# AN INVESTIGATION INTO THE ROLE OF

COLLECTINS IN TUBERCULOSIS INFECTION

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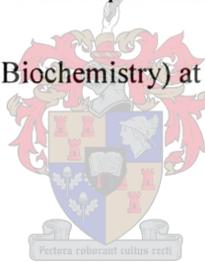
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Declaration

I, the undersigned, hereby declare that the work contained in this thesis is my own original work, and that I have not previously in its entirety or in part submitted it at any university for a degree.

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SUMMARY

Tuberculosis (TB) is a worldwide problem which is particularly serious in the developing countries. In South Africa, as with many other countries, HIV is a serious compounding factor in trying to control the spread of the disease. There is much evidence that host genetic factors influence the outcome of disease as only 10% of individuals who are infected with *Mycobacterium tuberculosis* (*M. tuberculosis*) will actually go on to develop active disease.

The innate immune response is the body's first line defence before the acquired immune response is initiated. It is probably in these early stages after inhalation of the bacilli that the fate of the bacterium is decided. The collectin molecules are an important part of the innate immune response particularly in the lung and include, most importantly, mannose binding lectin, and the surfactant proteins (SP) -A and -D. These three collectin molecules have been shown to play a role in the host defence with respect to *M. tuberculosis*.

In this investigation particular attention was paid to the surfactant proteins which seem to play important yet distinct roles in the response to the inhaled bacilli. SP-A acts to enhance attachment of *M. tuberculosis* to alveolar macrophages and increase phagocytosis whereas SP-D agglutinates *M. tuberculosis* thereby reducing phagocytosis of *M. tuberculosis* by macrophages.

Case-control genetic association studies have been performed using polymorphisms within these genes and have shown association with TB in numerous populations including the South African Coloured population in Cape Town. A polymorphism within the amino terminal region of the SP-D gene was associated with susceptibility to TB. A family-based study was therefore undertaken to replicate the result obtained in the case-control study. Discrepant results were obtained that appeared dependent on the family structure used. The number of families analysed was relatively small and therefore one cannot conclude that the association previously detected was spurious, and a larger study should be performed. The impact of this polymorphism was studied further to determine whether it affected the overall structure of the protein. We also examined the effect this polymorphism had on the concentration of SP-D in the serum of active TB patients compared to controls. We were not able to determine what role, if any, the polymorphism plays in the overall structure of the SP-D molecule. However, we showed that the serum concentration of SP-D was significantly increased in active TB patients compared to controls ($p < 0.0001$). Furthermore we

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demonstrated that the concentration of SP-D was significantly increased particularly in TB patients with a TT genotype compared to controls ($p < 0.0001$). The TT genotype was previously associated with susceptibility to TB in a case-control association study (T. Roos, unpublished data).

Numerous allelic variants have been identified in the SP-A genes (SP-A1 and SP-A2) which make up the fully functional SP-A protein. Polymorphisms that were recently identified within the collagen-like region of SP-A1 and SP-A2 (Madan et al., 2002) as well as one novel polymorphism identified in this study were investigated in the South African Coloured population. We found a significant association between a polymorphism in the collagen-like region of the SP-A2 gene and susceptibility to TB ($p = 0.007$).

Our study demonstrates the importance of human genetics in understanding human susceptibility to infectious diseases.

OPSOMMING

Tuberkulose (TB) affekteer die hele wêreld, maar dit is veral in ontwikkelende lande 'n groot probleem. In Suid-Afrika, soos in baie ander lande, veroorsaak die immuun-paraliserende uitwerking van HIV-koïnfeksie dat die TB-epidemie voortwoeker. Daar is bewyse dat genetiese faktore in die gasheer die uitkoms van die siekte bepaal, aangesien slegs 10% van die individue wat deur *Mycobacterium tuberculosis* (*M. tuberculosis*) geïnfekteer word, uiteindelik die aktiewe siekte ontwikkel.

Die aangebore immuunsisteem is die liggaam se eerste verdedigingslinie, waarna die verworwe immuunreaksie geïnisieer word. Die bakterium se lotgevalle word moontlik bepaal in hierdie vroeë stadium pas nadat dit ingeasem is. Die kollektien-molekule is veral in die long 'n belangrike deel van die aangebore immuunrespons en sluit die mannose-bindende lektien en die surfaktantproteïene A (SP-A) en D (SP-D) in. Dit is al aangetoon dat hierdie drie kollektien-molekule in die gasheer 'n rol speel in die verdediging teen *M. tuberculosis.*

In hierdie ondersoek is veral klem gelê op die surfaktantproteïene, wat voorkom asof dit belangrike en kenmerkende rolle speel in die reaksie teen die ingeasemde bakterieë. SP-A versterk die aanhegting van *M. tuberculosis* aan die alveolêre makrofage en verhoog fagositose, terwyl SP-D die bakterieë agglutineer en so verhoed dat dit deur die makrofage gefagositeer word.

