[31] Physicians should therefore be alert to the possibility that a drug-induced pulmonary reaction may originate from an inappropriate metabolism, especially in case of multiple drug use. The results in this study corroborate that genotyping a patient before drug initiation could lead to a more tailor-made dosing schedule that might protect the patient from the development of serious side effects at the start of the pharmacotherapy. Trial-and-error approaches could be reduced this way.[32]

In the present study we focused on *CYP* polymorphisms. The total 'genetic profile' of an individual patient should include genes expressing further polymorphic enzymes and other proteins involved in drug metabolism and response. For example, in the case of azathioprine indication, also used as treatment for IPF, testing for thiopurine methyltransferase (*TPMT*) variants

involved in the azathioprine metabolism is advised before starting treatment.[33-35] In the US, drug labels for azathioprine now include information on *TPMT* polymorphisms and recommend determining patients' phenotype or genotype prior to drug treatment.[36]

The finding that 87% of the studied DI-ILD patients received one or more drug(s) metabolized by an affected CYP metabolism route is consistent with the assumption that the interstitial lung reactions were drug-induced and that the case group in this study was well defined. In the other cases drug-drug interactions may have been responsible for the toxic drug effects or other pharmacogenetic factors might be involved, such as reduced TPMT activity involved in methotrexate metabolism.[37,38]

There are an increasing number of examples where pharmacogenetic studies have indicated that a genetic test prior to treatment may be useful either for setting the individual dose or making a decision to use a particular drug.[39] The ability to identify individuals who are susceptible to ADRs has the potential to reduce the personal and population costs of drug-related morbidity.[8]
In this retrospective study, the healthy volunteers used no medication and among the IPF group patients used fewer drugs compared with the DI-ILD patients. However, this does not imply that the healthy control patients and IPF patients are not at risk of developing a drug-induced pulmonary reaction. Persons with more than one *CYP* polymorphism and/or other relevant polymorphisms may be susceptible to develop DI-ILD when (multiple) drugs are prescribed.[9,35,37] To answer the question whether persons with one or more of these polymorphisms will develop a DI-ILD, whenever future drug prescription is mandatory, needs follow-up. However, since the *CYP* polymorphisms of these patients are already known, this can be taken into consideration when prescribing, thus avoiding (possible) adverse drug reactions. Moreover, a prospective case-control study deriving both treated patients and control patients from one population source of multi-drug users would be useful, in order to evaluate the cost effectiveness of the introduction of pharmacogenetic testing into routine healthcare. Such studies will help to identify factors that increase the risk of unwanted outcomes from drug therapy. They will also help to establish in which circumstances genotyping should be performed prior to commencing drug treatment and in tailoring drug treatment for individual patients.[8,9]

Conclusion

This study indicates that the presence of *CYP* variant genotypes appeared to be a substantial susceptibility risk factor in the development of drug-induced pulmonary adverse events. Therefore, genotyping prior to drug prescription may be clinical useful for the prediction and prevention of drug-induced pulmonary toxicity, especially in case of multiple drug use, where prior genotyping or phenotyping has the potential to contribute to the patients' safety. Both clinical and genetic risk stratification (pharmacogenomics) may lead to more accurate prevention of drug-induced damage in the future.

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Chapter 5

Depressive effect of an antidepressant: therapeutic failure of venlafaxine in a case lacking CYP2D6 activity

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Abstract

Background
Understanding the mechanisms of drug metabolism and interactions can help to prevent side-effects.

Study design
Not only drug interactions, environmental factors, disease processes and aging are factors in the inter-individual metabolic capacity variance, but also genetic factors probably play an important role, as is illustrated in the case presented. Besides therapeutic drug monitoring, genotyping some important cytochrome P450 (CYP) enzymes was of additional value in explaining why the patient developed severe adverse effects and, moreover, did not experience any therapeutical effect of venlafaxine.

Results
Results indicated that the patient was a poor metabolizer for CYP2D6, the most important phase I enzyme to metabolize venlafaxine. This corroborates that polymorphisms in the *CYP* gene influence the metabolic activity of the corresponding enzymes, thus affecting the subsequent serum drug levels and their metabolites.

Conclusion
This case highlights the potential benefit of both clinical and genetic risk stratification (pharmacogenetics) prior to treatment, either for setting the individual dose or for making a decision about using a particular drug.

Introduction

Adverse drug reactions (ADRs) pose a serious medical problem and are an important burden on health-care costs.[1,2] In general, the recognition of ADRs has become an increasingly important area in clinical practice. Clinical presentation of ADRs varies and to relate certain reaction with a drug, there must be a temporal relationship, appropriate clinical presentation and, ideally, improvement with discontinuation of the drug. Drug-induced reactions can cause any known pathological pattern. At present, there are no specific tests; rechallenge is unlikely to be helpful and is generally regarded to be dangerous. Drugs metabolism is influenced by drug-drug interactions, environmental factors, disease processes, food and ageing.[3] Furthermore, there is increasing evidence that interindividual difference in the genetic profile of phase I and II enzymes account for a substantial portion of the heterogeneity of the response to medication(s) and the development of ADRs.[4,5]

Antidepressants are widely used in clinical practice, although the effect is debated. Venlafaxine, for example, belongs to a new generation of antidepressants being a serotonin (5-HT)-norepinephrine (NE) reuptake inhibitor. Venlafaxine and its main active metabolite, *O*-desmethylvenlafaxine (ODV), inhibit the reuptake of both 5-HT and NE with a potency greater for the 5-HT than for the NE reuptake process. Venlafaxine is metabolized by CYP2D6 into its main active metabolite ODV and to a lesser extent by CYP3A4 into the inactive *N*-desmethylvenlafaxine, see also Figure 5.1.[6-8] The suggested therapeutic concentration range lies between 195 and 400 µg/l for the sum of venlafaxine and ODV, and depends mainly on the CYP2D6 activity.[8,9] Decreased CYP2D6 activity increases the risk of side-effects.[7]

Side-effects, due to overdosing, cause only mild symptoms in the majority of patients. However, severe toxicity is reported with the most common symptoms being central nervous system depression, serotonin toxicity, seizure or cardiac conduction abnormalities. Although the exact mechanism of injury is unclear, total serum concentrations of venlafaxine and its major active metabolite ODV of 900 µg/l or more combined are likely to cause toxicity.[6,10] Deaths have been reported following large doses and in combination with other medication and alcohol.[9,11]

Recently, we reported two cases of interstitial pneumonia with cardiac failure developing in patients treated with venlafaxine.[12] A strong relationship between the development of patients' illness and the initiation of venlafaxine treatment was identified. It was hypothesized that alterations in metabolic drug clearance as a result of genetic factors or drug-drug interactions might, in part, be responsible for the drug-induced toxicity. Unfortunately, no DNA was available of these two cases and therefore *CYP* variants could not be confirmed. However, this observation prompted us to evaluate a possible role of this

genetic variant in the presented case. We report a case with a severe refractory depression not responding to venlafaxine in which therapeutic drug monitoring (TDM) in combination with pharmacogenetics were performed in the diagnostic follow-up.

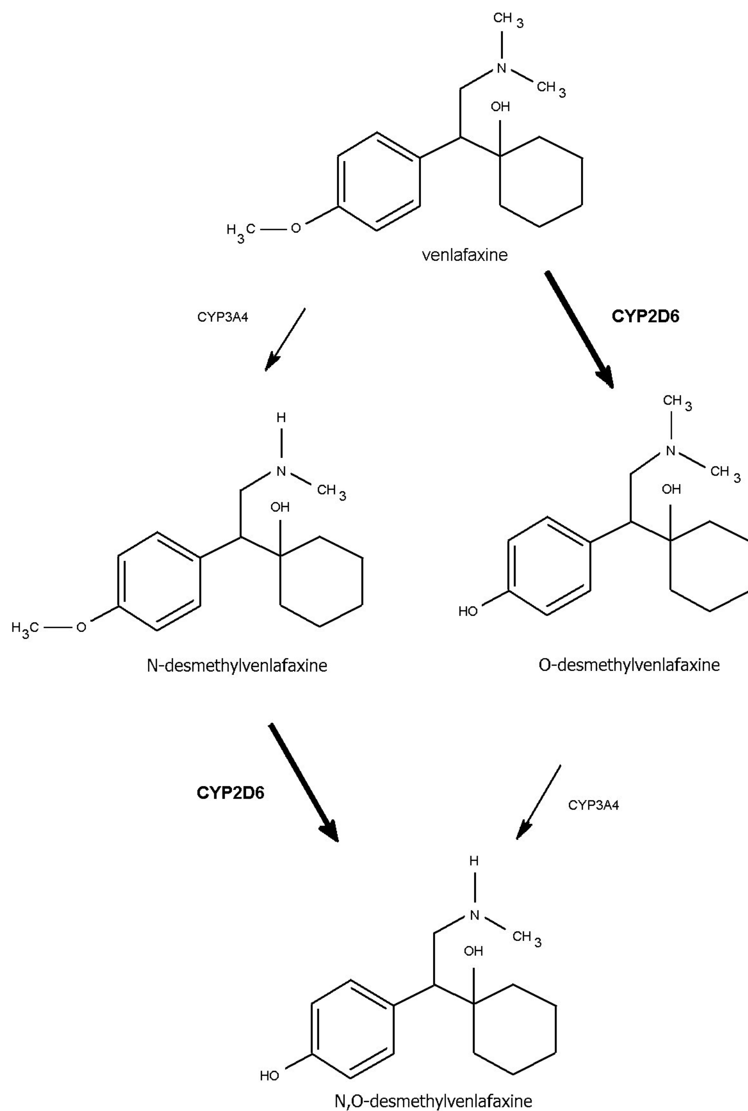

Figure 5.1 Main metabolic pathway of venlafaxine

Case report

A 42-year-old woman was visiting the outpatient hepatology clinic because of, since six weeks existing, progressive fatigue, dyspnoea, paresthesias of the fingers, hands and legs. She felt agitated and had an elevated heart rate in rest of 110 beats per minute. She was known with morbid obesity (body mass index [BMI] 46 kg/m^2), arterial hypertension, left ventricle hypertrophy with a decreased left ventricle ejection fraction (40% of predicted), depression and non-alcoholic steatohepatitis. The medication she used is summarized in Table 5.1. At that time haematological and biochemical laboratory investigations revealed no abnormalities. After her last suicide attempt, two months before presentation at the outpatient clinic, the psychiatrist started venlafaxine 75 mg to treat her depression. As no mental improvement occurred, the venlafaxine dose was increased gradually to 225 mg daily, but still no improvement was established. Moreover, her mental situation worsened and so did the other complaints she presented with at the outpatient clinic. The fore-mentioned adverse effects (especially the tachycardia and agitation) coincided the most with the side-effects listed for venlafaxine, see also Table 5.1. This prompted us to measure the serum venlafaxine level and of its main active metabolite ODV which were 1300 µg/l and less than 100 µg/l, respectively, well above the recommended combined concentration range of 195-400 µg/l.[8] At the same time, three common polymorphisms in the *CYP* genes (*CYP2C9*, *CYP2D6* and *CYP2C19*) were analyzed, as described previously.[13] These tests were performed to rule out or establish a genetic component. Moreover, the genotyping was used to investigate whether venlafaxine was the most likely cause for the ADR, or one or more of the prescribed co-medication not being metabolized properly by the investigated enzymes. For accumulation of other drugs might be causing additional drug-drug interaction problems. A homozygote variant of CYP2D6 (*CYP2D6**4/*4) was found indicating that no functional CYP2D6 enzyme and, therefore, no metabolic activity, was present. Furthermore, a heterozygote variant of CYP2C9 (*CYP2C9**1/*2) and a wild-type genotype of CYP2C19 (*CYP2C19**1/*1) were found. Mainly the fact that the patient appeared to be a poor metabolizer of CYP2D6 substrates explained the high toxic serum levels of venlafaxine without the presence of its main active metabolite ODV. Venlafaxine was stopped immediately and so was metoprolol, also metabolized by CYP2D6, see also Table 5.1.[14] Gradually, her complaints of agitation, dyspnoea and tachycardia disappeared.

Table 5.1 Specifics of the medication and dosages administered at time of presentation.

Drug and dose	Group	Main ADR	Metabolized by
Ursodeoxycholic acid 3 daily dose 300 mg	Dissolves cholesterol stones	Nausea, constipation	Not applicable
Furosemide 40 mg	Loop diuretic	Hypotension, nausea	Glucuronidation
Metoprolol 100 mg	β-blocker	Bradycardia, fatigue, dizziness, hypotension	CYP2D6
Simvastatin 40 mg	Lipid-lowering agent	Headache, asthenia, nausea	CYP3A4
Zopiclone 7.5 mg	Hypnotic agent	Confusion, clumsiness, anxiety	Major CYP3A4, minor CYP2C8
Valsartan 80 mg	Angiotensin II receptor antagonist	Headache, dizziness, asthenia	CYP2C9
Rosiglitazone 4 mg	Thiazolidinedione	Headache, fatigue, diarrhea	Major CYP2C8, minor CYP2C9
Pantoprazole 20 mg	Proton pump inhibitor	Headache, diarrhea, anxiety	CYP2C19
Venlafaxine 75 mg to 225 mg	Serotonin-nor epinephrine reuptake inhibitor	Tachycardia, fatigue, agitation, hypertension	Major CYP2D6, minor CYP3A4

Type of medication (group), main adverse reactions (ADR) and CYP route metabolism is depicted.

Discussion

This case highlights the importance of CYP genotyping in combination with serum drug monitoring to establish a relationship between ADR and drug metabolism. High toxic serum levels of venlafaxine without a detectable concentration of its main active metabolite ODV strongly indicated that no CYP2D6 enzyme activity was present. This was confirmed by genotyping. The complaints with which patient presented herself at the outpatient clinic could be attributed to high levels of venlafaxine and were a direct consequence of the rate of CYP metabolism.[7,15,16] Furthermore, this drug is known to cause serotonin-syndrome in some cases.[17] The complaints were not related to metoprolol, which is also metabolized mainly by CYP2D6, see also Table 5.1. Metoprolol, a selective adrenergic beta-1-blocking agent, was prescribed to the patient because of hypertension. The drug competes with adrenergic neurotransmitters such as catecholamines; resulting in a decreased heart rate, cardiac output and blood pressure. Toxic effects include bradycardia, hypotension, bronchospasm, and cardiac failure, which were not observed in our patient. Another rare side-effect of metoprolol therapy can be depressive symptoms, but as the patient was prescribed metoprolol years before the onset of the depression, this was unlikely.[18,19] A more plausible explanation is the fact that patients can also be non-responders to venlafaxine therapy, not linked to a certain CYP450 genotype, but in combination with a poor metabolizer the risk of a maintained depression and acquiring ADRs is increased substantially.[8,20]

Other more rare ADRs include pulmonary (e.g. interstitial pneumonia) and cardiovascular (e.g. agina pectoris, sinus arrythmia) problems and even sudden deaths have been reported, suggesting venlafaxine and/or its metabolites to be a possible proarrhythmic agent.[21-23] Direct toxic effect or hypersensitivity is likely to be the mechanism.
Venlafaxine is mainly metabolized by CYP2D6 and only for a very small amount by CYP3A4, see also Figure 5.1.[8,24] In particular, *CYP2D6* variants may manifest phenotypically as poor, intermediate, extensive or rapid metabolizers.[25,26] Administration of venlafaxine to poor metabolizers places them at risk of accumulation of the drug to toxic concentrations.[7] Moreover, poor metabolizers are more likely to experience ADR.[7] Also, co-administration of venlafaxine with drugs that inhibit the activity of CYP2D6, such as some antiarrhythmic agents, could provoke accumulation of the drug and predispose patients to drug-induced damage.[27] However, the venlafaxine datasheet states that co-prescription of venlafaxine and metoprolol, although it should be exercised with caution, poses no problem in a normal situation when checking the patient's blood pressure regularly.
Genotyping prior to administration of the drug probably prevents unnecessary medication for at least six weeks (period needed for evaluating the effect of the medication). Furthermore, and possibly even more important, it warns the psychiatrist that increasing the dose is no option, since the active metabolite will not be formed due to the inappropriate metabolism of venlafaxine. Moreover, it exposes the patient to an increased ADR risk, because of decreased clearance. The risk for development of drug toxicity, including drug-induced lung damage and cardiopulmonary effects, differ depending on a variety of host (including *CYP* polymorphisms) and drug factors.[23,28] Increased serum levels of drugs can be observed, not only after overdose administration of the drug, but also because of decreased clearance in some subjects, due to, as in this case, an incapability to metabolize or drug-drug interactions.[25]
The promise of pharmacogenetics, the study of the relationship between variants in a large collection of genes and variable drug effects, lies in its potential to identify the right drug and dose for each individual patient.[29] Thus, genotyping can provide therapeutic and pharmaco-economic benefits and should be considered in patients using (multiple) drugs.[29,30] The affected CYP450 enzyme, causing an inappropriate effect of the antidepressant, can also influence other drugs metabolized by the same CYP system. Therefore, physicians should prescribe combinations of certain drugs with caution.

Conclusion

Introduction of venlafaxine in our patient resulted in tachycardia and agitation without improvement of the depressive symptoms. It appeared that the patient was a poor metabolizer for CYP2D6, the most important phase I enzyme to metabolize venlafaxine. Discontinuation of this drug resulted in resolving of the symptoms. This case highlights the potential benefit of both clinical and genetic risk stratification (pharmacogenetics) prior to treatment, either for setting the individual dose or for making a decision about using a particular drug. To minimalize ADRs, genetic screening prior to prescription of certain 'risk drugs' and TDM during treatment would be an ideal situation.

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Chapter 6

Variant VKORC1 and CYP2C9 alleles in patients with diffuse alveolar haemorrhage caused by oral anticoagulants

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Abstract

Background
Diffuse alveolar hemorrhage (DAH) is a life threatening bleeding complication that can occur as a result of oral anti-coagulation therapy.
We hypothesized that in patients treated with coumarins, alveolar hemorrhage is associated with vitamin K epoxide reductase (*VKORC1*) and cytochrome P450 (CYP) 2C9 *(CYP2C9)* variant alleles. In addition, in the case of acenocoumarol use, *CYP2C19* allelic variants also play a role.

Study design
During a 7-year period patients using coumarins with confirmed DAH were gathered. Out of 173 confirmed DAH cases, 75 received oral anticoagulants, and 63 patients (84%) of these 75 were included because their DNA was available. For genotyping the *CYP2C9**2 (C430T), *CYP2C9**3 (A1075C), *CYP2C19**2 (G681A), *CYP2C19**3 (G636A), *VKORC1* (G-1639A) and *VKORC1* (C1173T) single nucleotide polymorphisms (SNPs), real-time PCR's were performed.