Gekontroleerde assosiasiestudies in pasiënte is gedoen deur polimorfismes in hierdie gene, wat geassosieer is met TB in ander bevolkings as ons eie, te bestudeer. 'n Polimorfisme in die amino-terminaal area van die SP-D-geen is positief geassosieer met vatbaarheid vir TB. 'n Familie-gebaseerde studie is ook gedoen om die resultate van die gekontroleerde assosiasiestudie te repliseer. Verskillende resultate is verkry en word moontlik bepaal deur die familiestruktuur wat gebruik is. Die aantal families wat bestudeer is, was relatief min en daarom kan daar nie afgelei word dat die assosiasie wat voorheen waargeneem is vals is nie. 'n Groter studie sal gedoen moet word. Die impak van hierdie polimorfisme is verder ondersoek om te bepaal of dit die totale struktuur van die proteïen beïnvloed. Die effekte van hierdie polimorfisme op die konsentrasie van SP-D in die serum van aktiewe TB-pasiënte is ondersoek en vergelyk met diè van kontroles. Ons kon nie vasstel watter rol, indien enige, die polimorfisme in die totale struktuur van die SP-D-molekule speel nie, maar ons het bewys dat die serumkonsentrasie van SP-D beduidend verhoog was in aktiewe TB-pasiënte in

vergelyking met kontroles (p < 0.0001). Verder het ons ook gedemonstreer dat die konsentrasie van SP-D beduidend verhoog was in die TT-genotipe van die aktiewe TB-pasiënte vergeleke met die kontroles (p < 0.0001). Die TT-genotipe is al voorheen positief geassosieer met vatbaarheid vir TB (T. Roos, ongepubliseerde inligting).

Verskeie alleliese variante is geïdentifiseer in die SP-A-gene (SP-A1 en SP-A2) wat saam die volle funksionele SP-A-proteïen vorm. Polimorfismes wat onlangs in die kollageen-agtige area van SP-A1 en SP-A2 gevind is (Madan et al., 2002) en een nuwe polimorfisme wat in hierdie studie geïdentifiseer is, is ondersoek in 'n Suid-Afrikaanse bevolking. Ons het beduidende positiewe assosiasie tussen 'n polimorfisme in die kollageen-agtige area van die SP-A2 geen en vatbaarheid vir TB (p = 0.007) aangetoon.

Ons bevindinge bewys die belangrikheid van die bestudering van mensgenetika, wat die immuunkompetensie rig, om vatbaarheid vir infektiewe siektes te verstaan.

Impossible is just a big word thrown
Around by small men who find it easier
To live in the world they've been given
Than to explore the power they have to change it.
Impossible is not a fact. It's an opinion.
Impossible is not a declaration. It's a dare.
Impossible is potential. Impossible is temporary.

Impossible is nothing.

Anon

CHAPTER 5: SEQUENCING OF THE AMINO TERMINAL, NECK AND PROXIMAL PROMOTER REGIONS OF THE SP-D GENE

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<u>ABBREVIATIONS</u>

ADC	albumin-dextrose-catalase mycobacterial growth supplement
$AgNO_3$	silver nitrate
AIDS	acquired immune deficiency syndrome
Ala	alanine
APR	acute phase response
araLAM	arabinofuranosyl-terminated LAM
Arg	arginine
APS	ammonium persulfate
ARDS	Acute Respiratory Distress Syndrome
ARMS	amplification refractory mutation system
Asn	asparagine
BALF	broncho alveolar lavage fluid
BCG	bacillus Calmette-Guérin
BLAST	Basic Local Alignment Search Tool
BSA	bovine serum albumin
$CaCl_2$	calcium chloride
CH_3COOH	acetic acid
CI	Confidence interval
CL-1	Collectin Liver 1
CL-43	Bovine Serum Lectin 43
COPD	chronic obstructive pulmonary disease
CR	complement receptor
CRD	Carbohydrate Recognition Domain
CRP	C-reactive protein
Cys	cysteine
D'	disequilibrium coefficient
dNTP	deoxynucleoside triphosphate
DOTS	Directly Observed Treatment Short-course
DTT	dithiothreitol
ECL	enhanced chemiluminescence

EDTA	ethylene diamine tetra-acetic acid
ELISA	enzyme-linked immunosorbent assay
FDR	false discovery rate
GBS	Group B streptococcus
gp-340	glycoprotein 340
HCl	hydrochloric acid
His	histidine
HIV	Human Immunodeficiency Virus
HLA	Human Leukocyte Antigen
HLA-DR2	Human Leukocyte Antigen locus DR2
HRR	Haplotype Relative Risk
HWE	Hardy-Weinberg equilibrium
IFN-γ	Interferon gamma
IFN-γR1	Interferon Gamma Receptor 1
IgG	Immunoglobulin G
IL	interleukin
iNOS	inducible nitric oxide synthase
IPCD	Interstitial pneumonia with collagen vascular disease
IPF	Idiopathic pulmonary fibrosis
KCl	potassium chloride
kDa	kilodalton
KH_2PO_4	potassium dihydrogen phosphate
LAM	lipoarabinomannan
LD	Linkage Disequilibrium
Leu	leucine
LJ	Lowenstein-Jensen
LM	lipomannan
M. smegmatis	*Mycobacterium smegmatis*
manLAM	mannosylated LAM
MBL	Mannose Binding Lectin
MDR	Multiple Drug Resistant
$MgCl_2$	Magnesium chloride
$MnCl_2$	manganese chloride

MR	mannose receptor
M.tuberculosis	*Mycobacterium tuberculosis*
NaCl	sodium chloride
Na_2CO_3	sodium carbonate
$NaHCO_3$	sodium hydrogen carbonate
Na_2HPO_4	sodium dihydrogen phosphate
NaOH	sodium hydroxide
NCBI	National Centre for Biotechnology Information
NRAMP1	Natural Resistant Associated Macrophage Protein 1
OD	Optical density
OR	Odds ratio
PA	polyacrylamide
PBS	Phosphate buffered saline
PBST	phosphate buffered saline containing 0.05% Tween 20
PCR	polymerase chain reaction
Pro	proline
RDS	Respiratory Distress Syndrome
rpm	revolutions per minute
rSP-D	recombinant SP-D
RSV	respiratory syncytial virus
SDS	sodium dodecyl sulfate
SLC11a1	Solute carrier family 11 member 1
PAGE	polyacrylamide gel electrophoresis
SNP	Single Nucleotide Polymorphism
SP	Surfactant Protein
SP-A	Surfactant Protein A
SP-A$^{-/-}$	Surfactant Protein A deficient
SP-D	Surfactant Protein D
SP-D$^{-/-}$	Surfactant Protein D deficient
S. pneumoniae	*Streptococcus pneumoniae*
T_A	annealing temperature
TB	Tuberculosis

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TBE	tris-boric acid-ethylene diamine tetra-acetic acid buffer
T_D	denaturation temperature
TDT	Transmission Disequilibrium Test
T_E	extension temperature
TE	tris-ethylene diamine tetracetic acid buffer
TEMED	NNN'N' -tetrametylethylenediamine
T_m	melting temperature
TNF-α	tumor necrosis factor alpha
Tris	Tris(hydroxymethyl)aminomethane
UBE3A	ubiquitin protein ligase 3A
UTR	untranslated region
UV	ultra violet
Val	valine
WHO	World Health Organisation
ZN	Zeihl-Neelsen

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Absolute Ethanol	Merck
Acrylamide/bis-Acrylamide	Sigma
ADC	Merck
Agarose	Whitehead Scientific
$AgNO_3$	Merck
APS	Promega
Boric acid	Merck
Brilliant Blue R250	Sigma
Bromophenol blue	Saarchem
BSA 98% pure	Sigma
Butanol	BDH
$CaCl_2$	Sigma
CH_3COOH	BDH
DTT	Sigma
EDTA	Fluka
Ethidium Bromide	Sigma
Ficoll	Sigma
Formaldehyde	Merck
Glycerol	BDH
Glycine	Saarchem
HCl	Merck
Maltose	Sigma
Maltose Agarose	Sigma
Methanol	Saarchem
Methylene Blue	BDH
Middlebrook 7H9 Broth	Difco
1M $MnCl_2$ solution	Sigma
Na_2CO_3	Merck
NaCl	Saarchem
$NaHCO_3$	Riedel-de Haën

Orange G	Merck
PBS pellets	Sigma
Phenol	Sigma
SDS	Sigma
Sodium azide	Sigma
Sodium Hydroxide Pellets	Merck
Sodium Thiosulphate	Merck
Streptavidin peroxidase conjugate	Sigma
Sulfuric acid	Saarchem
TEMED	BDH
TMB/Hydrogen peroxide	Bio-Rad
Tris-HCl	Fluka
Tween 20	Saarchem
Tween 80	Sigma
Urea	Saarchem

CHAPTER 1

Introduction

If we value the pursuit of knowledge, we must be free to follow wherever that search may lead us. The free mind is not a barking dog, to be tethered on a ten-foot chain.