Results
In 62 (98.4%) out of 63 DAH patients, variant genes were found. In 51 (81.0%) of the 63 patients *VKORC1* allelic variants (20 homozygote and 31 heterozygote) were present. In 31 (49.2%) of the 63 DAH cases *CYP2C9* variant alleles (three homozygote, 26 heterozygote, and two compound heterozygote) and in 20 (32.0%) of the 63 patients both allelic variants were observed.

Conclusion
Genotyping of four SNPs for *VKORC1* and *CYP2C9* polymorphisms is useful in predicting a high probability of the occurrence of DAH, in patients on oral anticoagulants. Early and timely use of genotyping is recommended to prevent a fatal outcome and to provide a safer and more individualized anticoagulant therapy.

Background

Coumarin-based oral anticoagulants act as vitamin K antagonists. They are the most commonly prescribed drugs for therapy (such as in venous thrombosis or pulmonary embolism) or prophylaxis (as in chronic atrial fibrillation, prosthetic heart valves and other cardiovascular diseases) of thromboembolic conditions. The primary goal of coumarin administration is to prevent clot formation and its expansion while carefully avoiding unintended adverse drug reactions (ADR) from over anticoagulation.[1] The effect of the therapy is monitored by the prothrombin-time international normalized ratio (INR). An INR of less than 2.0 is associated with an increased risk of thromboembolism, and an INR of 4.0 or more denotes an increased risk of bleeding.[2]

One of the bleeding complications occurring in patients receiving coumarins, is diffuse alveolar hemorrhage (DAH).[3] DAH may be fulminant and lead to death. Due to the severe effects of overdosing and the narrow therapeutic window, correct management of coumarins is challenging. A safe and effective dose has to be determined during the early phase of therapy, and maintenance doses need to be adjusted to compensate for changes in patients' weight, diet, disease state and concomitant use of other medication.[4] The challenge is becoming even more demanding because of the increased use of coumarins that is a consequence of the aging of populations in industrialised countries.

Despite the ability to closely monitor the therapeutic effect of coumarins, by means of the INR, there is a relatively high incidence of complications.[1] Since early treatment of these complications is life-saving and may result in complete recovery. Therefore, early diagnosis can be critical. At present the diagnosis of DAH is often made by an increased percentage of siderophages (>20%) in bronchoalveolar lavage fluid (BALF), indicated with Perl's staining.[5]

Instead of early diagnosis, prevention would of course be much more preferable. The relatively high inter-individual drug requirement indicates that genetic factors may impact the therapeutic effect of coumarins. The strongest predictors of coumarin induced anticoagulant effects appear to be genes encoding for the enzyme vitamin K epoxide reductase complex 1 (VKORC1), the target of vitamin K antagonists. The enzyme VKORC1 recycles vitamin K epoxide to the reduced form of vitamin K, an essential cofactor in the formation of active vitamin K dependent clotting factors II (prothrombin), VII, IX, and X through γ-glutamyl carboxylation, see also Figure 6.1.[6]

Another predictor appears to be cytochrome P450 (CYP) 2C9, the enzyme mainly responsible for the metabolism of coumarins.[4,7,8] For instance patients with the common, functionally defective, *2 and *3 allelic variants of the *CYP2C9* gene require significantly lower maintenance doses, take longer to achieve dose stabilization, and are at higher risk for serious and life-threatening bleeding than are patients without these variants.[8]

When using acenocoumarol as an oral anticoagulant, one might even consider CYP2C19, although its contribution to the metabolism of acenocoumarol is small compared with CYP2C9.[9]

This study evaluates the association between the occurrence of DAH in patients after initiating coumarin anticoagulant therapy and the presence of *VKORC1* and *CYP2C9* allelic variants.

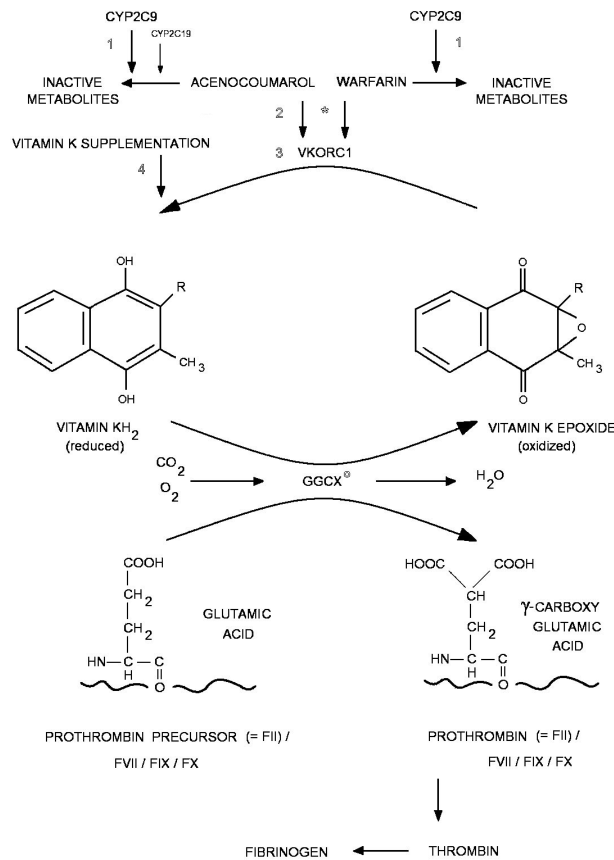

Figure 6.1 Interactions in the vitamin K cycle and coagulation. The vitamin K cycle plays an important role in the formation of functional vitamin K-dependent clotting factors (FII, FVII, FIX, and FX). Within the vitamin K cycle VKORC1 is responsible for the reduction of vitamin K epoxide (the inactive form) to vitamin KH_2 (the active form) and the target for oral anticoagulants. Interactions can occur on several levels: (1) If variant *CYP2C9* alleles are present, inadequate metabolism of coumarins by the affected CYP enzyme will result in more inhibition of VKORC1. (2) If variant *VKORC1* alleles are present, the VKORC1 enzyme will be more sensitive to inhibition by anticoagulants, resulting in over-anticoagulation. (3) Inhibition of VKORC1 by coumarins will prevent vitamin K epoxide to revert back to vitamin KH_2, slowing down the vitamin K cycle. This inhibits the formation of vitamin K-dependent clotting factors. High levels of coumarins cause over-anticoagulation. (4) Vitamin K supplementation stimulates the vitamin K cycle, thus preventing over-anticoagulation. * indicates antagonism.

Materials and methods

Setting and study population

Patients who were diagnosed with DAH at the Maastricht University Medical Centre (Maastricht, the Netherlands) from 2002 until 2009 were enrolled in the study. The inclusion criteria were bronchoalveolar lavage (BAL) performed in the diagnostic work-up and confirmed anticoagulant therapy initiated before the clinical episode of DAH. During this 7-year period, 1258 BAL analyses were carried out, and 252 cases with suspected DAH were identified. BAL was performed according to the hospital protocol, as reported previously.[10] A total of 200 macrophages were counted, the total number of iron-stain (Perl's stain)-positive macrophages were expressed as a percentage of the 200 cells counted. A percentage of >20% iron-positive macrophages was considered indicative for an alveolar hemorrhage.[5] Of the obtained BALF samples, 173 samples had >20% iron-stain-positive cells. In 75 of these 173 confirmed DAH cases, treatment with coumarins had been recently initiated. This study was a retrospective evaluation, and DNA was available only in 63 (84%) out of 75 cases. In 40 of these 63 DAH cases, either genotyping for *CYP2D6*, *CYP2C9*, and *CYP2C19* had been performed previously to evaluate whether there might be a drug-induced reaction involved in the observed clinical deterioration, or EDTA material was still available. In 23 cases, DNA was isolated from the cells in the BALF samples. All remaining samples were genotyped for CYP polymorphisms. In addition, for this study, *VKORC1* genotyping was performed in these 63 DAH cases.
A control population of 173 healthy, unrelated, Caucasian volunteers was also genotyped for the studied single nucleotide polymorphisms (SNPs).
The study was performed in accordance with the Declaration of Helsinki and its amendments. Written informed consent was obtained. The protocol was approved by the Medical Ethics Board of the Maastricht University Medical Centre.

Collection of clinical data

Inpatient and outpatient medical records of these 63 unrelated patients of Caucasian origin, presenting with DAH and using coumarins, were reviewed. Two patients received phenprocoumol, and the remaining 61 patients received acenocoumarol. Routine laboratory tests, chest X-rays, and high-resolution CT scans were reviewed in all patients. Appropriate and relevant biopsies were also evaluated when available (18 cases).

Genotyping

In addition to the previously determined polymorphisms, *VKORC1* genotyping was performed.
DNA was obtained from all subjects by using venous EDTA anticoagulated blood or BALF samples and isolated with a High Pure PCR Template Preparation Kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions.
For genotyping the *CYP2C9**2 (C430T), *CYP2C9**3 (A1075C), *CYP2C19**2 (G681A), *CYP2C19**3 (G636A), *VKORC1* (G-1639A), and *VKORC1* (C1173T) SNPs, three real-time PCR Fluorescence Resonance Energy Transfer (FRET) analyses were performed. FRET LightMix® assays (cat.-no. 40-0298-16, 40-0304-16, and 40-0302-16; TIB MOLBIOL, Berlin, Germany) on the LightCycler® (Roche Diagnostics) were used, according to the manufacturer's protocols. These three FRET assays simultaneously determined the two SNPs of *CYP2C9*, *CYP2C19* and *VKORC1*, respectively, in separate capillaries. Each assay consisted of a duplex reaction measuring the melting curves of the used specific fluorescent probes in two different channels, each with a distinct wavelength. Positive (heterozygote, provided with the kit) and negative controls were determined with each run.

Statistical analysis

Statistical analyses were performed with SPSS version 15.0 software for Windows (SPSS Inc., Chicago, IL, USA) for Windows. The chi-square test was used to test for statistically significant differences between groups. Odds ratios (ORs) with 95% confidence intervals (CIs) were derived from these tables to evaluate the strength of associations between genotypes and the DAH event. Actual allele distributions were compared against the expected frequencies that were calculated, using the Hardy–Weinberg equilibrium. Deviations from Hardy–Weinberg equilibrium were analysed using the chi-square test. A p-value of <0.05 (two-sided) was considered to indicate statistical significance. A Bonferroni correction was applied, if appropriate, to adjust for multiple comparisons ($p<0.01$, indicating statistical significance).

Results

The characteristics and summary of relevant clinical data of the studied patients (15 females and 48 males) with DAH are listed in Table 6.1. Data subdivided on the basis of the presence of the studied polymorphisms did not show substantial differences (Table 6.1).

Table 6.1 dwars

The reasons for the patients being on anticoagulants were as follows: atrial fibrillation or flutter (n=34); previous myocardial infarction (n=12); chronic heart failure (n=7); lung embolisms (n=4); valve replacement surgery (n=4); deep-vein thrombosis (n=2). The average dose of the coumarins was low (maximum 2 mg/day, with an initial dosing scheme of 6 mg/day for one day, then 4 mg/day for one day, and then 2 mg/day or 4 mg/day for two days and then 2 mg/day in elderly patients). The INR was above the therapeutic range within two weeks after initiation of anticoagulant treatment in all DAH cases (median 5.50, with 80% INR >4.00 and 40% INR >6.00). Most of the patients had several episodes of an increased INR during the follow-up, were difficult to normalize, and had to be kept on lower anticoagulant doses than is standard for the general population. Immunological analysis revealed no abnormalities, and no underlying systemic diseases were found. The high-resolution CT showed wide spread signs of DAH, with patchy bilateral or diffuse areas of ground-glass attenuation in all cases. A BAL was performed in all subjects, showing hemorrhagic BALF with a markedly positive iron staining (>22%, mean 59.3±25.7) and the presence of erythrocytes and pneumocytes type II, confirming the diagnosis DAH.[10]
The allele frequencies and genotype distribution in the DAH patients were determined and compared with the distribution of the same polymorphisms in a healthy, unrelated, Caucasian population from our hospital and from populations in the literature (Table 6.2).[11,12] Allele frequencies and genotype distributions of both control populations were in Hardy-Weinberg equilibrium. A *VKORC1* variant allele was found in 51 of the 63 patients with DAH (81.0%, p<0.025). This included 20 homozygotes (AA/TT), including the only two patients in our population receiving phenprocoumol, and 31 heterozygotes (GA/CT). A *CYP2C9* allelic variant was found in 31 of the 63 patients (49.2%, p<0.025), including three homozygotes, 26 heterozygotes, and two compound heterozygotes. Twenty (32.0%) out of the 63 DAH cases had both *VKORC1* and *CYP2C9* allelic variants. In 31 (49.2%) out of these 63 DAH cases, only a *VKORC1* allelic variant was found. Of the 12 DAH cases without a *VKORC1* allelic variant (GG/CC), five were *CYP2C9**1/*2 heterozygotes, four were *CYP2C9**1/*3 heterozygotes, and two had a compound heterozygous variant *CYP2C9**2/*3 genotype. The remaining subject had no variant of the studied alleles. When comparing patients with controls for the presence of a polymorphism (polymorphism present vs no polymorphism), a significant difference was found (OR=14.6, 95% CI: 1.96-109.3; p<0.001). Furthermore, a *CYP2C19* variant allele was found in one third of the DAH cases. In all of these patients, this coincided with the presence of a *CYP2C9* and/or *VKORC1* allelic variant (Table 6.3).

Table 6.2 Allele frequencies and polymorphism distribution in diffuse alveolar hemorrhage (DAH) patients compared with healthy volunteers and historical controls.

	Patients with DAH[a]			Healthy volunteers[b]			Controls from the literature[c]		
	CYP2C9 (%)	*VKORC1* (%)	*CYP2C19* (%)	*CYP2C9* (%)	*VKORC1* (%)	*CYP2C19* (%)	*CYP2C9*[11] (%)	*VKORC1*[11] (%)	*CYP2C19*[12] (%)
No variant allele	50.8	19.0	66.7	61.9	29.5	76.3	64.0	34.0	75.3
Variant allele	49.2[d]	81.0[e]	33.3[f]	38.1	70.5	23.7	36.0	66.0	24.7
Allele[g]									
*1	71.4		81.7	79.7		86.4	80.0		86.5
*2	15.9		18.3	13.9		13.6	13.5		13.3
*3	12.7		0.0	6.4		0.0	6.5		0.2
G/C		43.7			52.3			58.5	
A/T		56.3			47.7			41.5	

[a] n=63; sex 76.2% male, 23.8% female; age range=20–85 y; [b] n=173; sex 56.6% male, 43.4% female; age range=19–59 y; [c] For *CYP2C9* and *VKORC1*: n=200; sex 50% male, 50% female; age range =18–24 y; For *CYP2C19*: n=736; sex 82% male, 18% female; age range=18–79 y; [d] p=0.022 vs healthy volunteers; p=0.006 vs historical controls; [e] p=0.021 vs healthy volunteers; p=0.0015 vs historical controls; [f] p=0.024 vs healthy volunteers; p=0.046 vs historical controls; [g] For *CYP2C9*, *1=wild type; *2=430T, and *3=1075C; for *CYP2C19*, *1=wild type, *2=681A and *3=636A. *VKORC1* SNPs are G-1639A and C1173T; genotype G/C is wild type and A/T is variant.

Table 6.3 Influences on the coagulation: allelic variants and co-medication in patients (n=63) with diffuse alveolar hemorrhage.

Patient no.	*CYP2C9*[a] genotype	*CYP2C9*[a] influence	*VKORC1*[b] genotype	*VKORC1*[b] influence	*CYP2C19*[c] genotype	*CYP2C19*[c] influence	Influence of co-medication (yes/no)
3	*1/*3	Yes	AA/TT	Yes	*1/*2	Yes	2/1
1	*1/*2	Yes	AA/TT	Yes	*1/*2	Yes	1/0
1	*2/*2	Yes	AA/TT	Yes	*1/*1	No	0/1
1	*1/*3	Yes	AA/TT	Yes	*1/*1	No	1/0
2	*1/*2	Yes	AA/TT	Yes	*1/*1	No	2/0
2	*1/*1	No	AA/TT	Yes	*2/*2	Yes	1/1
3	*1/*1	No	AA/TT	Yes	*1/*2	Yes	2/1
7	*1/*1	No	AA/TT	Yes	*1/*1	No	3/4
2	*2/*2	Yes	GA/CT	Yes	*1/*1	No	1/1
6	*1/*3	Yes	GA/CT	Yes	*1/*1	No	5/1
4	*1/*2	Yes	GA/CT	Yes	*1/*1	No	3/1
11	*1/*1	No	GA/CT	Yes	*1/*2	Yes	8/3
8	*1/*1	No	GA/CT	Yes	*1/*1	No	4/4
2	*2/*3	Yes	GG/CC	No	*1/*1	No	1/1
1	*1/*3	Yes	GG/CC	No	*1/*2	Yes	0/1
3	*1/*3	Yes	GG/CC	No	*1/*1	No	2/1
5	*1/*2	Yes	GG/CC	No	*1/*1	No	2/3
1	*1/*1	No	GG/CC	No	*1/*1	No	1/0

[a] *CYP2C9* SNPs are C430T and A1075C; allele designations: *1=wild type; *2=430T, and *3=1075C; [b] *VKORC1* SNPs are -G1639A and C1173T; genotype GG/CC is homozygous wild type and AA/TT is homozygous variant; [c] *CYP2C19* SNPs are G681A and G636A; allele designations: *1=wild type, *2=681A and *3=636A.

The influence of allelic variants and co-medication on the anticoagulation in each individual case is summarized in Table 6.3. In about 60% of all the patients, co-medication was prescribed that might have influenced the coagulation. In only one patient - the one without any variant alleles in the *VKORC1* or *CYP2C9* - the co-medication might have caused the bleeding. In this patient, a drug-drug interaction was most likely, with no fewer than four drugs (amiodarone, paroxetine, pantoprazole, and clopidogrel), being used that could have interacted with the anticoagulant. The drugs prescribed to the patients that might have influenced the anticoagulation are listed in Table 6.4.
Since May 2007, genotyping for the *VKORC1* polymorphism, together with the earlier described CYP polymorphisms, has been available on request for clinicians in our hospital. Subsequently, in the period between May 2007 and December 2008, 11 patients with DAH were identified and included in this study. In all of these patients, vitamin K supplementation (1 mg/day, orally) was started. Out of the 11 patients, 10 responded quite well, recovered, and are still alive. Of the 63 studied DAH cases, 37 patients died, primarily because of complications related to heart failure in combination with DAH.