Adlai E. Stevenson Jr. (1900 - 1965)

1.1 – IMPACT OF TUBERCULOSIS WOLDWIDE

Tuberculosis (TB) is an infectious disease caused by the tubercle bacillus, *Mycobacterium tuberculosis* (*M. tuberculosis*). TB is the leading cause of death due to a single infectious agent and although it is considered by some authorities to be less aggressive than smallpox, the bubonic plague, leprosy or acquired immune deficiency syndrome (AIDS), it is responsible for the largest number of deaths in history. It is estimated that between 2002 and 2020, nearly 1000 million people will be newly infected, over 150 million people will become diseased and 36 million will die of TB (The World Health Organisation (WHO), http://www.who.int/en/) (**Figure1.1**). Although the largest number of cases and the highest number of deaths occurs in the South East Asia region the highest mortality per capita is in the Africa region. This is because the human immunodeficiency virus (HIV) has led to dramatic increases in the incidence of TB as well as increasing the likelihood of dying from TB (WHO Report 2004). South Africa has an incidence of 558/100 000 population and a TB mortality rate of 79/100 000 population (WHO Report 2004). It is alarming to note that 60% of the adult population (15 – 49 years) who are infected with TB are also HIV positive and 1.5% of the new cases reported are multiple drug resistant (MDR) TB cases (WHO Report 2004). The Western Cape, South Africa, has one of the highest incidences in the world (up to 636/100 000) (WHO).

1.1.1   *M. tuberculosis* – The pathogen

M. tuberculosis is a facultative, intracellular pathogen that has developed specialised mechanisms to survive within the macrophage. This is different from most other pathogens that avoid entering the macrophage (obligate extracellular pathogens) to escape killing (Ernst, 1998). Entry into, and survival within the macrophage is the distinguishing factor between pathogenic and non-pathogenic mycobacteria (Ramakrishnan et al., 2000).

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FIGURE 1.1: The estimated global TB incidence rates in 2002. (Taken from www.who.int/gtb/country_info/index.htm).

M. tuberculosis along with *M. bovis*, *M. bovis* Bacille Calmette-Guérin (BCG), *M. aficanum* and *M. microti* belong to the *M. tuberculosis* complex and cause TB. The genus *mycobacterium* are non-motile, rod shaped and do not form spores and are classified as acid fast bacilli as they are able to retain carbol-fuchsin stain after washing with acid, alcohol or both (Goodfellow and Minnikin, 1984; Kubica, 1984). The *M. tuberculosis* bacterium is characterised by its slow growth, dormancy and complex cell envelope. The cell wall structure is different from that of other Gram-positive bacteria. Like other bacteria it has a cell membrane and a peptidoglycan layer, but there is also a mycolyl arabinogalactan layer, consisting of mycolic acids esterified to the cell wall. There are also numerous lipids and glycolipids non-covalently associated with the cell envelope. These include trehalose dimycolate, phthiocerol dimycocerate and lipoarabinomannan (LAM), many of which have been suggested to play a role in virulence (Glickman and Jacobs, Jr., 2001). Although it is known that there is a whole array of entities in the cell envelope, it is still poorly understood how these molecules act in pathogenesis of disease and the roles they play in virulence (Glickman and Jacobs, Jr., 2001).

1.1.2   Treatment of TB

1.1.2a. TB Control Programme (DOTS)

The internationally recommended approach to TB control is Directly Observed Treatment Short-course (DOTS). The DOTS strategy has five key elements, according to the WHO (www.who.int.gtb):

- Government commitment to sustained TB control.
- Detection of TB cases through sputum smear microscopy among people with symptoms.
- Regular and uninterrupted supply of high quality TB drugs.
- 6 – 8 months of regularly supervised treatment (including direct observation of drug taking for at least two months).
- Reporting systems to monitor treatment progress and programme performance.

To ensure the patient's progress is monitored throughout the treatment, sputum smear testing is repeated after two months and then again at the end of treatment. In 1991 DOTS was introduced and since then more than 13 million patients have received treatment under this

strategy. By the end of 2002, all 22 countries with the highest incidences of TB (including South Africa), together accounting for 50% of the world's estimated cases, had adopted DOTS.

In South Africa DOTS was implemented in 1996 with the goal of extending TB control services to the whole country. DOTS now reaches 180 districts (98%), however the quality of DOTS has deteriorated in some areas. There are several reasons for this deterioration and why targets are not met. There is sometimes a lack of sustained commitment to the DOTS programme and in some instances there is insufficient staff and TB managers in certain districts and provinces due to the high workloads and because of the increasing number of TB patients who are HIV positive. To help alleviate this problem home-based caregivers and community health workers are being trained in the management of both TB and HIV. DOTS aims to detect 70% of all new infectious TB cases and to cure 85% of those detected by 2005. In 2002, 18 countries had already achieved these targets (WHO Report 2004).

1.1.2b. Vaccines and Drugs

The conventional drugs used for treating TB include; ethambutol, isoniazid, pyrazinamide and rifampicin, which are effective against actively growing bacilli but may be ineffective against bacilli that have entered the latent or slow growth phase. When *M. tuberculosis* is confronted with these drugs it is able to slow its growth and enter stationary phase allowing the bacilli to persist for extended periods of time (Butler, 2000). To eliminate slow growing bacteria drugs need to be developed that directly target against persistence genes and thereby prevent bacteria from entering the latent phase and possibly reactivating after completion of therapy.

There is a drastic need for new and effective vaccines and drug treatments for TB; furthermore it is essential that newly developed vaccines are superior to the currently used *M. bovis* BCG vaccine which has not been improved upon since 1921. Despite the long-term use of BCG it has had no effect on decreasing the incidence of TB (Collins, 2000). No new specific anti-TB drug has been introduced since rifampicin in the early 1970's. More recently the development of new vaccines for TB has been receiving worldwide attention mostly focussing on live and non-living vaccines (Mustafa, 2002). Live vaccines include genetically modified *M. bovis* BCG, genetically attenuated strains of the *M. tuberculosis* complex as well

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as genetically engineered vaccinia virus and *Salmonella* strains (Collins, 2000). Non-living candidates include killed mycobacterial species, protein subunits and DNA vaccines. With the sequencing of the *M. tuberculosis* genome and identification of potential virulence genes of the bacterium there is a better understanding of the mechanisms *M. tuberculosis* employs to gain access to the macrophage and how it persists. This has helped scientists to try and use genes that are specific to the various stages of the *M. tuberculosis* infection and use them in potential vaccines.

Naked DNA immunisation provided the first evidence that selected antigens protected against *M. tuberculosis*. Examples of antigens that protect mice include Mtb8.4 (Coler et al., 2001), Ag85 (Huygen et al., 1996; Baldwin et al., 1999; Ulmer et al., 1997), Mtb41 (Skeiky et al., 2000), Mtb39 (Dillon et al., 1999), MPT-63 and –83 (Morris et al., 2000), hsp65 (Lowrie et al., 1999), PstS-3 (Tanghe et al., 1999), ESAT-6 and MPT64 (Brandt et al., 2000; Kamath et al., 1999) and the 36kD lipoprotein (Fonseca et al., 2001). Although DNA vaccines have been proven to be effective in mice they have not had the same efficacy in larger animals, such as non-human primates. Therefore more work is needed before this approach can be used in humans. Results obtained thus far are promising and over the next 10 years it is hoped that once the large number of candidates has been reduced to a few suitable candidates, phase I clinical trials and thereafter Phase II clinical studies can be done (Reed et al., 2003).

1.1.3 Drug Resistant TB

In recent years the emergence of drug resistant and MDR TB strains has become a major concern to health authorities worldwide. Some strains of TB are resistant to all major anti-TB drugs. The most common anti-TB drugs are isoniazid, rifampicin, pyrazinamide, streptomycin and ethambutol. Drug resistant TB is thought to arise initially from inconsistent or partial treatment. There are various reasons for this. Sometimes patients cease taking medicine as they start to feel better, healthcare workers and doctors may prescribe the wrong drug regimes, or it could be because drug supply is unreliable. Drug resistant TB is still treatable but it requires extensive chemotherapy (up to two years of treatment) that is often expensive as well as being more toxic to patients. MDR TB is defined as a TB bacillus that is resistant to at least isoniazid and rifampicin, which are the two most powerful anti-TB drugs. Preventing the emergence and controlling the spread of MDR strains can only be achieved by ensuring

that patients receive the correct and full course of medication (Gleissberg, 1999). In more recent years, HIV has become a significant compounding factor in the spread of MDR TB. In areas where there is a high incidence of HIV, TB transmission rates are also high and this, coupled with poor living standards, decreases the chances of controlling TB in the near future (Chintu and Mwinga, 1999).