Table 6.4 Medication influencing the coagulation in the patients in which this was relevant (n=39).

Administered co-medication	No. of patients
amiodarone	15
amitriptyline	1
aspirin	5
atorvastatine	7
carvedilol	5
clopidogrel	2
colchicine	1
esomeprazol	1
felodipine	1
fluoxetine	1
insulin	2
isoniazid	1
levothyroxine	1
nifedipine	1
omeprazole	5
pantoprazole	15
paroxetine	4
prednisone	8
ranitidine	1
rifampin	1
simvastatin	2
trimethoprim/sulfamethoxazole	2
valproic acid	2
verapamil	2

Discussion

Anticoagulants can cause fatal pulmonary hemorrhage. Barnett et al.[13] reported a case of DAH due to superwarfarin ingestion. More recently, Erdogan et al.[3] reported a case of DAH associated with coumarin therapy. We described a case of DAH in a patient who had malnutrition and was taking antibacterials and anticoagulants; at that time, genotyping was not yet available.[14] DAH results in accumulation of iron in the lungs and, in turn, iron causes oxidative stress and inflammation. It has been suggested that oxidative damage plays a role in the pathophysiology of various diseases.[15] It is important to prevent or recognize DAH at an early stage to avoid irreversible damage. Particularly, in critically ill patients with unexplained infiltrates, DAH should be considered. DAH events can occur as a result of over-anticoagulation due to coumarin sensitivity, caused by *VKORC1* or *CYP2C9* polymorphisms, resulting in a relative vitamin K deficiency. Prophylactic administration of vitamin K to patients at risk can prevent severe damage.[16-18] Just recently, the information gathered from genotyping in this study has become available to clinicians. Subsequently, vitamin K supplementation (1 mg/day) was initiated in 11 DAH cases, resulting in a stabilization of the INR and a positive outcome in 10 of these 11 DAH cases. This supports the concept that the use of new pharmacogenetic-based dosing schemes and the concomitant application of low-dose vitamin K with coumarins will greatly improve coumarin drug safety.[18,19]

Pharmacogenomics uses the tools of human genetics to tailor medical treatment to an individual's genetic make-up. To this end, phenotypic manifestations, a therapeutic outcome, or ADRs are considered in relation to the underlying genetic background of a patient.[7,20] The identification of the molecular target of coumarins, VKORC1, has greatly improved the understanding of coumarin treatment and illuminated new perspectives for a safer and more individualized oral anticoagulation therapy. Rieder et al.[4] previously demonstrated that the *VKORC1* genotype appeared to be the most important genetic factor determining variability in coumarin dose; its effect was approximately three times higher than that of the *CYP2C9* genotype. More recently, in line with this, Schwarz et al.[16] concluded that the initial variability was more strongly associated with genetic variability in the pharmacogenetic target of coumarins, VKORC1, than with CYP2C9.

Variations and SNPs within the translated and non-translated regions of the *VKORC1* gene have been shown to cause coumarin resistance and sensitivity, respectively.[21] A frequent SNP within the *VKORC1* promoter (G-1639A) has been identified as a major determinant of coumarin sensitivity, reducing vitamin K epoxide reductase enzyme activity to 50% of wild type (GG = fully functional). Homozygous carriers of the *VKORC1* -1639A allele (AA) are

strongly predisposed to coumarin sensitivity and require lower coumarin dosages. However, the link between DAH and the presence of *VKORC1* and *CYP2C9* variant alleles has never been made before.

To the best of our knowledge, our study is the first one evaluating the association between the occurrence of a serious adverse reaction of anticoagulant therapy, DAH, and the presence of relevant polymorphisms. We found that in 62 (98.4%) of the patients in our study population, a variant allele was present. In 81.0% (51/63) of the studied patients, the bleeding complication could be explained by the *VKORC1* haplotype (61.0% without and 39.0% with a *CYP2C9* allelic variant) alone. As also shown in Table 6.2, in only 19.0% of the patients had no *VKORC1* 1173T/-1639A variant alleles, compared with 29.5% in a healthy volunteer population (n=173) and 34.0% in a historical control population (n=200) from the literature.[11] In 11 of the 12 DAH cases without a *VKORC1* variant the *CYP2C9* variant allele could explain the problems in reaching an appropriate INR. The present *CYP2C9* functionally defective allelic variant required a 34% lower maintenance dose for the *1/*3 genotype and a 61% reduction for the *2/*3 genotype, compared with 13% for the *CYP2C9**1/*2. In the one remaining patient without any variant of the alleles studied, the high INR and subsequent DAH event seemed attributable to drug-drug interactions. One third of the patients had an extremely high risk as they appeared to have both genetic risk factors that are known to stratify patients into low dose/high-risk cases.[16] Furthermore, the patients with *VKORC1* and/or *CYP2C9* allelic variants present need longer times before dose stabilization and are at a higher risk for serious and life-threatening bleeding, including DAH, than patients without these variants.[8,22]

Our observation confirms that genotyping four SNPs, namely of the *VKORC1* and the *CYP2C9* genes, predicts a high risk of overdosing with coumarins (warfarin, acenocoumarol, phenprocoumon).[23] Although other studies have reported a strong linkage disequilibrium between the SNPs in the *VKORC1* gene, our own experience shows that sometimes only one of the examined SNPs can display a variant allele.[24,25] Accordingly, this implies a potential risk factor could be missed if only one SNP is examined. Moreover, in this study, the *VKORC1* results were obtained in one run, using a reagent combining primers and probes for both SNPs, without any extra time or costs. In the case of oral anticoagulation with acenocoumarol, genotyping for the *2 (G681A) and *3 (G636A) allelic variants in the *CYP2C19* gene could be performed. Although acenocoumarol is mainly metabolized by CYP2C9 it is also partly metabolized by CYP2C19 (Figure 6.1).[9] Polymorphisms in this enzyme system could therefore present additional anticoagulation problems. In our population, however, this polymorphism was of minor importance. All of the subjects with a *CYP2C19* variant allele (33.3% of all of the patients) also displayed one or both of the other two studied polymorphisms.

One of the limitations of this study was the fact that DNA was available in only 63 of the 75 subjects who used oral anticoagulants and were diagnosed with DAH. Therefore, conclusions from this case series should be interpreted with care, and prospective studies should be conducted to evaluate the cost effectiveness of genotyping. Moreover, confirmation of our findings in other populations is mandatory. However, the fact that all but one of the included patients with DAH demonstrated at least one of the studied genetic defects makes the association highly likely. The merits of genotyping before starting treatment involving drugs such as coumarins, the effectiveness of which depends on genetic variants of *CYP2C9* and *VKORC1*, is still an area of debate between regulatory authorities and clinicians. Even though genotyping four SNPs is relatively cheap (about $US 200-250 in 2009) and needs to be performed only once in a lifetime, until its cost effectiveness is established, one could choose to only genotype patients who experience unstable INRs, in order to avoid serious complications. Nevertheless, as of August 2007, the US FDA issued a recommendation to genotype *CYP2C9* and *VKORC1* in warfarin product labeling, to optimize dosing schedules when prescribing warfarin.[24,26] Furthermore, it is tempting to speculate that by using individualized dose adaptation, a significant reduction of bleeding complications, including DAH, can be expected, especially in the initial drug-saturation phase.[4]

Conclusion

In all but one of the studied patients with DAH treated with coumarins, an association with either a *VKORC1* or a *CYP2C9* variant allele, or both, was found. Early and timely use of appropriate genotyping is important in case of coumarin treatment, because of the potential fatal outcome of over-anticoagulation and the fact that simple vitamin K supplementation can be life-saving. Therefore, in concordance with the FDA, genotyping of only four SNPs for *VKORC1* and *CYP2C9* allelic variants is recommended in order to provide a safer and more individualized anticoagulant therapy.

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Chapter 7

The role of TNF-α G-308A polymorphisms in the course of pulmonary sarcoidosis

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Abstract

Background
This study was designed to evaluate the relationship between the presence of tumor necrosis factor (TNF) polymorphisms, human leukocyte antigen (HLA)-DRB1*03 linkage and the prognosis of sarcoidosis.

Study design
In a retrospective case-control study, *TNF-α* G-308A, *TNF-α* G-238A, *lymphotoxin-α* (*LTA*) and HLA-DRB1*03 were genotyped in 625 sarcoidosis patients. These patients were classified into 298 patients with persistent disease and 327 patients with non-persistent disease using chest X-ray (CXR) appearances and lung function parameters after at least two years of follow-up.

Results
The *TNF-α* -308A variant allele was observed in 25.5% of patients with persistent disease compared with 44.0% of patients with non-persistent disease. The corresponding odds ratio (OR) was 0.43 with a 95% confidence interval (CI) of 0.30-0.61. A strong linkage was found between *TNF-α* G-308A and HLA-DRB1*03 (OR=0.03, 95% CI: 0.02-0.05). For *TNF-α* G-238A and *LTA NcoI* A252G, there were no statistically significant differences in the distribution of genotypes between the groups with and without persistent disease.

Conclusion
The data indicate that presence of a *TNF-α* -308A variant allele and HLA-DRB1*03 were associated with a favorable prognosis. Because of the strong linkage between *TNF-α* G-308A and HLA-DRB1*03, genotyping of one simple and less expensive TNF-α single nucleotide polymorphism can be used to predict the prognosis of pulmonary sarcoidosis in clinical practice.

Introduction

Sarcoidosis is a multisystem granulomatous disorder with distinct immunopathologic features. The disease is most likely the product of genetic susceptibility and an appropriate environmental antigen.[1-3] The clinical presentation and outcome of sarcoidosis varies considerably. Therapeutic options range from no treatment to a variety of therapeutic agents.
It is well known that the outcome of sarcoidosis varies considerably.[4] The presence of hilar adenopathy alone[5,6] or patients presenting with Löfgren's syndrome more often have a favorable outcome as compared with those with parenchymal lung disease.[7-9] By contrast, the presence of pulmonary fibrosis is associated with chronic disease.[5] Pulmonary disease is the most common manifestation of sarcoidosis, and pulmonary symptoms are the most common reason for treatment.[1,3] It is important to identify the patients who are likely to have a poor prognosis, to ensure the appropriate individual treatment regimen without delay.[10] Genetic polymorphisms contribute to clinical phenotypes.[6,9,11] Association of sarcoidosis and class I and II human leukocyte antigens (HLA) antigens is well known.[3,6,12] Grunewald et al. reported an influence of both HLA class I and class II alleles on the disease course in patients with sarcoidosis. They found that 76.8% of patients with resolving disease, appeared to be HLA-DRB1*03 positive, in contrast to only 9.2% of patients with persistent disease.[6] Tumor necrosis factor alpha (TNF-α), a potent pro-inflammatory cytokine that plays a pivotal role in inflammatory and immune responses, regulates and sustains granuloma formation in sarcoidosis.[3] TNF-α production is an innate host characteristic that varies between individuals and is associated with certain HLA-D receptor (HLA-DR) alleles.[13] The TNF gene locus, comprising of the *TNF-α*, *lymphotoxin-α* (*LTA*, formerly also referred to as *TNF-β*) and *lymphotoxin-β* genes, is located in the class III region of the major histocompatibility complex (MHC). Genetic analysis has showed a number of polymorphisms in these genes and new polymorphisms with potential functional consequences.[7,14-16] Of genes in the MHC III region, the *TNF-α* polymorphisms have been extensively studied. Several single nucleotide polymorphisms (SNPs) are identified in the *TNF-α* gene. Among these, a common polymorphism in the promoter region, a G to A substitution at position -308, has been associated with variation of TNF-α production. Especially the A-allele of the *TNF-α* G-308A gene is associated with higher TNF-α serum levels and an acute course of sarcoidosis.[17] The existence of a strong linkage disequilibrium between HLA-DRB1*03 and the *TNF-α* -308A variant allele has been showed.[6,9,18] In addition to the *TNF-α G-308A* polymorphism, others found that the *TNF-α* G-238A and *LTA NcoI* A252G SNPs were associated with variations in TNF-α production and suggested a possible role in the course of sarcoidosis.[7,8,11,17]

The aim of the present study was to assess the association among *TNF-α* G-308A*, TNF-α* G-238A and *LTA NcoI* A252G variant alleles and clinical outcome in sarcoidosis. In addition, the linkage between *TNF-α* G-308A and HLA-DRB1*03 and its influence on disease course in our population was studied.

Materials and methods

Patients

From January 2000 to July 2008, 625 consecutive Dutch Caucasian sarcoidosis patients, attending the outpatient referral clinic of the Sarcoidosis Management Center of the Department of Respiratory Medicine of the Maastricht University Medical Centre (MUMC) were included in this study. The time since diagnosis to inclusion and/or follow-up for all patients was at least two years. The diagnosis was based on a positive biopsy in 71% of cases. In patients with typical features of Löfgren's syndrome and characteristic features of bronchoalveolar lavage (BAL) fluid analysis results, no biopsy was obtained. This policy is consistent with the World Association of Sarcoidosis and Other Granulomatous diseases (WASOG) guidelines.[1]

The study was performed in accordance with the Declaration of Helsinki and its amendments. The protocol was approved by the Medical Ethics Board of the MUMC. Written informed consent for participation in this study was obtained from all subjects.

Collection of clinical data

Clinical course of patients with sarcoidosis was defined using changes in CXR stage and lung function parameters during follow-up. All CXR films were graded by a single observer, who was not aware of the clinical data. Five stages of the radiographical abnormality were recognized: stage 0 (normal CXR), stage I (bilateral hilar lymphadenopathy [BHL]), stage II (BHL and parenchymal abnormalities), stage III (parenchymal abnormalities without BHL) and stage IV (end stage lung fibrosis).

For the main analysis, patients were categorized into groups with and without persistent sarcoidosis. Persistent disease was defined as worsening of the CXR stage to stage II or higher, or remaining at CXR stage II or III, at least two years after diagnosis. Non-persistent disease cases were those who remained at or regressed to stage 0 or I.

At inclusion, the forced vital capacity (FVC) and forced expiratory volume in 1 s (FEV_1) were measured with a pneumotachograph (Masterlab, Jaeger, Würzburg, Germany). The diffusing capacity for carbon monoxide (DLCO) was

measured by the single-breath method (Masterlab). Values were expressed as a percentage of predicted values. The cut-off value for the DLCO, FEV_1 and FVC was <80% of predicted (≥80% is normal).[19]

TNF and HLA typing

TNF

DNA was obtained using venous EDTA anti-coagulated blood and isolated with a High Pure PCR Template Preparation Kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions.
All patients were genotyped for two *TNF-α* promoter polymorphisms (G-308A and G-238A) and one *LTA* polymorphism (*LTA NcoI* A252G, also known as *lymphotoxin-α*, *LTA_NcoI*, *LTA+252,* or *TNF-β NcoI* A329G).[20,21] For genotyping *TNF-α* G-308A, *TNF-α* G-238A and *LTA NcoI* A252G SNPs, real-time PCR Fluorescence Resonance Energy Transfer (FRET) assays (TIB MOLBIOL, Berlin, Germany) were performed as described by Bestmann et al.[20] on the LightCycler® (Roche Diagnostics, Mannheim, Germany). The person who performed the analyses was blinded to the clinical data that were used for the classification of subjects according to disease course.

HLA

Genomic DNA was isolated with QIA-AMP kits following the supplier's protocol (Qiagen, Westburg, Leusden, The Netherlands). Concentration and purity of DNA samples were measured at 260 nm and 260/280 nm.
Low-resolution typing of HLA-DRB1 was obtained by Luminex reverse SSO, using bead kits from One Lambda (One Lambda, Bethesda, MD) or by PCR-SSP using 45 in-house primer mixes as described previously.[22]

Statistical analysis

Statistical analyses were performed with SPSS 15.0 (SPSS Inc., Chicago, IL) for Windows.
Cross tables were used to compare the observed percentages with each genotype between groups of patients differing in prognosis. The chi-square test was used to test for statistical significant differences between groups. Odds ratios (ORs) with 95% confidence intervals (CIs) were also derived from these tables to evaluate the strength of associations between genotypes and the course of sarcoidosis. Multivariate logistic regression models were used to adjust for differences in baseline characteristics between compared groups. A p-value of <0.05 (two sided) was considered to indicate statistical significance. Deviations from the Hardy–Weinberg equilibrium were analysed using the chi-square test.

Results

Patient characteristics of the studied total population and within groups of patients with and without persistent sarcoidosis are summarized in Table 7.1. Patients with persistent disease more often were male, smoked less often and were more frequently treated with corticosteroids. At diagnosis, the percentage with CXR stage 0 or I was much lower in patients with persistent disease than in the patients with non-persistent disease. Persistent disease was also associated with lower mean values of DCLO, FEV_1 and FVC at diagnosis.

Table 7.1 Patient characteristics for the total population and sorted by disease persistence.

		Total population (n=625)	Non-persistent (n=327)	Persistent (n=298)	p[a] value
Gender	Female	280 (44.8)	178 (54.4)	102 (34.2)	
	Male	345 (55.2)	149 (45.6)	196 (65.8)	<0.001
Age at diagnosis	Year ± SD	40.2 ± 11.7	40.5 ± 12.6	39.8 ± 10.6	
	(range)	(12-84)	(17-84)	(12-76)	
	<40 year	353 (56.5)	185 (56.6)	168 (56.4)	
	≥40 year	272 (43.5)	142 (43.4)	130 (43.6)	1.00
Smoking	No	556 (89.1)	282 (86.2)	275 (92.3)	
	Yes	68 (10.9)	45 (13.8)	23 (7.7)	0.020
Time since diagnosis	<5 year	154 (24.6)	82 (25.1)	72 (24.2)	
	≥5 year	471 (75.4)	245 (74.9)	226 (75.8)	0.78
Corticosteroid use	No	281 (45.0)	185 (56.6)	96 (32.2)	
	Yes	344 (55.0)	142 (43.4)	202 (67.8)	<0.001
CXR at diagnosis	0	59 (9.4)	59 (18.1)	0	
	I	188 (30.1)	178 (54.4)	10 (3.4)	
	II	244 (39.1)	65 (19.9)	179 (60.1)	
	III	134 (21.4)	25 (7.6)	109 (36.5)	<0.001[c]
CXR at follow-up	0	270 (43.2)	270 (82.6)	0	
	I	57 (9.1)	57 (17.4)	0	
	II	142 (22.7)	0	142 (47.7)	
	III	74 (11.9)	0	74 (24.8)	
	IV	82 (13.1)	0	82 (27.5)	<0.001[d]
DLCO[b]	Mean ± SD	81.2 ± 17.3	86.9 ± 15.1	75.0 ± 17.5	
	(range)	(23-129)	(37-129)	(23-121)	
	≥80%	341 (56.0)	228 (71.9)	113 (38.7)	
	<80%	268 (44.0)	89 (28.1)	179 (61.3)	<0.001
FEV_1[b]	Mean ± SD	89.8 ± 21.5	99.8 ± 15.6	78.7 ± 21.7	
	(range)	(23-140)	(54-140)	(23-128)	
	≥80%	439 (72.4)	289 (91.2)	150 (51.9)	
	<80%	167 (27.6)	28 (8.8)	139 (48.1)	<0.001
FVC[b]	Mean ± SD	98.7 ± 19.1	106.0 ± 15.5	90.5 ± 19.4	
	(range)	(25-152)	(66-152)	(25-148)	
	≥80%	509 (84.8)	301 (95.3)	208 (73.2)	
	<80%	91 (15.2)	15 (4.7)	76 (26.8)	<0.001

n, number; SD, standard deviation; CXR, chest X-ray; DLCO, diffusing capacity of carbon monoxide; FEV_1, forced expiratory volume in 1 s; FVC, forced vital capacity. Missing: 16/19/25 for DLCO/FEV_1/FVC, respectively. All values presented are absolute numbers with percentages in parentheses unless otherwise specified. [a] Non-persistent versus persistent; [b] % of predicted (<80% is abnormal); [c] CXR 0 + I versus II + III; [d] CXR IV versus 0 + I + II + III.

Table 7.2 shows the distribution of polymorphisms for *TNF-α* G-308A, *TNF-α* G-238A, *LTA* and HLA-DRB1*03 in patients with and without persistent disease. The *TNF-α* -308A variant allele was more often present in the patients with non-persistent disease (44.0%) when compared with patients having persistent disease (25.5%). This difference was statistically significant ($p<0.001$). HLA-DRB1*03 was comparable with the *TNF-α* G-308A with 34.9% of patients with non-persistent disease being DRB1*03 positive and of the patients with persistent sarcoidosis 13.4% having DRB1*03, see Table 7.2.

Table 7.2 Genotype distributions for the total population and sorted by disease persistence.

	Genotype	Total population (n=625)	Non-persistent (n=327)	Persistent (n=298)	p[a] value
TNF-308	GG	405 (64.8)	183 (56.0)	222 (74.5)	
	GA	200 (32.0)	129 (39.4)	71 (23.8)	<0.001[b]
	AA	20 (3.2)	15 (4.6)	5 (1.7)	
TNF-238	GG	562 (89.9)	290 (88.7)	272 (91.3)	
	GA	63 (10.1)	37 (11.3)	26 (8.7)	0.29 [b]
	AA	0	0	0	
LTA	AA	281 (45.0)	130 (39.8)	151 (50.7)	
	AG	279 (44.7)	158 (48.3)	121 (40.6)	0.09 [b]
	GG	65 (10.3)	39 (11.9)	26 (8.7)	
DRB1*03	Neg	471 (75.4)	213 (65.1)	258 (86.6)	
	Pos	154 (24.6)	114 (34.9)	40 (13.4)	<0.001

TNF-308, *TNF-α* G-308A; TNF-238, *TNF-α* G-238A; LTA, *LTA NcoI* A252G; DRB1*03, HLA-DRB1*03; GG, wild type for TNF-308 and TNF-238; for LTA wild type, AA. All values presented are absolute numbers with percentages in parentheses unless otherwise specified. [a]Non-persistent versus persistent. [b]No variant allele versus variant allele.

For *TNF-α* G-238A and *LTA* there were no statistically significant differences in the distribution of genotypes between the groups with and without persistent disease; therefore, these polymorphisms were not used for further analysis. However, for the *LTA* there was a trend towards a higher G-allele percentage in the group with non-persistent disease (60.2%) compared with the group with a persistent course (49.3%).

In Table 7.3, patients were classified according to genotype distribution and next to persistent versus non-persistent disease outcome or HLA-DRB1*03 presence, alternative clinical parameters for clinical course, such as CXR stage and lung function test results are shown. Patients with poor prognosis were compared with patients with good prognosis with respect to the distribution of the *TNF-α* -308A variant allele. Those with CXR stage 0 and lung function parameters ≥80% represented the group with good prognosis and were used as reference. The ORs with 95% CIs were consistently and in most comparisons significantly lower than 1, indicating a protective effect of the presence of a *TNF-α* -308A variant allele. Presence of the *TNF-α* -308A variant

allele is associated with good prognosis: in the groups with favorable clinical outcome this allele was observed significantly more often than in the groups with a less favorable clinical outcome. The OR was 0.43 with 95% CI: 0.31-0.61. After adjustment for age, gender, corticosteroid use and smoking, the odds ratio remained the same: OR=0.43 (95% CI: 0.30-0.61).

Table 7.3 Prognosis by genotype distribution of the *TNF-α* G-308A variant.

		GG (n=405)	GA (n=200)	AA (n=20)	OR[a] (95%CI) carriage -308A	p[a] value
Persistence	No	183 (56.0)	129 (39.4)	15 (4.6)	1	
	Yes	222 (74.5)	71 (23.8)	5 (1.7)	0.43 (0.30-0.61)	<0.001
DRB1*03	Pos	18 (11.7)	118 (76.6)	18 (11.7)	1	
	Neg	387 (82.2)	82 (17.4)	2 (0.4)	0.03 (0.02-0.05)	<0.001
CXR stage	0	150 (55.6)	109 (40.4)	11 (4.0)	1	
	I	33 (57.9)	20 (35.1)	4 (7.0)	0.91 (0.51-1.63)	0.75
	II	105 (74.0)	34 (23.9)	3 (2.1)	0.45 (0.28-0.68)	<0.001
	III	47 (63.5)	25 (33.8)	2 (2.7)	0.72 (0.41-1.23)	0.23
	IV	70 (85.4)	12 (14.6)	0	0.19 (0.09-0.38)	<0.001
CXR improving	No	286 (70.1)	111 (27.2)	11 (2.7)	1	
	Yes	119 (54.8)	89 (41.0)	9 (4.2)	1.93 (1.37-2.72)	<0.001
CXR stable	No	197 (63.8)	103 (33.3)	9 (2.9)	1	
	Yes	208 (65.8)	97 (30.7)	11 (3.5)	0.91 (0.66-1.27)	0.62
CXR worsening	No	327 (61.3)	186 (34.9)	20 (3.8)	1	
	Yes	78 (84.8)	14 (15.2)	0	0.28 (0.16-0.52)	<0.001
DLCO[b]	Mean ± SD	80.3 ± 17.0	82.2 ± 18.1	89.2 ± 14.9		
	(range)	(37-128)	(23-129)	(57-121)		
	≥80%	208 (61.0)	116 (34.0)	17 (5.0)	1	
	<80%	188 (70.2)	77 (28.7)	3 (1.1)	0.68 (0.48-0.97)	0.031
FEV_1[b]	Mean ± SD	87.6 ± 21.7	93.0 ± 20.9	100.4 ± 16.1		
	(range)	(26-140)	(23-136)	(71-128)		
	≥80%	272 (62.0)	149 (33.9)	18 (4.1)	1	
	<80%	122 (73.1)	43 (25.7)	2 (1.2)	0.61 (0.40-0.93)	0.020
FVC[b]	Mean ± SD	97.2 ± 18.9	100.6 ± 19.3	109.1 ± 15.6		
	(range)	(33-152)	(25-147)	(83-148)		
	≥80%	320 (62.9)	170 (33.4)	19 (3.7)	1	
	<80%	69 (75.8)	22 (24.2)	0	0.56 (0.33-0.95)	0.031

n, number; OR, Odds ratio corrected for age, gender, corticosteroid use and smoking; SD, standard deviation; DRB1*03, HLA-DRB1*03; CXR, chest X-ray; DLCO, carbon monoxide diffusing capacity; FEV_1, forced expiratory volume in 1 s; FVC, forced vital capacity; -308A, *TNF-α* -308A variant allele; GG, wild type; GA, heterozygote; AA, homozygote variant. Missing: 16/19/25 for DLCO/FEV_1/FVC, respectively. All values are absolute numbers with percentages in parentheses unless otherwise specified. [a]No variant allele versus variant allele; [b] % of predicted (<80% is abnormal).

The OR for the HLA-DRB1*03 positive *vs* negative cases compared with the presence of a *TNF-α* -308A variant allele was 0.03 (95% CI: 0.02-0.05) indicating a strong association.

Table 7.3 also shows an increase in mean values of lung function test result parameters as the number of A-alleles in the *TNF-α* G-308A genotype increases (e.g. mean % DCLO GG=80.3 < GA=82.2 < AA=89.2).
In the total population the proportions of patients without or with a *TNF-α* G-308A variant allele were 64.8% (GG) and 35.2% (32.0% GA and 3.2% AA), respectively (chi2=0.61, p=0.74). These findings were in accordance with the Hardy-Weinberg equilibrium. The observed allele frequencies for all 625 patients were 80.8% for the *TNF-α* G-308 and 19.2% for the *TNF-α* -308A allele (p=0.18). The frequency distribution is similar to the distribution reported in healthy Dutch controls (75.0% for the G-allele and 25.0% for the A-allele) from the literature.[23]

Discussion

In this study, we observed that the presence of a *TNF-α* -308A variant allele had a favorable impact on radiologic evolution and prognosis in sarcoidosis. Patients without a *TNF-α* -308A variant allele had a significantly higher risk of developing persistent sarcoidosis with progression to a higher CXR stage and worsening of lung function, particularly the DLCO and FVC. Furthermore, the existence of a strong linkage disequilibrium between the *TNF-α* -308A variant allele and the presence of HLA-DRB1*03 was confirmed.
In our current study, the presence of a *TNF-α* -308A variant allele was associated with a lower risk of worsening of the disease. No association between the *TNF-α* G-308A polymorphism and sarcoidosis in general was found as all genotype and allele frequencies of the 625 studied sarcoidosis patients were in Hardy-Weinberg equilibrium, similar to control populations in literature and in concurrence with findings from earlier studies.[8,11,23,24] In a meta-analysis by Medica et al., it was concluded that the presence of the *TNF-α* -308A variant allele increased the susceptibility to and risk of sarcoidosis by as much as 47%, and it was suggested that the polymorphism could be involved in the clinical presentation of sarcoidosis.[17]
When sorting the sarcoidosis patients into persistent and non-persistent categories a clear association did emerge between the absence of the *TNF-α* -308A variant allele and sarcoidosis persistence (OR=0.43, p<0.001). In earlier studies, it was found that carriers of the *TNF-α* -308A variant allele were more prone to go through the more acute form (i.e. Löfgren’s syndrome) of sarcoidosis with frequent spontaneous remission.[8,9,11] This is accordance with our finding that presence of the *TNF-α* -308A variant allele is more prevalent in the non-persistent group.
Categorizing the patients according alternative parameters for clinical course, instead of grouping them into patients with persistent and non-persistent

disease, also showed strong associations with the distribution of the *TNF-α G*-308A genotype. Looking at the results from this perspective revealed that the *TNF-α* -308A variant allele was absent in the large majority of patients who had evolved to CXR stage IV at the end of follow-up (OR=0.19, p<0.001). Previous studies are hard to compare with our data as not only sample size and characteristics of the participants (ethnicity) displayed significant heterogeneity, but sarcoidosis patients were almost always only categorized into Löfgren and non-Löfgren patients, and not according to the different CXR stages. Therefore, previously no conclusions could be drawn about associations or differences in genotype distribution between the various CXR stages.[6,8,11] The categorization according to alternative parameters of clinical outcome also showed that the mean lung function test results were worse in the wild type (*TNF-308* GG) category (Table 7.3). The finding that presence of the variant A-allele is strongly and consistently associated with a more favorable prognosis, irrespective of the definition of good prognosis, lends support to the validity of this association. Moreover, the large sample size in this study also makes it very unlikely that the findings are due to chance.

When examining the selected TNF polymorphisms, only the *TNF-α* G-308A SNP showed significant association with disease progression. The *TNF-α* G-238A polymorphism did not show a different distribution for the non-persistent and persistent disease groups. In accordance to others, we observed that presence of the *LTA NcoI* 252G variant allele only tended to be higher in patients who did not progress, ruling it out as a prognostic factor.[25] In a Japanese population, however, as studied by Yamaguchi et al., the A-allele of the *LTA NcoI* A252G polymorphism was found to be a marker for prolonged clinical course.[26] This is an important reminder of the fact that populations of different ethnicity, as in this case Japanese versus Caucasian, can display different associations to the same polymorphisms. However, in line with Mrazek et al., we also found a slight increased but not statistically significant *LTA NcoI* 252G variant allele presence (34.6%; chi2=1.52, p=0.22) in CXR stage I at diagnosis compared with CXR stages 0, II and III.[7]

In the current study, it was also found that smoking appeared to protect against progressing to persistent sarcoidosis, for fewer smokers were present in the persistent disease group. This was in accordance with findings from earlier studies.[27-30]

Interestingly, in our population, men more often showed progression to persistent sarcoidosis. It is tempting to speculate that this might be explained by the fact that males were more frequently exposed to occupational and/or environmental antigenic triggers than females (67.4% versus 31.2%, respectively). It is known that these triggers, beside genetic factors, are involved in the pathogenic concept of sarcoidosis.[3,31,32]

The frequency of use of systemic corticosteroids was higher in patients with persistent disease and may be a confounder for clinical course. However, after adjustment for differences in baseline characteristics between the groups with persistent and non-persistent disease using multivariate logistic regression, the OR associated with the presence of the *TNF-α* -308A variant allele did not change and was still indicative of a halving of the risk of persistent disease.
In the clinical management of sarcoidosis patients, it is important to identify those cases that might have a chance to develop a more severe manifestation of sarcoidosis. In those particular cases, treatment aimed at avoiding irreversible damage should be started early.[33] Corticosteroids are still the first drug of choice to treat sarcoidosis in most cases, but it is well known that long-term use is associated with significant toxicity.[33] Since a few years anti-TNF-α agents are being used more frequently, especially in chronic cases.[10,34] Failure of patient response to conventional therapy or the presence of unacceptable side effects from other available drugs, especially prednisone, constitute the most common reasons for prescribing an anti-TNF-α agent for sarcoidosis.[34] When considering anti-TNF-α treatment it can be very important to have information that might be helpful in predicting whether a patient will be a responder or non-responder. Studies into responders and non-responders to anti-TNF-α therapy have been carried out, e.g. in rheumatoid arthritis (RA) patients by Mugnier et al.[35] They found that patients without a *TNF-α* G-308A allelic variant were the overall better responders. As shown in the present study, patients without a *TNF-α* -308A variant allele were also more prone to develop the persistent form of sarcoidosis. Interestingly, in the infliximab trial by Baughman et al., it was found that the patients with a more severe or longer duration of (i.e. persistent) pulmonary disease including reduced FVC, severe dyspnoea as well as a more impaired quality of life were more likely to respond to anti-TNF-α treatment.[36] This might suggest that they were most likely cases without a variant allele. In accordance to experiences with RA patients, it seems to be practical and helpful to monitor sarcoidosis patients without a *TNF-α* G-308A allelic variant early and initiate treatment accordingly. So it appears that one simple and less expensive SNP test prior to treatment could not only render very useful information about progression. Given that this was beyond the scope of this study, future study should evaluate whether a single SNP test (*TNF-α* G-308A*)* might also be helpful in deciding to start anti-TNF-α treatment or not. Moreover, when anti-TNF-α treatment is considered genotyping *TNF-α* G-308A may be a useful tool to differentiate between responders and non-responders of anti-TNF-α treatment in sarcoidosis.

Conclusion

Our results clearly show that genotyping for the *TNF-α* G-308A polymorphism is helpful in predicting prognosis in sarcoidosis patients. The risk of progressing to a more severe pulmonary involvement is higher in the absence of a *TNF-α* G-308A allelic variant and HLA-DRB1*03. Because of the strong linkage between *TNF-α* G-308A and HLA-DRB1*03 genotyping of one simple *TNF-α* SNP is useful in predicting the prognosis of pulmonary sarcoidosis. The findings in this study can be used as a base for further clinical validation of the use of *TNF-α* G-308A genotyping to predict the clinical course of sarcoidosis in individual patients. Research must continue to depict the role of *TNF* genes in the immunogenetics and clinical management of sarcoidosis.

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Chapter 8

Butyrophilin-like 2 in pulmonary sarcoidosis: a factor for susceptibility and progression?

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Abstract

Background
The aims of this study were to assess the association of *BTNL2* G16071A with the course of pulmonary sarcoidosis and verify association with disease predisposition. In addition, the linkage between *BTNL2* G16071A and certain HLA-DRB1/DQB1 types was investigated.

Methods
In a retrospective case-control study *BTNL2* G16071A, HLA-DQB1 and DRB1 were typed in 632 sarcoidosis patients. These patients were classified into 304 patients with persistent and 328 patients with non-persistent sarcoidosis.

Results
The *BTNL2* 16071A variant allele was significantly more often present in patients with persistent disease (92.4%; 281/304) compared with patients demonstrating a non-persistent course (86.6%; 284/328); odds ratio (OR) =1.89 with 95% confidence interval (CI 95%): 1.11-3.22.
Furthermore, *BTNL2* 16071A variant allele carriers have an increased risk (OR=1.85, CI 95%: 1.19-2.88) to develop sarcoidosis. Moreover, the strong linkage between variant allele and HLA-DRB1*15 presence (OR=8.43, CI 95%: 3.02-23.5) was confirmed.

Conclusion
The presence of a *BTNL2* G16071A variant allele almost doubles the risk of progressing to persistent pulmonary sarcoidosis, besides increasing the risk to develop sarcoidosis. Presumably, these increased risks are due to the strong linkage between *BTNL2* G16071A and DRB1*15. The choice between determining *BTNL2* G16071A SNP or the HLA-DRB1 type depends on the ability and/or availability to perform either test.