1.1.4   HIV and TB

HIV and TB form a dangerous partnership with each speeding the other's progress. HIV weakens the immune system leaving an individual susceptible to infectious diseases. A person who is HIV positive is more likely to develop TB disease than someone who is infected with TB and is HIV negative. TB results in an increase of HIV replication leading to elevated viral load (Lawn et al., 1999) (Bekker et al., 2000). Patients who are HIV positive have an increased risk of getting primary or reactivation TB (Wood et al., 2000). TB accounts for approximately 13% of AIDS related deaths worldwide (WHO). HIV positive individuals contracting MDR TB show a much faster progression from diagnosis to death, with a known case fatality rate of approximatley 80% (Bloom and Murray, 1992). Africa has one of the highest incidences of HIV and therefore it is not surprising that it is the single most important factor in the increased incidence of TB in the past 10 years (**Figure 1.2**).

FIGURE 1.2: Estimated HIV prevalence in TB cases.

1.2 – HOST SUSCEPTIBILITY TO TUBERCULOSIS

TB is a major problem in numerous developing countries, including South Africa. It is estimated that 5 - 10% of individuals infected with *M. tuberculosis* will develop the disease and the question that arises is: why are some people more susceptible to developing active disease than others? Before the discovery of *M. tuberculosis*, physicians were convinced that TB was a hereditary disease as it was seen frequently in members of the same family, for example the Brontë family. However with the discovery of the *M. tuberculosis* bacterium in 1882 by Robert Koch (Koch R, 1882), scientists' attention moved away from host genetics to focusing more on the role of the pathogen. Although *M. tuberculosis* is necessary for developing disease it is not the only factor influencing progression. There is evidence that interactions between environmental factors, pathogenic variation and host genetic factors play a role in TB progression as well as susceptibility (Schluger and Rom, 1998). The most important environmental factor, which impacts on a global scale, is co-infection with HIV, as discussed above in **Section 1.1.4**. Co-infection with HIV increases the risk of developing TB from a lifetime risk of 10% to an annual risk of 10% (Corbett and De Cock, 1996). Socio-economic and nutritional status is also likely to have an impact on susceptibility to TB (Cegielski and McMurray, 2004). Genetic variation of the bacterium itself is another important factor influencing host susceptibility, although sequence diversity of *M. tuberculosis* is lower than other pathogens (McShane, 2003; Lopez et al., 2003; Sreevatsan et al., 1997).

In the late 1800's and early 1900's it was recognized that there were racial differences in susceptibility to TB (Budd W, 1867; Cummins SL, 1908; Millar JG, 1908). More recently this was supported by Stead *et al.* (1990) who showed that black individuals were twice as likely to become infected with *M. tuberculosis* than white individuals. They were all living in the same nursing home. This suggested that there is a genetic basis for susceptibility to TB as these patients were living in the same environment (Stead et al., 1990). Evidence that host genetics played a role in TB susceptibility also came from the Qu'Appelle Indian Reservation, Saskatchewan in 1890. When the population was first exposed to TB the annual death rate was approximately 10% of the population. Over half of the families died over the next 40 years and the annual death rate during this time decreased to 0.2% (Motulsky, 1960). It is speculated that this decrease in the annual death rate resulted from selective pressure against TB susceptibility genes supporting a role for host genetics in disease susceptibility. The most

compelling evidence for a host genetic component to susceptibility to TB comes from twin studies (Kallmann FJ, 1942; Comstock, 1978). These studies showed that there is a much higher concordance for disease among monozygous (identical) twins than dizygous (non-identical) twins which suggests that genetic factors are involved, as environment within a family is similar.

With advances in technology the identification of disease genes has progressed at a very rapid rate. The use of the polymerase chain reaction (PCR) in screens for polymorphisms, mutations and in linkage studies has simplified the methods used to identify genes. Infectious diseases can be influenced by a number of different susceptibility genes with each gene making a contribution to the outcome of the disease. There are a number of methods for identifying susceptibility genes including linkage analyses which require large families, genome wide scans which can be performed in case-control cohorts or utilise many families with affected sibling pairs (Blackwell et al., 1997; Bellamy et al., 2000a), and association studies with candidate genes that are potentially involved in the host response to infection. These methods are described in detail in **Section 1.3**. The identification of susceptibility or resistance genes is important as it could lead to a better understanding of disease progression, which in turn could ultimately lead to the development of new treatments for diseases such as TB.

Insights into host genetics have been gained through studies involving animals, in particular mice. This is demonstrated by work on natural resistance associated macrophage protein 1 (NRAMP1, now known as solute carrier family 11 member 1 (SLC11a1)) which was identified by studies on mice that were genetically susceptible to BCG infection (Gros et al., 1981; Vidal et al., 1993; Malo et al., 1994). Although a number of studies in humans had reported association between human leukocyte antigen (HLA) markers and TB, for example HLA-DR2 (Singh et al., 1983), the first non-HLA gene associated with susceptibility to TB was SCL11a1. The initial report by Bellamy *et al.* (Bellamy et al., 1998) showing association in a case-control study in the Gambia, has subsequently been supported by studies in African (Awomoyi et al., 2002; Cervino et al., 2000; Hoal et al., 2004), Asian (Ryu et al., 2000; Gao et al., 2000; Delgado et al., 2002) and in North American populations (Ma et al., 2002).

The interferon gamma (IFN-γ) signaling pathway has also provided valuable information regarding host susceptibility to TB (Jouanguy et al., 1996; Newport et al., 1996). Mice with

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disruptions in the IFN-γ gene as well as the receptor have been shown to be susceptible to mycobacterial infections (Cooper et al., 1993; Kamijo et al., 1993). A family in Malta was identified who appeared to have a single gene defect that was inherited recessively resulting in increased susceptibility to mycobacterial infection (Levin et al., 1995; Newport et al., 1995). A genome search using microsatellite markers identified a region on chromosome 6q in which the gene for IFN-γ receptor 1 (IFN-γR1) is located and appeared to be the likely candidate (Newport et al., 1996). It was found that this susceptibility was caused by a mutation in the IFN-γR1 gene. The mutation produces a premature stop codon resulting in a truncated protein being formed. Immunologic studies revealed that the affected children are unable to produce tumour necrosis factor alpha (TNF-α) in response to endotoxin as well as being unable to upregulate this cytokine in response to IFN-γ (Levin et al., 1995).

A number of other genes have been associated with susceptibility to TB including; mannose binding lectin (MBL) (Selvaraj et al., 1999; Hoal-van Helden et al., 1999), the vitamin D receptor (Bellamy et al., 1999), Surfactant protein A1, -A2 and -D (Floros et al., 2000), Interleukin (IL) 8 (Ma et al., 2003b) the NFkappaB binding site in the IFN-γ gene (Lio et al., 2002; Lopez-Maderuelo et al., 2003; Rossouw et al., 2003) and most recently IL-12B (Tso et al., 2004). There have also been reports of protective associations against TB. For example it was reported that a nucleotide substitution in the promoter region of the *mbl2* gene resulted in low levels of MBL which was shown to be a protective factor against *M. tuberculosis* infection (Soborg et al., 2003) and another allele in the coding region of the gene (allele B, codon 54) has been associated with protection against TB meningitis (Hoal-van Helden et al., 1999). A single nucleotide polymorphism (SNP) in the promoter region of the P2X7 gene was also found to be associated with protection against TB (Li et al., 2002). However, these genes explain only part of the susceptibility to TB and it is assumed that there are other genes involved that remain to be identified and investigated. Numerous candidate genes that have been selected as potential genes involved in susceptibility to TB have shown no association with TB (Ma et al., 2003a; Ma et al., 2003b), but many of these studies may have been under – powered.

1.3   IDENTIFYING SUSCEPTIBILITY GENES

There are currently numerous methods employed to identify susceptibility genes. Genetic susceptibility factors have been identified through twin and adoptee studies. Twin studies assess the concordance rates in monozygotic and dizygotic twins to establish the extent of genetic contribution to susceptibility and adoptee studies determine concordance of disease with adoptive versus biological family members providing convincing evidence that there is a considerable genetic component to infectious disease susceptibility (Hill, 1998). Other methods for identifying susceptibility genes are linkage studies and association studies of candidate genes.