Introduction

Sarcoidosis is a multisystem granulomatous disorder of which clinical presentation and outcome vary considerably.[1-3] The assumption that genes contribute to the etiology of sarcoidosis comes from the observation that prevalence and incidence rates of sarcoidosis are different between ethnic groups and that the disease tends to cluster in families. Next to family clustering, disease clustering in time and place, often with co-workers, was also noted.[4] Therefore, shared common environmental exposures must be considered as well. However, the etiology may not prove to be a single, known exposure, and the confirmation of interactions of exposure with genetic predispositions would have important implications for the understanding of immune responses as well as the pathogenesis of sarcoidosis.[4]

In previous studies the association between single nucleotide polymorphisms (SNPs) in the butyrophilin-like 2 (*BTNL2*) gene and the risk to develop sarcoidosis has been shown.[5-8] The *BTNL2* G16071A (rs2076530) polymorphism showed the strongest association with sarcoidosis.[6] Arnett et al. found that the functional BTNL2 reduces proliferation and cytokine production from activated T-cells, suggesting a role for BTNL2 as a negative co-stimulatory molecule with implications for inflammatory disease.[9] The A-allele of the *BTNL2* G16071A polymorphism causes a premature truncation of the protein, disrupting insertion in the cell membrane, a necessary process for downregulating activated T-cells (Th1).[5,10] The truncated protein increases the risk of developing sarcoidosis independent of HLA-DRB1 risk alleles.[5,6] However, a possible association with the prognosis of sarcoidosis was not investigated. Until now an association with chronic sarcoidosis only has been suggested, because of an established strong linkage of the *BTNL2* 16071A variant allele with HLA-DRB1*15 (DRB1*15).[6] Association of sarcoidosis and class I and II HLA antigens is well known and several studies established the risk of progression of sarcoidosis and presence or absence of specific HLA-types.[11-15] In addition, DRB1*15 and the DQB1*0602/DRB1*1501 haplotype in particular, was associated with more severe sarcoidosis, whereas DRB1*03 has been associated with good prognosis.[11,13,16,17]

Pulmonary disease is the most common manifestation of sarcoidosis and pulmonary symptoms are the most common reason for treatment.[1,18] Numerous studies have confirmed the utility of chest X-ray (CXR) stage as a prognostic guide.[1] The presence of hilar adenopathy alone (CXR stage I) is usually associated with good prognosis.[1,11,19] By contrast, presence of parenchymal infiltrates (CXR stages II-IV) is associated with chronic disease.[1,19] Spontaneous remissions occur in 55-90% of patients with stage I, 40-70% of patients with stage II, 10-20% of patients with stage III, and in 0% of patients with stage IV disease.[1] Because of recognized value, CXR staging

was used in several studies to categorize sarcoidosis patients into subgroups with different pulmonary manifestations.[1,20,21]

The aims of this study were to assess the association of *BTNL2* G16071A with the course of pulmonary sarcoidosis and to verify the association with disease predisposition. In addition, the linkage between *BTNL2* G16071A and certain HLA-DRB1/DQB1 types was investigated.

Materials and methods

Patients

From January 2000 to July 2008, 632 consecutive Dutch Caucasian sarcoidosis patients, attending the outpatient referral clinic of the ild care center of the Department of Respiratory Medicine of the Maastricht University Medical Centre (MUMC) The Netherlands, were included in this study. Time between diagnosis or inclusion and end of follow-up for all patients was at least two years. Diagnosis was in 71% of cases based on a positive biopsy (lung, skin, lymph node or liver). In patients with typical features of Löfgren's syndrome and characteristic features of bronchoalveolar lavage (BAL) fluid analysis results (including lymphocytosis), no biopsy was obtained.[22,23] This policy is consistent with the World Association of Sarcoidosis and Other Granulomatous diseases (WASOG) guidelines.[1]
A control group consisting of 1474 local, healthy, unrelated Dutch Caucasian volunteers, hospital workers, and blood or stem cell donors (age: 48.8±10.3 (21-86); 48.6% female) was used for HLA typing. Out of this control group 200 samples were randomly selected and typed for the *BTNL2* SNP.
The study was performed in accordance with the Declaration of Helsinki and its amendments. The protocol was approved by the Medical Ethics Board of the MUMC. Written informed consent for participation in this study was obtained from all subjects.

Collection of clinical data

At inclusion forced vital capacity (FVC) and forced expiratory volume in one second (FEV_1) were measured with a pneumotachograph (Masterlab, Jaeger, Würzburg, Germany). The diffusing capacity for carbon monoxide (DLCO) was measured by the single-breath method (Masterlab). Values were expressed as a percentage of predicted values. Missing values for DLCO/FEV_1/FVC were 17/21/26, respectively. The cut-off value for the DLCO, FEV_1, and FVC was <80% of predicted (≥80% is normal).[24] Respiratory functional impairment (RFI) was defined as DLCO<80%, FVC<80% or FEV1<80% (percentage of

predicted). Patients without RFI were those for whom all three indices were normal (≥80%).[25]
Clinical course of patients with sarcoidosis was defined using changes in CXR stage during follow-up. All CXRs were graded by a single experienced observer, who was not aware of the clinical data. Five stages of radiographical abnormality were recognized: stage 0 (normal CXR), stage I (bilateral hilar lymphadenopathy [BHL]), stage II (BHL and parenchymal abnormalities), stage III (parenchymal abnormalities without BHL), and stage IV (end stage lung fibrosis).
For the analysis with respect to prognosis, patients were categorized into groups with persistent and non-persistent sarcoidosis. Persistent disease was defined as worsening of the CXR stage to stage II or higher, or remaining at CXR stage II or III, at least two years after diagnosis. Non-persistent disease cases were those who remained at or regressed to stage 0 or I.
In addition to the categorization into persistent/non-persistent sarcoidosis, CXR stages were used combined with lung function test results to classify the patients into groups with and without progressive disease. No progression was defined as remaining at stage 0, I, II or III or regressing to stage 0 or I. Progression was defined as worsening of the CXR stage or remaining at stage I, II or III in combination with RFI.

BTNL2 and HLA typing

DNA isolation

DNA was obtained using venous EDTA or heparin anti-coagulated blood and isolated with a High Pure PCR Template Preparation Kit (Roche Diagnostics, Mannheim, Germany), or with QIA-AMP kits (Qiagen, Westburg, Leusden, The Netherlands) according to the manufacturer’s instructions.

BTNL2

Patients and controls were genotyped for the *BTNL2* G16071A (rs2076530) polymorphism using a real-time PCR Fluorescence Resonance Energy Transfer (FRET) assay (TIB MOLBIOL, Berlin, Germany) on the LightCycler® (Roche Diagnostics).

HLA

Low resolution typing of HLA-DRB1 and DQB1 was obtained by Luminex reverse SSO, using bead kits from One Lambda (One Lambda, Bethesda, USA) or by PCR-SSP, using 45 in-house primer mixes as described previously.[26]
High resolution typing of DRB1 and DQB1 was obtained by sequencing exon 2 and if needed exon 3, as previously described.[26,27] Either solid phase

sequencing was used as described,[27,28] or cycle sequencing was performed using Big Dye Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, USA). In cases of ambiguous typing, allele specific sequencing was performed, using suitable allele specific amplification primers in combination with adjusted sequencing primers as described.[13]

Statistical analysis

The Chi-square test was used to test for statistical significant differences between groups. Odds ratios (OR) with 95% confidence intervals (CI) were derived from cross tables to evaluate strength of associations. Multivariate logistic regression models were used to adjust for differences in baseline characteristics (sex, smoking, and corticosteroid use) between compared groups. A $p<0.05$ (two sided) was considered to indicate statistical significance. Deviations from the Hardy–Weinberg equilibrium were analysed using the Chi-square test. Statistical analyses were performed with SPSS 15.0 (SPSS Inc., Chicago, USA) for Windows.

Results

Patient characteristics of the total sarcoidosis population and of the subgroups with persistent and non-persistent sarcoidosis are summarized in Table 8.1. Patients with persistent disease more often were male, smoked less often and were more frequently treated with corticosteroids. At the time of diagnosis, the percentage with CXR stage 0 or I was much lower in patients with persistent disease than in the patients with non-persistent disease. Persistent disease was also associated with lower mean values of DCLO, FEV_1 and FVC at diagnosis.
In Table 8.2 an overview of *BTNL2* G16071A allele and HLA-DRB1*15 phenotype frequencies found in our entire sarcoidosis patient group (n=632) and in sarcoidosis patients with persistent and non-persistent disease is shown. The local healthy Dutch Caucasian control population (n=1474 for HLA and n=200 randomly selected out of the total 1474 for *BTNL2* G16071A establishing that our 'local' healthy population did not differ from historic controls with the same ethnicity from literature regarding the *BTNL2*), and several patient and control populations taken from the literature, are also listed for comparison.

Table 8.1 Patient characteristics for the total sarcoidosis population and sorted by disease persistence.

		Total population n=632	Non-persistent n=328	Persistent n=304	p[a] value
gender	female	283 (44.8)	177 (54.0)	106 (34.9)	
	male	349 (55.2)	151 (46.0)	198 (65.1)	<0.001
age at diagnosis	yr±SD (range)	40.2±11.7 (12-84)	40.5±12.6 (17-84)	39.9±10.7 (12-76)	
	< 40 yr	356 (56.3)	185 (56.4)	171 (56.2)	
	≥ 40 yr	276 (43.7)	143 (43.6)	133 (43.8)	NS
smoking	no	562 (88.9)	283 (86.3)	279 (91.8)	
	yes	70 (11.1)	45 (13.7)	25 (8.2)	0.03
corticosteroid use	no	285 (45.1)	187 (57.0)	98 (32.2)	
	yes	347 (54.9)	141 (43.0)	206 (67.8)	<0.001
CXR at diagnosis	0	66 (10.4)	66 (20.1)	0	
	I	186 (29.4)	172 (52.5)	14 (4.6)	
	II	245 (38.8)	66 (20.1)	179 (58.9)	
	III	135 (21.4)	24 (7.3)	111 (36.5)	<0.001[b]
CXR at follow-up	0	282 (44.6)	282 (86.0)	0	
	I	46 (7.3)	46 (14.0)	0	
	II	141 (22.3)	0	141 (46.4)	
	III	76 (12.0)	0	76 (25.0)	
	IV	87 (13.8)	0	87 (28.6)	<0.001[c]
DLCO[d]	mean±SD (range)	81.4±17.3 (23-129)	87.0±15.0 (37-129)	75.4±17.5 (23-121)	
	≥80%	353 (57.4)	235 (73.9)	118 (39.7)	
	<80%	262 (42.6)	83 (26.1)	179 (60.3)	<0.001
FEV_1[d]	mean±SD (range)	90.2±21.5 (23-140)	100.5±15.3 (54-140)	79.0±21.8 (23-128)	
	≥80%	448 (73.3)	294 (92.5)	154 (52.6)	
	<80%	163 (26.7)	24 (7.5)	139 (47.4)	<0.001
FVC[d]	mean±SD (range)	99.0±19.1 (25-152)	106.6±15.3 (66-152)	90.7±19.4 (25-148)	
	≥80%	516 (85.1)	303 (95.6)	213 (73.7)	
	<80%	90 (14.9)	14 (4.4)	76 (26.3)	<0.001

[a]= non-persistent versus persistent, [b]= CXR 0+I versus II+III, [c]= CXR IV versus 0+I+II+III, [d]= % of predicted (<80% is abnormal), n=number, yr=years, NS=not significant, SD=standard deviation, CXR=chest X-ray, DLCO=diffusing capacity of carbon monoxide, FEV_1=forced expiratory volume in one second, FVC=forced vital capacity. Missing: 17/21/26 for DLCO/FEV_1/FVC, respectively. All values presented are absolute numbers with percentages in parentheses unless otherwise specified.

In the healthy controls the proportions of persons without or with a *BTNL2* 16071A variant allele were 18.0% (GG) and 82.0% (44.0% GA and 38.0% AA), respectively (chi2=1.39, p=0.24). These findings were in accordance with the Hardy-Weinberg equilibrium.

Table 8.2 Frequencies of *BTNL2* G16071A and HLA-DRB1*15 sarcoidosis patients and controls.

Population	number	*BTNL2* G-allele %	*BTNL2* A-allele %
Sarcoidosis patients	632	33.9	66.1
Non-persistent sarcoidosis	328	36.0	64.0
Persistent sarcoidosis	304	31.6	68.4
UK+Dutch patients[45]	288	33.2	66.8
German patients[5]	904	31.4	68.6
German patients[7]	210	30.7	69.3
Local controls	200	40.0	60.0
Dutch controls[41]	351	41.2	58.8
German controls[5]	427	42.7	57.3
German controls[7]	202	38.1	61.9
UK+Dutch controls[45]	446	42.6	57.4
		HLA-DRB1*15 neg %	HLA-DRB1*15 pos %
Sarcoidosis patients	632	68.2	31.8
Non-persistent sarcoidosis	328	74.1	25.9
Persistent sarcoidosis	304	61.8	38.2
Local controls	1474	77.1	22.9
Dutch controls[46]	207	69.1	30.9
Dutch controls[47]	2400	74.0	26.0
Dutch controls[48]	700	76.2	23.8
UK controls[49]	537	78.1	20.9

Table 8.3 shows the distributions of the studied *BTNL2* polymorphism and HLA types in healthy controls, the total sarcoidosis population, and subgroups with and without persistent disease or progression. The *BTNL2* G16071A polymorphism occurs more frequently in the total sarcoidosis population than in the healthy local controls (OR=1.85; p=0.007).
The *BTNL2* 16071A variant allele was more often present in patients with persistent disease when compared with patients having non-persistent disease (OR=1.89; p=0.02). After multivariate correction for sex, smoking, and corticosteroid use the OR remained similar (OR=1.84, CI 95%: 1.06-3.21; p=0.03). The ORs for the subgroups no progression and progression were similar to those with and without persistent disease (Table 8.3).

Significant differences with respect to the HLA types DRB1*03 and DRB1*15 (with or without DQB1*06) were also observed. For the DRB1*15 a significantly larger proportion of patients with persistent sarcoidosis was positive, 38.2% versus 25.9% in the non-persistent group (OR=1.76; p=0.001). The presence of DRB1*15 in combination with DQB1*06 (15Q6 haplotype) was also associated with an increased risk of poor prognosis: OR of 1.63 (p=0.006). From all but 60 patients high resolution typing of the DQB1*06 allele was available, and presence of the DQB1*0602 allele was

associated with poor prognosis: OR=2.36, CI 95%: 1.59-3.49; p<0.001 (data not shown).

Table 8.3 Distributions for the controls, total sarcoidosis population, and sorted by disease persistence.

	Type	Controls n=200/1474[c]	Total population n=632	Odds Ratio (range)	p[a] value	Non-persistent n=328	Persistent n=304	Odds Ratio (range)	p[b] value
BTNL 71	GG	36 (18.0)	67 (10.6)			44 (13.4)	23 (7.6)		
	GA	88 (44.0)	294 (46.5)	1.85 (1.19-2.88)	0.007[d]	148 (45.1)	146 (48.0)	1.89 (1.11-3.22)	0.02[d]
	AA	76 (38.0)	271 (42.9)			136 (41.5)	135 (44.4)		
DRB1*03	neg	1140 (77.3)	476 (75.3)			215 (65.5)	261 (85.9)		
	pos	334 (22.7)	156 (24.7)	0.89 (0.72-1.11)	NS	113 (34.5)	43 (14.1)	0.31 (0.21-0.46)	<0.001
DRB1*15	neg	1136 (77.1)	431 (68.2)			243 (74.1)	188 (61.8)		
	pos	338 (22.9)	201 (31.8)	1.57 (1.27-1.93)	<0.001	85 (25.9)	116 (38.2)	1.76 (1.26-2.47)	0.001
15Q6	neg	1145 (77.7)	441 (69.8)			245 (74.7)	196 (64.5)		
	pos	329 (22.3)	191 (30.2)	1.51 (1.22-1.86)	<0.001	83 (25.3)	108 (35.5)	1.63 (1.16-2.29)	0.006

[a]= controls versus total population, [b]= non-persistent versus persistent, [c]= 200 healthy, unrelated, Dutch Caucasian volunteers for the *BTNL2* and 1474 for the HLA typing, [d]= no variant allele versus variant allele, NS=not significant, BTNL 71=*BTNL2* G16071A (rs2076530), DRB1*03=HLA-DRB1*03, DRB1*15=HLA-DRB1*15, 15Q6=HLA-DQB1*06/DRB1*15 haplotype. All values presented are absolute numbers with percentages in parentheses unless otherwise specified.

Within the healthy Dutch Caucasian controls who were DRB1*15 positive, DQB1*06 was absent in 0.6% (9/1474). In the DRB1*15 positive sarcoidosis patients with non-persistent disease a comparable percentage without a DQB1*06 (0.6%; 2/328) was found, whereas in the subgroup with persistent disease 2.6% (8/304) of the patients possessed a DRB1*15 type without bearing a DQB1*06. Within the DRB1*15 bearing group, DQB1*06 showed a negative association with progressive disease, but due to the small numbers the confidence interval around the odds ratio is very wide. (OR=0.18, CI 95%: 0.02-1.54; p=0.14). These findings indicate a trend towards an effect of DRB1*15 on the course of sarcoidosis independent of DQB1*06. In contrast to the DRB1*15 results, the presence of a DRB1*03 allele was associated with a good prognosis (OR=0.31; p<0.001).

The presence of DRB1*15 was strongly associated with the presence of a *BTNL2* 16071A variant allele: OR=8.43 (CI 95%: 3.02-23.5; p<0.001).Within the subgroup of patients who were positive for the DRB1*15 (n=201) only 2% (n=4) did not possess a *BTNL2* 16071A variant allele, compared with 14.6% (n=63) within the subgroup of DRB1*15 negative patients.

Possessing neither a DRB1*15 nor the *BTNL2* G16071A variant allele compared with both the DRB1*15 and a *BTNL2* 16071A variant allele being present in patients displaying persistent disease, resulted in OR=2.61 (CI 95%: 1.45-4.72; p=0.001) for the DRB1*15/*BTNL2* positive haplotype. The OR associated with presence of the BTNL2 G16071A in absence of DRB1*15 for persistence is

1.53 (CI 95%: 0.88-2.67; p=0.17) and this OR indicates that the effect of the BTNL2 is independent of DRB1*15.
The OR obtained for the combined presence of the 15Q6 haplotype and the *BTNL2* 16071A variant allele were comparable to the results of the DRB1*15 and *BTNL2* 16071A variant allele: OR=7.79 (CI 95%: 2.79-21.7; p<0.001). The observed association between the presence of DRB1*03 and *BTNL2* 16071 A-allele presence was in the opposite direction: OR=0.08, CI 95%: 0.02-0.32; p<0.001. Of the subgroup of patients who were positive for the DRB1*03 (n=156) only 1.3% (n=2) did not possess a *BTNL2* 16071A variant allele, compared with 13.7% (n=65) within the subgroup of DRB1*03 negative patients.

Discussion

This study is the first that demonstrates that the presence of the *BTNL2* 16071A variant allele increases the risk of progressing to more severe and persistent pulmonary sarcoidosis. Previously, an association between the presence of the *BTNL2* 16071A variant allele and a susceptibility towards the chronic form of sarcoidosis was suggested and an increase in A-allele frequency in non-Löfgren patients was observed.[7,29] However, the A-allele was not considered a risk factor in the progressing of pulmonary sarcoidosis. Furthermore, it is confirmed in accordance with others, that the *BTNL2* 16071A variant allele presence almost doubles the risk of developing sarcoidosis. In addition, the strong linkage of the *BTNL2* G16071A with HLA-DRB1*15 is confirmed. This explains why DRB1*15 bearing also is found to be a risk factor for disease susceptibility as well as for disease course.

It is well known that the outcome of sarcoidosis varies considerably.[30] Inconsistency exists on how to define severity. The course of sarcoidosis is mainly monitored by assessing clinical features and using auxiliary diagnostic tests. In the past decade, insight has been provided into the genetic risk for sarcoidosis and how the genetic make-up of a patient determines the clinical presentation and outcome. Two more provocative studies in sarcoidosis include the recognition of the *BTNL2* gene as a candidate sarcoidosis susceptibility gene[5,6] and the identification of mycobacterial catalase-peroxidase as a potential sarcoidosis antigen[31] that drives granuloma formation.[32] The *BTNL2* gene is located close to and in linkage with HLA-DRB1, which in turn is implicated in the etiology of sarcoidosis.[21,33-35] The *BTNL2* G16071A polymorphism in particular has been linked with an increased susceptibility risk for developing sarcoidosis.[5-7] However, other than the suggestion that susceptibility might be preferential towards the chronic form of sarcoidosis[7],

there were no studies done exploring the possibility that BTNL2 G16071A could also be associated with an increased risk of progression. In this study it was found that possessing the A-allele almost doubles (OR=1.89, CI 95%: 1.11-3.22) the risk of a persistent course of sarcoidosis. When also using lung function test results to categorize the sarcoidosis patients into groups with and without progression the OR remains similar (OR=1.75, CI 95%: 1.01-3.04). Next to other progression markers, such as DRB1*03 or DRB1*15 phenotypes[11,13,36] or the TNF-α G-308A polymorphism[14,37,38], one could argue that BTNL2 G16071A does not add much. However, especially in families with sarcoidosis patients, this SNP could provide information about the risk of developing sarcoidosis and when present, about the course of the disease. Moreover, as was found in a study by Coudurier et al. examining a family of sarcoidosis patients, the A/A genotype of BTNL2 G16071A can be defined as a putative prognosis and/or predictive factor of recurrent and severe sarcoidosis.[8] Therefore, genotyping before developing sarcoidosis in persons with a relative with the disease, might give additional information about disease risk and course, and can facilitate disease management. Prospective family-based studies should be performed to confirm this.

In our study the A-allele of the *BTNL2* G16071A also appeared to increase the risk of developing sarcoidosis almost twofold, in agreement with results found by Valentonyte et al.[5] No significant association was established in a study performed on other diseases, despite the fact that these were also Th1 dominated granulomatous diseases similar to sarcoidosis, and a role for MHC class II (HLA-DR/DQ) genes was found in these conditions.[35,39-44] A possible explanation for the lack of a significant association between *BTNL2* and sarcoidosis in general might be the result of differences in the patient and healthy volunteer populations studied. This once again draws the attention to the importance of selection and description of the used populations in genetic association studies.[45,46] In other studies the association between *BTNL2* and HLA-DRB1/DQB1 was found, but because of the strong linkage disequilibrium attributed to HLA-DR/DQ haplotypes.[35,47] Indeed, several previous studies in other diseases have convincingly and consistently shown association between HLA-DRB1 (more specifically the 15Q6 haplotype or DRB1*15) and disease susceptibility.[48-50]

In the present study, the strong linkage disequilibrium for the association between DRB1*15 and *BTNL2* G16071A was confirmed (OR=8.43, CI 95%: 3.02-23.5). Moreover, of the DRB1*15 bearing sarcoidosis patients only four did not possess a *BTNL2* 16071A variant allele. Comparing the healthy Dutch Caucasian controls (n=1474) with the total sarcoidosis population (n=632) for the DRB1*15 resulted in a significant difference indicating an increased risk for

developing sarcoidosis when DRB1*15 positive. In addition, an association between presence of DRB1*15 and persistent sarcoidosis (OR=1.76, CI 95%: 1.26-2.47) was established. This was in agreement with results from previous studies.[5,6,13] This indicates that DRB1*15 positivity can be considered as an important factor in both the susceptibility to sarcoidosis and the severity of the disease, comparable with the presence of a *BTNL2* 16071A variant allele. In addition, the *BTNL2* 16071A/DRB1*15 positive haplotype increases the risk of a persistent course of pulmonary sarcoidosis almost threefold. However, the possibility remains that due to the strong linkage, other functional variations on this *BTNL2*/DRB1*15 haplotype may be the true or additional causal determinants.[35] Nevertheless, the strong linkage disequilibrium between *BTNL2* and HLA-DR/DQ requires that *BTNL2* G16071A should be considered relevant to any immune-related disease associated with HLA-DR/DQ.[35]

Previously, an association of the 15Q6 haplotype with the severity of sarcoidosis was found.[13] However, because of the rather limited number of patients (n=156) and controls (n=418) and the strong linkage disequilibrium between DRB1*15 and DQB1*06, it could not be concluded whether the association was with either DRB1*15 or with DQB1*06.[13] In the present study, with an extended number of patients and controls, it was found that the number of DRB1*15 bearing individuals without DQB1*06 was nine out of 1474 for the healthy controls and 10 out of 632 for the patients. Furthermore, it was two out of 328 out of the non-persistent group and eight out of 304 out of the persistent group. Together with a higher OR observed for the DRB1*15, this implicates that typing for HLA-DRB1 and HLA-DQB1 to ascertain the 15Q6 haplotype does not give any extra information, and that typing for DRB1 is sufficient.

In conclusion, the presence of a *BTNL2* 16071A variant allele was found to be associated with an almost twofold increased risk of progressing to more severe and persistent pulmonary sarcoidosis in Caucasians. Furthermore, the predisposition to develop sarcoidosis was confirmed, as well as the strong linkage between the *BTNL2* 16071A variant allele and DRB1*15 positivity. It also became apparent that typing for DRB1 is sufficient because of the lack of additional information obtained by typing the DQB1*06, to establish the 15Q6 haplotype. Whether or not to determine the DRB1 type or test the *BTNL2* G16071A SNP therefore, depends on the ability and/or availability to perform either test. Additional research will be necessary to explore the role of these findings in the clinical management of sarcoidosis patients.

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Chapter 9

Summary, general discussion and directions for future research

Summary

Many acute and chronic lung diseases can cause pulmonary fibrosis and are commonly referred to as interstitial lung disease (ILD) or diffuse lung disease. In most cases the origin of ILD is not known. Genetic predisposition and environmental antigens are indicated as risk factors in the majority of these ILD. Several studies have explored the association of genetic polymorphisms or the presence of certain variant alleles, with the occurrence and/or progression of ILD of unknown etiology.[1-7] Moreover, the search for more specific 'markers' still continues.

The aim of the studies presented in this thesis was to evaluate the clinical and prognostic importance of genetic testing in a group of patients with drug-induced ILD (DI-ILD), a group of patients with oral anticoagulants induced diffuse alveolar hemorrhage (DAH), and a group of sarcoidosis patients. All these patients were referred to the ild care center of the Maastricht University Medical Centre, the Netherlands.

The analyses performed were executed on whole blood samples of drug-induced ILD, idiopathic pulmonary fibrosis (IPF), and sarcoidosis patients. Of patients suffering from diffuse alveolar hemorrhage (DAH) analyses were done on whole blood samples and cells present in bronchoalveolar lavage fluid (BALF). For the analyses performed on healthy volunteer (HV) groups, whole blood and buccal swab (BS) samples were used. In afore mentioned populations the value of genotyping cytochrome P450 (CYP) enzymes (i.e. CYP2D6, CYP2C9 and CYP2C19) and/or vitamin K epoxide reductase (VKORC1) was examined. In sarcoidosis patients, next to tumor necrosis factor alpha (TNF-α -308) and butyrophiline-like 2 (BTNL2 G16071A), human leukocyte antigen (HLA) DRB1 and DQB1 were typed.

Chapter 1, the general introduction, presents a summary of various ILDs, with an emphasis on drug-induced interstitial lung disease (DI-ILD), diffuse alveolar hemorrhage (DAH), and sarcoidosis. Furthermore, the term polymorphism and the possible ways in how to detect them are pointed out. In addition, the fluorescence resonance energy transfer (FRET) detection method used in this thesis is explained more in detail.

In **chapter 2** the possible role of cytochrome P450 (CYP) enzymes in ILD, especially in drug-induced ILD (DI-ILD) was reviewed. The CYP enzyme family plays an important role in the metabolization of a variety of ingested, injected or inhaled xenobiotic substances. Polymorphisms in the *CYP* genes can

influence the metabolic activity of the subsequent enzymes, which in turn may lead to localized (toxic) reactions and tissue damage. Drug toxicity can be due to either absence or to very poor enzyme activity. In case of reduced enzyme activity, dose reduction or prescribing an alternative drug metabolized by a different, unaffected CYP enzyme is recommended to prevent toxic side effects. This is particularly important in multi drug-use. Knowing a patient's CYP profile before drug prescription could prevent drug-induced ILD and other drug-induced toxicity. Moreover, it might be helpful in explaining serious adverse effects from inhaled, injected or ingested xenobiotic substances.

Chapter 3 describes an easy DNA isolation method to be used prior to genetic testing. Several commercial DNA isolation kits are available for extracting genomic DNA from whole blood samples, but these procedures are time consuming and expensive. An alternative technique could be dried blood spot (DBS) sampling. With the subsequent simple isolation method DNA isolation is faster, cheaper, and logistics are easier. This DBS DNA isolation method, an alternative to the commercially available DNA isolation kits, is practical and it discriminates between genotypes. It can also be used for buccal swabs resulting in good DNA yields and giving completely concurrent results with samples isolated using commercial DNA isolation kits. This expands the possibilities of this quick and easy DNA isolation procedure, especially in combination with the noninvasive patient friendly buccal swab sampling method. In addition, buccal swab sampling appeared to be a good alternative to invasive sampling methods.

Chapter 4 describes drug-induced pulmonary toxicity, a serious and expanding problem. Many drugs are metabolized by cytochrome P450 (CYP) enzymes. To establish whether allelic variation in *CYP* polymorphic genes contributes to variability in drug response and unexpected toxicity, a case-control study was conducted. The cases consisted of patients with drug-induced interstitial lung disease. Furthermore, two control groups were used: one group of healthy volunteers and one group of patients with idiopathic pulmonary fibrosis (IPF). Of the patients with drug-induced ILD 91.5% had at least one of the studied variant genes compared with 70.5% of the healthy volunteers and 69.1% of the IPF patients (both p's<0.001). The percentage of individuals with one or more variant *CYP* genes was higher in the drug-induced ILD group. A significant association between the development of drug-induced ILD and the presence of one or more variant *CYP* genes was found. Drug-induced ILD was associated with the presence of at least one variant *CYP* allele. This study supports the potential usefulness of 'personalized' medicine by genotyping, aiming to improve efficacy, tolerability and drug safety.

The case presented in **chapter 5** illustrates that understanding the mechanisms of drug metabolism and interactions may help to prevent side effects. The patient was a poor metabolizer for CYP2D6 corroborating that polymorphisms in this *CYP* gene influenced the metabolic activity of the corresponding enzymes. In addition, this affected the subsequent serum drug levels of venlafaxine and to a much lesser degree metoprolol and their metabolites. This achieved that toxic serum levels of venlafaxine were present and no active metabolite (*O*-desmethylvenlafaxine) could be detected. Besides therapeutic drug monitoring, genotyping some important cytochrome P450 (CYP) enzymes was of additional value in explaining why the patient developed severe adverse effects. It also helped us to understand why the patient did not experience any therapeutical effect of the prescribed venlafaxine. This case highlights the potential benefit of clinical and genetic risk stratification (pharmacogenetics) before starting treatment.

In **chapter 6** diffuse alveolar hemorrhage (DAH) is highlighted. DAH is a serious bleeding complication that can occur as a result of, among others, oral anticoagulation therapy. This study explored the hypothesis that in patients treated with coumarins DAH may be associated with vitamin K epoxide reductase complex1 (*VKORC1*) and cytochrome P450 (*CYP*) variant alleles in *CYP2C9*, and in case of acenocoumarol use also with *CYP2C19* variants. Clinical information of patients using coumarins with at least one episode of DAH was gathered retrospectively during a seven year period. Out of 173 confirmed DAH cases, 75 had received oral anticoagulants and 63 of these 75 (84%) patients were included because DNA was available. Of these samples *CYP* and *VKORC1* single nucleotide polymorphisms (SNPs) were genotyped. In 62 out of 63 DAH cases *VKORC1*, *CYP2C9* variant genes, or both were found. This indicates that genotyping appears to be useful in predicting a high risk of serious side effects related to oral anticoagulant use, including DAH. Consequently, in concurrence with the American Food and Drug Administration (FDA), we recommend this genotyping, in order to provide a safer and more individualized anticoagulant therapy.

Chapter 7 describes a study designed to evaluate the relationship between the presence of tumor necrosis factor alpha (*TNF-α*) polymorphisms and the prognosis of sarcoidosis. In a retrospective case-control study *TNF-α* G-308A, *TNF-α* G-238A and *lymphotoxin-α* (*LTA*) were genotyped in 625 sarcoidosis patients. These patients were classified into 327 patients with non-persistent (regressing to or stable at chest X-ray stage 0 or I) disease and 298 patients with persistent disease using chest X-ray appearances and lung function test results after a period of at least two years of follow-up. The *TNF-α* -308A variant allele was observed in 25.5% of patients with persistent disease

compared with 44.0% of patients with non-persistent disease. Consequently, the presence of a *TNF-α* -308A variant allele is associated with a favorable prognosis. Because of the strong linkage between *TNF-α* G-308A and HLA-DRB1*03, genotyping of one relatively simple TNF-α SNP is useful in predicting the prognosis of pulmonary sarcoidosis.

In **chapter 8** the association between butyrophiline-like 2 (BTNL2) G16071A with the course of pulmonary sarcoidosis was assessed and the association with disease predisposition was verified in 632 sarcoidosis patients. In addition, the linkage between *BTNL2* G16071A and certain HLA-DRB1 and HLA-DQB1 types was investigated. It appeared that the *BTNL2* 16071A variant allele was significantly more often present in patients with persistent disease (92.4%) compared with patients having non-persistent disease (86.6%). The presence of a *BTNL2* 16071A variant allele was found to be associated with an almost twofold increased risk of progressing to more severe and persistent pulmonary sarcoidosis. Furthermore, the predisposition to develop sarcoidosis was confirmed, as well as the strong linkage between the *BTNL2* 16071A variant allele and DRB1*15 positivity. It also became apparent that typing for DRB1 is sufficient because of the lack of additional information obtained by typing the DQB1*06, to establish the 15Q6 haplotype. Whether or not to determine the DRB1 type or test the *BTNL2* G16071A SNP therefore, depends on the ability and/or availability to perform either test. Additional research will be necessary to explore the role of these findings in the clinical management of sarcoidosis patients.

General discussion

Both genetic and non-genetic information is important in the susceptibility, development, cause, and treatment response of diseases. The more we know about a patient's genes and context, the better disease management decisions can be made.[8] Nevertheless, often the cause of a disease appears to be not only associated with a single nucleotide polymorphism (SNP), but is much more complicated. Some patients will continue to react unpredictably to therapy even though, according to obtained tests results, problems were not expected. Extending mapping of transcription factor binding SNPs and structural variants associated with transcription factor binding might gather useful information about the role of the genetics in the phenotypic diversity in humans and provide insight into genetic causes of human disease.[9,10]

The success of modern medicine is partly the result of effective medical treatment. Although the overall advantage of many drugs outweighs the side effects, substantial costs are still made as a result of complications of drug therapy. The variability in drug response among patients is multi-factorial. These factors include extrinsic features like environmental aspects or co-medication, but also genetic and intrinsic factors that affect the disposition (absorption, distribution, metabolism and excretion) of individual drugs. The existence of large population differences with small intra-patient variability is consistent with inheritance as determinant of drug response. It is estimated that genetics can account for 20-95% of variability in drug disposition and effects.[11] Together clinical and genetic risk stratification (pharmacogenetics) may lead to more accurate prevention of drug-induced damage in the future.

In a recent editorial the results of almost ten years after revealing a draft sequence of the human genome were assessed. Although detailed maps of genetic markers of human variation, have facilitated many remarkable genomewide efforts to associate known SNPs with disease predisposition, more than one decade of genomics will be required to understand the inborn risks of most common disorders.[8] Nevertheless, reviews that highlight successful applications of gene-based medicine might hasten adoption of the beneficial changes in medicine that will eventually, if gradually, come from gene-based sciences.[8] All the same, our increased understanding of the interactions between the entire genome and non-genomic factors that result in health and disease is paving the way for an era of 'genomic medicine'.[12]

There are an increasing number of examples where pharmacogenetic studies have indicated that a genetic test prior to treatment may be useful either for setting the individual dose or choosing a certain drug.[13-18] The ability to identify

individuals who are susceptible to adverse drug reactions has the potential to reduce the personal and population costs of drug-related morbidity and the potential to attribute to the patients' safety. Especially persons with more than one cytochrome P450 (*CYP*) polymorphism and/or other relevant polymorphisms may be susceptible to develop adverse effects, such as drug-induced interstitial lung disease (ILD), when (multiple) drugs are prescribed.[19] Moreover, other pharmacogenetic factors might be involved, such as those involved in methotrexate induced pneumonitis.[20-22]

One of the major conclusions of this thesis is that genetic screening prior to drug prescription may potentially prevent serious adverse effects such as diffuse alveolar hemorrhage (DAH) or drug-induced ILD. Another important finding is that the results obtained by genetic testing appeared to be useful in disease management, because of the prognostic value of the absence or presence of specific polymorphisms.

Polymorphisms in drug-induced lung diseases

Not only drug interactions, environmental factors, disease processes, and aging are factors in the inter-individual metabolic capacity variance, genetic factors also play an important role in developing adverse effects in general. Considering the fact that bio-(in)activation by CYP enzymes play an important role in human drug toxicity, polymorphisms in the CYP enzyme system may result in large inter-individual variations in the metabolism and toxicity of xenobiotics. The presence of *CYP* variant alleles adds a substantial susceptibility risk factor to the development of drug-induced pulmonary adverse events.[19,23,24] In the study presented in chapter 6, genetic allelic variants appeared to be one of the determinants of variability in sensitivity to coumarins. Furthermore, others found that even in patients with stable international normalized ratios (INRs) without adverse (bleeding) effects these polymorphisms had a profound influence on dose requirements and time to reach therapeutic INRs.[15,25] Despite numerous attempts to standardize the management of oral anticoagulant therapy, a high proportion of patients are still inadequately anticoagulated and the optimal means to initiate the therapy is still a matter of debate. It has been suggested that dosing algorithms incorporating genetics might outperform usual care. However, a major obstacle to the widespread adoption of genetic based coumarin dose modelling is that access to timely genetic testing is currently not widely available.[26] Nevertheless, even despite its limitations, prospective genotyping for *CYP2C9* and *VKORC1* of patients taking oral anticoagulants has the clear potential to significantly optimize the safety of drug therapy and set a promising example of 'personalized' medicine.[10]

Drugs, oxidative stress and pulmonary damage

Beside anticoagulants, D-penicillamine, nitrofurantoin, amiodarone, propylthiouracil, cocaine, or sirolimus, and/or exposure to toxic agents such as trimellitic anhydride, insecticides, and pesticides may also cause bleeding complications including DAH events.[27] It has been suggested that oxidative damage plays a role in the pathophysiology of various diseases, including DAH and pulmonary fibrosis.[28,29] DAH is characterized by pulmonary alveolar cell death, inflammation, and hyaline membranes composed of fibrin and cellular debris.[30] Idiopathic pulmonary fibrosis (IPF) is a chronic, progressive and frequently fatal form of ILD, characterized by impaired fibrinolysis and a complex pathogenesis.[29,31,32] As a result of diffuse alveolar bleeding events iron is released in the lung. In turn this free toxic iron causes oxidative stress, inflammation, and finally irreversible damage or fibrosis.[30,33] Moreover, people with *VKORC1* and/or *CYP2C9* variant alleles might have a predisposition for a greater risk of bleeding events in case they use oral anticoagulants, which might make them more susceptible to iron-catalysed toxicity caused by oxidants. This is important as anticoagulant therapy is considered to be an additional new strategy to treat IPF patients.[34] In contrast, Thomassetti et al. did not find a beneficial effect of anticoagulants.[35] It is tempting to speculate that an association with *VKORC1* and/or *CYP2C9* variant alleles might even be a risk factor for the development or exacerbation of IPF. Furthermore, it was accentuated that in DAH cases early recognition of the presence of one of the studied polymorphisms is important, because of a potential lethal outcome and the fact that simple vitamin K supplementation can be life-saving.[36] Increased availability of vitamin K through daily supplementation, is expected to diminish the inhibitory activity and the relative day-to-day variability of coumarins and can significantly improve anticoagulation control in unstable patients.[36,37]

Importance of various genetic variants

It was emphasized that early recognition of the presence of certain polymorphisms is important.[36,37] Not only in case of anticoagulant use, also in psychiatry, pharmacogenetic testing has become more common practice in the United States (US) with FDA approval of the AmpliChip CYP450 and CYP2D6 and CYP2C19 testing helping patients with a history of excessive difficulties with antidepressants.[38-40] This was also illustrated by the case described in chapter 5. Besides *CYP* and *VKORC1* polymorphisms other genetic variants are also important. Two main immunosuppressive drugs that are often used in the treatment of severe ILD are methotrexate (MTX) and azathioprine. MTX is frequently used in the treatment of autoimmune diseases such as rheumatoid arthritis, psoriasis, Crohn's disease, as well as in sarcoidosis. MTX toxicity and efficacy is associated with a number of polymorphic enzymes and testing for

variants was found to be predictive of response to and safety of MTX treatment.[17,21,22,41] Moreover, when a patient develops an adverse drug reaction after starting MTX treatment, testing the C677T and A1298C variants in the methylenetetrahydrofolate reductase (*MTHFR*) gene involved in the MTX metabolism, should be considered.[42] In case of an azathioprine indication, also used as treatment for certain ILD including IPF, testing thiopurine methyltransferase (*TPMT*) variants involved in the azathioprine metabolism is advised before starting treatment.[43-45] In the US, drug labels for azathioprine now include information on TPMT polymorphisms and recommend determining patients' phenotype or genotype prior to drug treatment.[46]

Sarcoidosis

Sarcoidosis is a chronic granulomatous disorder of unknown cause with a highly variable course, characterized by activation of T-lymphocytes and macrophages. In an effort to determine the etiology of sarcoidosis the multicenter study A Case Control Etiologic Study of Sarcoidosis (ACCESS) was designed.[47] One of the lessons learned from ACCESS was that genetic associations with sarcoidosis were observed and appeared to be stronger predictors of the course of sarcoidosis than environmental factors.[48] The assumption that genes contribute to the etiology of sarcoidosis comes from the observation that prevalence and incidence rates of sarcoidosis are different between ethnic groups and that the disease tends to cluster in families. Interactions of exposures with genetic predispositions would have important implications for the understanding of immune responses as well as the pathogenesis of sarcoidosis.[47] Prognostic factors of sarcoidosis are very important because certain obstacles confound the accurate prediction of the prognosis of sarcoidosis, such as the lack of reliable activity markers, the intensity level of granulomatous response, and the inability to differentiate the response from the dys-regulated repair process leading to fibrosis.[47]

The role of genes in the etiology of sarcoidosis

Association of sarcoidosis and class I and II HLA antigens is well known and in several studies the risk of progression of sarcoidosis, and the presence or absence of a polymorphism has been established.[1,3,24,49,50] In addition to HLA types, co-stimulatory molecules of the immunoglobulin superfamily are also necessary to activate T-cells. The butyrophiline-like 2 (*BTNL2*) gene is located close to and in linkage with HLA-DRB1, which in turn is implicated in the etiology of sarcoidosis.[51-54] Moreover, the presence of the *BTNL2* 16071A variant allele was recently associated with an increased risk of developing sarcoidosis.[55-58] In T-helper cell 1 (Th1) dominated granulomatous diseases similar to sarcoidosis, an association between the presence of *BTNL2* G16071A

and disease predisposition was found. This association was attributed to the strong linkage with HLA-DRB1/DQB1 haplotypes.[54,59] Nevertheless, because of this strong linkage between *BTNL2* and HLA-DRB1/DQB1, *BTNL2* G16071A should be considered relevant to any immune-related disease associated with HLA-DRB1/DQB1.[54] In the study presented in this thesis, the strong linkage between the *BTNL2* 16071A variant allele and HLA-DRB1*15 was confirmed. Another conclusion was that the presence of the BTNL2 A-allele was also associated with an increased risk of progressing to a persistent form of pulmonary sarcoidosis. Another SNP that was confirmed to influence sarcoidosis susceptibility is cyclooxegenase-2 (*COX2*) T8473C, however, no association with progression was found.[60] Therefore, determining the *BTNL2* G16071A genotype even prior to the development of complaints, can be beneficial, for example when there is a family history of sarcoidosis.[61] Not only to establish whether or not someone is more prone to develop sarcoidosis, but also whether there is an additional risk to progress to a more persistent form. Recently, strong evidence was found for the postulation that several SNPs in the vascular endothelial growth factor (*VEGF*) and its receptor genes *VEGFR-1* and *VEGFR-2* also possessed the ability to predict predisposition and progression of sarcoidosis, but further studies are needed to evaluate the clinical relevance.[62]

The influence of genes on the course of sarcoidosis

Recently, it was found that serum amyloid A plays an important role in granuloma formation in sarcoidosis as well as tumor necrosis factor (TNF), interleukin-10 (IL-10), interferon-gamma (INF-γ), and Toll-like receptor 2 (TLR2).[63] In turn, these cytokines and enzymes not only influence the serum amyloid A production, but their expression can also be influenced by possible polymorphisms in their coding or non-coding sequence, and thus influence the cause or course of sarcoidosis.[7,50] The potent pro-inflammatory cytokine TNF-alpha (TNF-α) for instance plays a pivotal role in inflammatory and immune responses, and regulates and sustains granuloma formation in sarcoidosis.[64] Several SNPs are identified in the *TNF-α* gene and especially the variant A-allele of the TNF-α G-308A gene is associated with higher TNF-α serum levels and an acute course of sarcoidosis.[65] The findings presented in chapter 7 of this thesis demonstrate that it is sometimes beneficial to possess a variant allele (i.e. *TNF-α* -308A), opposed to not having a polymorphism, in the course of sarcoidosis.[50] The risk of progressing to a more severe pulmonary involvement or persistent form of sarcoidosis was found to be higher in the absence of a *TNF-α* G-308A allelic variant, corroborating findings of other studies conducted on smaller populations.[66,67] In line with this, bearing the HLA-DRB1*03 type also predicted a more favorable outcome, as was previously established in sarcoidosis patients with Löfgren's syndrome, the

acute form of sarcoidosis with predominantly spontaneous remission.[1,2,66,68] What is more, both *TNF-α* G-308A and HLA-DRB1*03 typing appear to be interchangeable as far as the outcome or prediction of the course of sarcoidosis is concerned, consequently confirming the strong linkage between both genes.[68,69]

The role of genes in therapeutic management

Because of the strong linkage disequilibrium found between several genes and HLA-types, the choice of whether or not to test for a simple SNP (for example *BTNL2* or *TNF-α* gene SNPs) or perform HLA typing appears to depend more on the availability of the technique or test. Conclusions drawn from studies presented in this thesis can be useful in furthering the clinical validation of applying genotyping in predicting the clinical course of sarcoidosis in individual patients and fine-tune disease management. Since available sarcoidosis therapies are not without risk and sometimes even toxic, patient care would benefit from the ability to predict the progression of the disease.[70] Therefore, identifying those cases that might have a chance to develop a more severe manifestation of sarcoidosis is important and treatment aimed at avoiding irreversible damage can be started early.[71] Furthermore, if the course of the disease has already progressed to severe or refractory sarcoidosis it would be of great clinical relevance to be able to select those cases who might benefit from a certain drug and who will not. For instance, when corticosteroids, most often the first drug of choice in sarcoidosis, are not effective, MTX might be considered. It has been beneficial in certain cases, however, it has a variety of clinical efficacies and toxicities, which are difficult to predict.[22,72] Several reports have suggested that the use of pharmacogenetics might help to improve the understanding of drug efficacy and toxicity. However, studies in rheumatoid arthritis or psoriasis patients showed conflicting data whether or not these side effects were caused by the altered expression of genes by MTX.[22,73,74]

Recently, anti-TNF-α therapy has proven to be useful in the treatment of refractory sarcoidosis. However, response to this therapy is not always as promising as expected.[70,75] Moreover, it takes some time before the effect of these so-called biologicals is apparent, substantial side-effects are reported and additionally, this therapy is expensive. In refractory sarcoidosis, bearing a *TNF-α* G-308A variant allele appears to be a disadvantage. In previous studies conducted in rheumatoid arthritis patients not possessing a variant allele seem to respond to the therapy in contrast to variant allele carriers.[76,77] Insight into the patient's probable response might provide important information, especially for those patients, who very likely will not benefit from this anti-TNF-α therapy, and therefore should be treated with alternative medication without delay.

Directions for future research

There is still a need for well designed prospective clinical trials that measure patient-oriented outcomes of selected genomic applications, and studies that evaluate the role of genomic variation in disease susceptibility, predicting prognosis, treatment response and in tailoring drug treatment for individual patients. These investigations are aimed to help to bridge the gap between 'personalized' and 'evidence-based' medicine[12]

In order to ascertain anticoagulation levels quicker and safer, without the risk of serious side effects and identify coumarin sensitive cases, genotyping for *VKORC1*, *CYP2C9*, and *CYP2C19* polymorphisms would be a preferable cause of action. This genotyping should be performed ideally prior to oral anticoagulant therapy, but certainly in case of high and unstable or even overshot target INRs. A cost-effectiveness study, evaluating whether or not screening for *VKORC1* and *CYP2C9* polymorphisms prior to administering oral anticoagulant therapy is of clinical relevance, is therefore highly recommended.

Furthermore, a prospective study to determine if, when variant alleles are present, simultaneous oral vitamin K supplementation can prevent diffuse alveolar hemorrhage (DAH) events is needed. Whether or not an association exists between *CYP* and *VKORC1* polymorphisms and the development and/or progression of pulmonary fibrosis is another important issue for future studies. Hypothesizing that fibrosis could be associated with repeated episodes of diffuse alveolar damage to a certain extent is intriguing. Prospective clinical trials are needed to clarify the role of anticoagulant therapy and other alveolar bleeding initiating agents in the development of pulmonary fibrosis. Further investigations are also needed to assess if anticoagulant therapy is a friend or foe in the therapeutic strategies of pulmonary fibrosis. Therefore, it would be interesting to evaluate whether screening of patients before initiating anticoagulant therapy might be of clinical relevance.

MTX, frequently used in sarcoidosis in combination with or without anti-TNF-α therapy, is also influenced by the presence of variant alleles. For that reason, a study into the influence of these polymorphisms on the efficiency and efficacy of this drug in sarcoidosis therapy should certainly be considered. Moreover, these studies might even give an answer to the question about which patients would benefit from MTX or biologicals and for whom no advantage can be achieved or for whom the result will even be detrimental. It might be fascinating to develop a screening system based on both clinical and genetic information.

Next to the information about the progression of sarcoidosis, a patient's TNF-α status or genetic make-up can also play a significant role in therapeutic disease management. Assessing a possible association in individual cases between the absence of a *TNF-α* -308A variant allele and being a responder or non-responder to anti-TNF-α therapy would be of great clinical relevance. The results could provide important information about whether or not a patient would possibly benefit from anti-TNF-α therapy. Therefore, research should continue to depict the role of *TNF* genes in the immunogenetics and clinical management of sarcoidosis.

The ability to identify individuals who are susceptible to adverse drug reactions, with the inclusion of both clinical and genetic risk stratification, may lead to a more accurate prevention of drug-induced damage and has the potential to reduce the personal and population costs of drug-related morbidity.[78,79] The introduction of a genetic medical passport for each patient aimed to achieve a more appropriate individual pharmacotherapy seems promising in therapeutic drug monitoring. However, whether this might reduce the risk of side effects and related medical consumption needs to be evaluated. Collaboration between medical specialists, clinical pharmacists, pharmacologists and laboratory specialists will be necessary to accomplish individualize pharmacotherapy based on the pharmacogenetic profile.[80]

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Appendix

Appendix

In the appendix drugs and other substances metabolized by and influencing (inhibit or induce) the most important CYP enzymes are listed. The drugs are subdivided into substrates, inhibitors, and inducers. The term substrate refers to a drug or other substance that is metabolized (activated or de-activated) by an enzyme. An inhibitor is a substance that inhibits the normal function of an enzyme and may result from non-competitive or competitive inhibition by a second drug or other substance, an effect that may occur rapidly. The result of non-competitive inhibition by the addition of a second agent is a slower metabolism of the first substance, higher plasma concentrations, and toxicity risk. In case of competitive inhibition, the metabolism of both substances can be reduced, resulting in higher than expected and possible toxic concentrations of both parent agents. An inducer is a substance that may result in increased CYP enzyme synthesis, faster drug metabolism, subtherapeutic drug concentrations and the risk for ineffective drug therapy. The promptness of the induction depends on the half-life of the inducing substance as well as the rate of synthesis of new enzymes.

The drugs and other substances are alphabetically ordered by CYP enzyme. The list of substrates metabolized by a specific (affected) enzyme and possible present inducers and/or inhibitors might be helpful to prevent possible problems.

This appendix is not comprehensive as new information is constantly being identified.[1-11]

Drugs and other substances metabolized by and influencing CYP enzymes.

Substrates

1A2		**2C9**		**2C19**	
Acetaminophen	Paroxetine	Aceclofenac	Lornoxicam	Acenocoumarol	Primidone
Aminophylline	Pentazocine	Acenocoumarol	Losartan	Alprazolam	Progesterone
Amitriptyline	Perphenazine	Acetaminophen	Mefenaminic acid	Amiodarone	Proguanil
Anagrelide	Phenacetin	Amitriptyline	Meloxicam	Amitriptyline	Propranolol
Bendamustine	Pimozide	Bosentan	Mephenytoin	Carisoprodol	Rabeprazole
Caffeine	Pirfenidone	Candesartan	Mestranol	Chloramphenicol	Ranitidine
Chlordiazepoxide	Primaquine	Cannabinol	Mirtazapine	Cilostazol	Ritonavir
Chlorpromazine	Propafenone	Carmustine	Montelukast	Citalopram	R-Mephobarbital
Cimetidine	Propranolol	Carvedilol	Naproxen	Clobazam	R-Warfarin
Cinacalcet	Ramelteon	Celecoxib	Nateglinide	Clomipramine	Sertraline
Citalopram	Ranitidine	Chloramphenicol	Nicoumalone	Clopidogrel	S-Mephenytoin
Clomipramine	Rasagiline	Chlorpheniramine	Omeprazole	Cyclophosphamide	Temazepam
Clopidogrel	Riluzole	Chlorporpamide	Paclitaxel	Desipramine	Teniposide
Clozapine	Ritonavir	Clomipramine	Paroxetine	Diazepam	Thioridazine
Cyclobenzaprine	Ropinirole	Clopidogrel	Phenobarbital	Diclofenac	Tolbutamide
Dacarbazine	Ropivacaine	Cotrimoxazol	Phenytoin	Divalproex	Trimipramine
Desipramine	R-Warfarin	Dapsone	Pirfenidone	Efavirenz	Valproic acid
Diazepam	Sertraline	Desogestrel	Piroxicam	Escitalopram	Venlafaxine
Duloxetine	Tacrine	Diazepam	Ritonavir	Esomeprazole	Voriconazole
Erlotinib	Tamoxifen	Diclofenac	Rosiglitazone	Flunitrazepam	
Estradiol	Theophylline	Dicoumarol	R-Warfarin	Fluoxetine	
Ethinyl estradiol	Thioridazine	Divalproex sodium	Seratrodast	Fluvoxamine	
Febuxostat	Thiothixene	Dronabinol	Sertraline	Formoterol	
Fluoxetine	Tizanidine	Efavirenz	Sildenafil	Hexobarbital	
Fluphenazine	Toremifene	Febuxostat	Sulfamethoxazole	Ibuprofen	
Flutamide	Trifluoperazine	Fluoxetine	Sulfaphenazole	Ifosfamide	
Fluvoxamine	Verapamil	Flurbiprofen	Sulfinpyrazone	Imipramine	
Frovatriptan	Zileuton	Fluvastatin	Sulfonamides	Indomethacin	
Haloperidol	Ziprasidone	Fluvoxamine	Suprofen	Lansoprazole	
Imipramine	Zolmitriptan	Formoterol	S-Warfarin	Loratidine	
Isotretinoin	Zolpidem	Glibenclamide	Tamoxifen	Mephenytoin	
Levobupivacaine		Gliclazide	Tenoxicam	Mephobarbital	
Lidocaine		Glimepiride	Tertrahydrocannabinol	Methsuximide	
Melatonin		Glipizide	Testosterone	Moclobemide	
Mesoridazine		Glyburide	Tolbutamide	Nelfinavir	
Methadone		Hexobarbital	Torsemide	Nevirapine	
Mexiletine		Ibuprofen	Valdecoxib	Nilutamide	
Mibrefradil		Idarubicin	Valproic acid	Omeprazole	
Mirtazapine		Imipramine	Valsartan	Pantoprazole	
Naproxen		Indomethacin	Vardenafil	Paroxetine	
Nortriptyline		Irbesartan	Voriconazole	Pentamidine	
Olanzapine		Irinotecan	Zafirlukast	Phenobarbital	
Ondansetron		Ketoprofen	Zileuton	Phenytoin	
Oxtriphylline		Leflunomide	Zolpidem	Pirfenidone	

Substrates

2D6

Acetaminophen
Alprenolol
Amiodarone
Amitriptyline
Amphetamine
Aripiprazole
Atomoxetine
Benztropine
Bisoprolol
Bufuralol
Captopril
Carvedilol
Cevimeline
Chlorpheniramine
Chlorpromazine
Chlorpropamide
Cinacalcet
Citalopram
Clemastine
Clomipramine
Clozapine
Codeine
Cyclobenzaprine
Darifenacin
Debrisoquine
Delavirdine
Desipramine
Dexfenfluramine
Dextroamphetamine
Dextromethorphan
Diltiazem
Dimenhydrinate
Dimethoxyamphetamine
Diphenhydramine
Dolasetron
Donepezil
Doxepin
Doxorubicin
Duloxetine
Encainide
Escitalopram
Ethylmorphine
Fenfluramine
Fentanyl
Flecainide
Flunarizine
Fluoxetine
Fluphenazine
Fluvoxamine
Formoterol
Galantamine
Guanoxan
Haloperidol
Hydrocodone
Hydrocortisone
Hydroxyzine
Idarubicin
Imipramine
Indinavir
Indoramin
Labetalol
Lidocaine
Loratadine
Maprotiline
MDMA (ecstasy)
Meperidine
Mequitazine
Methadone
Methamphetamine
Methoxyamphetamine
Metoclopramide
Metoprolol
Mexiletine
Minaprine
Mirtazapine
Moclobemide
Morphine
Nebivolol
Nefazodone
Nifedipine
Nisoldipine
Nortriptyline
Olanzapine
Ondansetron
Oxycodone
Paroxetine
Penbutolol
Perhexiline
Perphenazine
Phenacetin
Phenformin
Pindolol
Pirfenidone
Procainamide
Promethazine
Propafenone
Propoxyphene
Propranolol
Pyrantel
Quetiapine
Quinidine
Ranitidine
Ranolazine
Risperidone
Ritonavir
Ropivacaine
Selegiline
Sertindole
Sertraline
Sparteine
Tacrine
Tamoxifen
Tamsulosine
Thioridazine
Timolol
Tolterodine
Tramadol
Tranylcypromine
Trazodone
Trimipramine
Tripelennamine
Tropisetron
Venlafaxine
Yohimbine
Zuclopenthixol

Substrates

3A4-5

Acetaminophen
Alfentanil
Alfuzosin
Aliskiren
Almotriptan
Alprazolam
Amiodarone
Amitriptyline
Amlodipine
Amprenavir
Anastrozole
Aprepitant
Aranidipine
Aripriprazole
Astemizole
Atazanavir
Atorvastatin
Azithromycin
Bepridil
Bexarotene
Bicalutamide
Bosentan
Bromocriptine
Budesonide
Buprenorphine
Buspirone
Busulfan
Cabergoline
Caffeine
Cannabinoids
Carbamazepine
Carvedilol
Cerivastatin
Cevimeline
Chlordiazepoxide
Chloroquine
Chlorpheniramine
Chlorpromazine
Cilnidipine
Cilostazol
Cimetidine
Cinacalcet
Ciprofloxacin
Cisapride
Citalopram
Clarithromycin
Clindamycin
Clobazam
Clomipramine
Clonazepam
Clopidogrel
Clozapine
Cocaine
Codeine
Colchicine
Conjugated estrogens
Cortisol
Cyclobenzaprine
Cyclophosphamide
Cyclosporine
Cytarabine
Danazol
Dapsone
Darifenacin
Darunavir
Dasatinib
Daunorubicin
Delavirdine
Desogestrel
Dexamethasone
Dextromethorphan
Diazepam
Diclofenac
Dihydroergotamine
Diltiazem
Dirithromycin
Disopyramide
Docetaxel
Dofetilide
Dolasetron
Domperidone
Donepezil
Doxazosine
Doxepin
Doxorubicin
Doxycycline
Dronabinol
Dutasteride
Ebastine
Efavirenz
Eletriptan
Enalapril
Eplerenone
Ergots
Erlotinib
Erythromycin
Escitalopram
Esomeprazole
Estazolam
Estradiol
Eszopiclone
Ethinyl estradiol
Ethosuximide
Ethylmorphine
Etonogestrel
Etoposide
Exemestane
Felbamate
Felodipine
Fentanyl
Fexofenadine
Finasteride
Fluconazole
Flunitrazepam
Fluoxetine
Flupenthixol
Flurazepam
Flutamide
Fluticasone
Fluvestrant
Fluvoxamine
Fomepizole
Fosamprenavir
Galantamine
Gefitinib
Gestodene
Glyburide
Granisetron
Halofantrine
Haloperidol
Hydrocannabinol
Hydrocodone
Hydrocortisone
Ifosfamide
Imatinib
Imipramine
Indinavir
Irinotecan
Isotretinoin
Isradipine
Itraconazole
Ketamine
Ketoconazole
Lamotrigine
Lansoprazole
Lercanidipine
Letrozole
Levobupivacaine
Levomethadyl
Lidocaine
Lilopristone
Loperamide
Lopinavir
Loratadine
Losartan
Lovastatin
Loxapine
Medroxyprogesterone
Mefloquine
Meloxicam
Meperidine
Methadone
Methsuximide
Methylprednisolone
Methysergide
Metoprolol
Mexiletine
Mibefradil
Miconazole
Midazolam
Mifepristone
Mirtazapine
Mitotane
Modafinil
Mometasone
Montelukast
Nateglinide
Navelbine
Nefazodone
Nelfinavir
Nevirapine
Nicardipine
Nifedipine
Nilotinib
Nimodipine
Nisoldipine
Nitrazepam
Nitrendipine
Nordazepam
Norethindrone
Omeprazole
Onapristone
Ondansetron
Oral contraceptives
Oxybutynin
Oxycodone
Paclitaxel
Pantoprazole
Paroxetine
Pergolide
Perphenazine
Phencyclidine
Pimozide
Pioglitazone
Pravastatin
Praziquantel
Prednisolone
Prednisone
Primaquine
Progesterone
Progestin
Proguanil
Propafenone
Propoxyphene
Propranolol
Quetiapine
Quinidine
Quinine
Rabeprazole
Ramelteon
Ranitidine
Ranolazine
Rapamycin
Reboxetine
Repaglinide
Rifabutin
Rifampin
Risperidone
Ritonavir
Rokitamycin
Ropinirole
R-Warfarin
Salmeterol
Saquinavir
Selegiline
Seratrodast
Sertraline
Sibutramine
Sildenafil
Simvastatin
Sirolimus
Solifenacin
Sorafenib
Sparfloxacin
Sufentanil
Sunitinib
Tacrolimus
Tadalafil
Tamoxifen
Tamsulosin
Taxol
Telithromycin
Temazepam
Teniposide
Terazosin
Terfenadine
Testosterone
Theophylline
Tiagabine
Timolol
Tinidazole
Tipranavir
Tolcapone
Tolterodine
Toremifene
Tramadol
Trazodone
Tretinoin
Triamcinolone
Triazolam
Trimetrexate
Trimipramine
Trofosfamide
Troleandomycin
Valdecoxib
Valproic acid
Vardenafil
Venlafaxine
Verapramil
Vesnarinone
Vinblastine
Vincristine
Vindesine
Vinorelbine
Voriconazole
Zaleplon
Zileuton
Ziprasidone
Zolpidem
Zonisamide
Zopiclone
Zuclopenthixol

Inhibitors

1A2

Acyclovir
Amiodarone
Amitriptyline
Anastrozole
Atazanavir
Caffeine
Cimetidine
Ciprofloxacin
Citalopram
Clarithromycin
Diltiazem
Duloxetine
Echinacea
Enoxacin
Erythromycin
Estradiol
Ethinyl estradiol
Famotidine
Fluoxetine
Fluphenazine
Flutamide
Fluvoxamine
Furafylline
Gatifloxacin
Grapefruit (juice)
Imipramine
Interferon
Isoniazid
Ketoconazole
Levofloxacin
Levonorgestrel
Lidocaine
Lomefloxacin
Methoxsalen
Mexiletine
Mibefradil
Moclobemide
Moxifloxacin
Nalidixic acid
Nelfinavir
Norethindrone
Norfloxacin
Ofloxacin
Omeprazole
Oral contraceptives
Paroxetine
Perphenazine
Phenacetin
Pirfenidone
Propafenone
Ranitidine
Rifampin
Ritonavir
Ropinirole
Sparfloxacin
St. John's Wort
Tacrine
Tegaserod
Ticlopidine
Tocainide
Troleandomycin
Verapamil
Zafirlukast
Zileuton

2C9

Amiodarone
Anastrozole
Atazanavir
Benzbromarone
Capecitabine
Chloramphenicol
Cimetidine
Clopidogrel
Cotrimoxazole
Delavirdine
Devil's claw
Diclofenac
Disulfiram
Efavirenz
Fenofibrate
Fluconazole
Fluorouracil
Fluoxetine
Flurbifprofen
Fluvastatin
Fluvoxamine
Garlic
Gemfibrozil
Grapefruit (juice)
Imatinib
Isoniazid
Itraconazole
Ketoconazole
Ketoprofen
Leflunomide
Lovastatin
Methoxsalen
Metronidazole
Mexiletine
Modafinil
Nalidixic acid
Norethindrone
Norfloxacin
Omeprazole
Oral contraceptives
Paroxetine
Phenylbutazone
Pirfenidone
Probenicid
Ranitidine
Ritonavir
Rosuvastatin
Sertraline
St. John's Wort
Stiripentol
Sulfamethoxazole
Sulfaphenazole
Sulfinpyrazone
Sulfonamides
Tacrine
Tamoxifen
Teniposide
Ticlopidine
Tipranavir
Trimethoprim
Troglitazone
Troleandomycin
Valproic acid
Voriconazole
Wolfberry
Zafirlukast
Zileutin

2C19

Amitriptyline
Artemisinin
Chloramphenicol
Cimetidine
Citalopram
Delavirdine
Devil's Claw
Efavirenz
Esomeprazole
Felbamate
Fluconazole
Fluoxetine
Fluvastatin
Fluvoxamine
Garlic
Imipramine
Indomethacin
Interferon
Isoniazid
Ketoconazole
Lansoprazole
Letrozole
Modafinil
Norfluoxetine
Omeprazole
Oral contraceptives
Oxcarbazepine
Pantoprazole
Paroxetine
Pirfenidone
Probenicid
Rabeprazole
Ranitidine
Ritonavir
Sertraline
St. John's Wort
Sulfaphenazole
Telmisartan
Ticlopidine
Tolbutamide
Topiramate
Tranylcypromine
Troglitazone
Valdecobix
Voriconazole

Inhibitors

2D6

Amiodarone
Amitriptyline
Anti-histamine
Aripiprazole
Black cohosh
Black pepper
Buproprion
Cajuput
Celecoxib
Chloroquine
Chlorpheniramine
Chlorpromazine
Cimetidine
Cinacalcet
Cinnamon
Citalopram
Clemastine
Clomipramine
Clove
Clozapine
Cocaine
Darifenacin
Desipramine
Diphenhydramine
Doxepin
Doxorubicin
Duloxetine
Escitalopram
Febuxostat
Fluoxetine
Fluphenazine
Fluvoxamine
Ginger
Ginseng
Goldenseal
Halofantrine
Haloperidol
Hydroxychloroquine
Hydroxyzine
Imatinib
Imipramine
Indinavir
Lansoprazole
Levomepromazine
Lomustine
Lopinavir
Methadone
Methotrimeprazine
Methylphenidate
Metoclopramide
Mibefradil
Midodrine
Moclobemide
Nefazodone
Norfloxacin
Norfluoxetine
Orphenadrine
Paroxetine
Perphenazine
Pimozide
Pomegranate
Propafenone
Propoxyphene
Propranolol
Quinacrine
Quinidine
Quinine
Ranitidine
Ranolazine
Risperidone
Ritonavir
Sandalwood
Sertindole
Sertraline
St. John's Wort
Tegaserod
Terbinafine
Thioridazine
Ticlopidine
Tipranavir
Tripelennamine
Valproic acid
Venlafaxine
Vinblastine
Vinorelbine
Yohimbine

3A4-5

Acitretin
Amiodarone
Amprenavir
Anastrozole
Androstenedione
Aprepitant
Atazanzvir
Black cohosh
Bromocriptine
Cat's claw
Chloramphenicol
Chloroquine
Cimetidine
Ciprofloxacin
Cisapride
Clarithromycin
Clomipramine
Clotrimazole
Cyclosporine
Dalfopristin
Danazol
Delavirdine
Devil's claw
Diltiazem
Dithiocarbamate
Doxycycline
Echinacea
Efavirenz
Enoxacin
Erythromycin
Ethinyl estradiol
Ezetimibe
Fluconazole
Fluoxetine
Fluvoxamine
Garlic
Gestodene
Ginseng
Goldenseal
Grapefruit (juice)
Haloperidol
Imatinib
Indinavir
Isoniazid
Itraconazole
Ketoconazole
Khella
Lopinavir
Methadone
Methylprednisolone
Methylprednisone
Metronidazole
Mibefradil
Miconazole
Mifepristone
Nafimidone
Nefazodone
Nelfinavir
Nevirapine
Nicardipine
Nifedipine
Norethindrone
Norfloxacin
Norfluoxetine
Omeprazole
Oral contraceptives
Oxiconazole
Paroxetine
Phenobarbital
Pimozide
Pirfenidone
Posaconazole
Prednisone
Primaquine
Propofol
Propoxyphene
Quinidine
Quinine
Quinupristin
Ranitidine
Ranolazine
Ritonavir
Roxithromycin
Saquinavir
Sertindole
Sertraline
Sparfloxacin
St. John's Wort
Star fruit
Tacrolimus
Tamoxifen
Telithromycin
Troglitazone
Troleandomycin
Valproic acid
Venlafaxine
Verapamil
Voriconazole
Zafirlukast
Zileuton

Inducers

1A2	2C9	2C19	2D6	3A4-5
beta-Naphthoflavone	Aprepitant	Artemisinin	Dexamethasone	Aminoglutethimide
Barbituates	Barbituates	Barbiturates	Rifampin	Amprenavir
Broccoli	Bosentan	Carbamazepine		Aprepitant
Brussel sprouts	Carbamazepine	Ginko Biloba		Barbiturates
Cabbage	Cyclophosphamide	Lopinavir		Bosentan
Caffeine	Ethanol	Norethindrone		Butalbital
Carbamazepine	Ginseng	Orphenadrine		Cannabinoids
Cauliflower	Glipizide	Phenobarbital		Carbamazepine
Charbroiled meats	Ifosfamide	Phenytoin		Cisplatin
Clotrimazole	Pentobarbital	Prednisone		Clotrimazole
Esomeprazole	Phenobarbital	Primidone		Cyclophosphamine
Griseofulvin	Phenytoin	Rifabutin		Dexamethasone
Insulin	Primidone	Rifampin		Efavirenz
Lansoprazole	Rifabutin	Ritonavir		Ethosuximide
Marijuana	Rifampin	St John's Wort		Felbamate
Mebendazole	Rifapentine	Valproic acid		Garlic supplements
Methylcholanthrene	Ritonavir			Ginseng
Modafinil	Secobarbital			Glucocorticoids
Moricizine	St John's Wort			Glutethimide
Nafcillin	Valproic acid			Griseofulvin
Nicotine				Ifosfamide
Omeprazole				Lopinavir
Phenobarbital				Methadone
Phenytoin				Methylprednisolone
Primidone				Modafinil
Psoralen				Nafcillin
Rifampin				Nevirapine
Ritonavir				Oxcarbazepine
St John's Wort				Pentobarbital
Tobacco				Phenobarbital
				Phenylbutazone
				Phenytoin
				Pioglitazone
				Prednisone
				Primidone
				Rifabutin
				Rifampin
				Rifapentine
				Ritonavir
				St. John's Wort
				Sulfinpyrazone
				Topiramate
				Troglitazone
				Troleandomycin

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List of publications

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Dankwoord

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Curriculum Vitae

Curriculum Vitae

Petal (Petra Albertine Hildegard Maria) Wijnen was born on July 26th, 1963 in Heerlen, The Netherlands. From 1975 until 1981 she attended secondary school (Atheneum B) at the Albert Schweitzer Scholengemeenschap (ASA) in Geleen. After graduation, she started the higher laboratory education (HLO) at the Zuidlimburgse Laboratorium School (ZLS) in Sittard. She specialized in clinical chemistry and haematology and graduated in 1985. Thereafter, she started working as a general laboratory technician at the Department of Clinical Chemistry of the Maastricht University Medical Centre (MUMC). Her specialisation at that time was protein chemistry and high pressure liquid chromatography (HPLC). From 2003 she has been working as a research technician in the field of Molecular Diagnostics (PCR/DNA analyses). In that capacity, she cooperated in several research projects and assisted several researchers in completing their thesis. In 2007 she started the work presented in this thesis, supervised by Prof. Dr. M. Drent, Prof. Dr. M.P. van Dieijen-Visser, and Dr. O. Bekers. She is a member of the CYP task force and the ild care team of the MUMC.