1.3.1   Linkage Studies

Linkage analysis involves constructing a transmission model to explain the inheritance of a disease in pedigrees. This model is straightforward for simple Mendelian traits but can become very complicated for complex traits. These studies are carried out using large families and linkage of a genotype to a genetic marker and infectious disease may be specific for a particular family and therefore cannot produce an association in the general population (Daly and Day, 2001).

As yet there has not been a major TB susceptibility gene identified. Two putative TB susceptibility loci were identified in a genome wide linkage analysis using African families. This resulted in the identification of a locus on chromosome 15q, containing numerous candidate genes. The other locus identified was on chromosome Xq27 which could explain the higher incidence of male TB patients (Bellamy et al., 2000a). The region on chromosome 15 was examined further by an association study discussed in **Section 1.3.2b**. Linkage has also been observed between TB and chromosome 2q35 which includes SLC11a1 in a large aboriginal Canadian Family (Greenwood et al., 2000).

1.3.2   Association Studies

Association studies have the advantage in that they have the power to detect genetic contributors having minor effects. They are mostly case-control studies that follow the distribution of a certain marker allele in unrelated affected and unaffected individuals (Abel and Dessein, 1997; Bellamy, 2000b; Strachen and Read, 2000). Associations can be detected

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whether the polymorphism being investigated is the actual cause of the genetic susceptibility, or if it is in linkage disequilibrium (LD) with the true susceptibility polymorphism (Mathew, 2001). There are two approaches to conducting an association study, population based case-control association studies or family based studies. There are advantages and disadvantages to both approaches and these are highlighted in **Table 1.1**.

TABLE 1.1: A summary of the advantages and disadvantages of family-based and case-control association studies.

	Advantages	Disadvantages
Family-based study	Uses parents as internal controls Not influenced by population stratification Not subject to type I errors	Specific individuals are required Possible selection bias of susceptibility genes Difficulty in obtaining parental samples especially in a late onset disease Lower statistical power
Population-based Case-control study	Comparatively easy to obtain samples Power does not depend on allele frequency Detect small gene effects	Population stratification may bias results Subject to type I errors

1.3.2a. Population-based case-control association study

This approach involves genotyping unrelated individuals with and without the disease in question and subsequently comparing the allele and genotype frequencies in these two groups. Association of a particular allele with disease is purely a statistical finding and is not necessarily an indication of genetic linkage. There are some important issues to consider when one is designing a case-control association study including the choice of candidate gene

and polymorphism to be studied, recruitment methods, matching of the controls and the number of individuals to be studied (Daly and Day, 2001). One of the most important considerations is choosing the control group that optimally should be as similar as possible to the cases with the important criteria that they do not have the disease of interest and come from the same community. One way to make cases and controls more comparable is to match them for some variables for example sex and age. Matching implies that each case is individually matched to a control individual. Matching however is only useful for variables that are strongly related to both the exposure and the outcome of interest (Altman, 1991b).

There are a number of advantages to this approach including the fact that these studies may have sufficient power to detect relative risks as low as 1.5 which is usually not possible in family studies (Risch, 2000). The major disadvantage of this approach is that false positive or negative results are occasionally obtained, due to type I and II errors respectively (Altman, 1991b). When a type I error occurs the null hypothesis is rejected incorrectly. This type of error is the most frequent error in case-control association studies due to population stratification. In order to ensure that these types of errors do not occur one should consider the following. As mentioned above, the choice of controls should be done carefully, ensuring that they are from the same ethnic group.

Another important consideration is the Hardy-Weinberg equilibrium (HWE) within the control group. The HWE relates genotype frequencies to allele frequencies and failure to attain HWE in a case-control study could be due to genotyping error, inbreeding, genetic drift, mutation or population stratification (Gillespie, 1998). Lastly, corrections for multiple comparisons should be made using one of the numerous tests which include the Bonferroni, Bonferroni Stepdown (Holm), Westfall and Young Permutation and the Benjamini and Hochberg False Discovery Rate (FDR) (www.siliconegenetics.com) (Altman, 1991a). The Bonferroni method is the most stringent and the Benjamini and Hochberg FDR the least stringent. Using a highly stringent test such as the Bonferroni method the rate of false negatives (genes that are reported as non-significant when in fact they are) is very high (www.siliconegenetics.com) (Altman, 1991a). It is also advisable to replicate the results obtained in a case-control study using a second, independent sample, or another method such as a family-based study.

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The results of association studies on a particular gene may differ between populations. At the population level, the IFN-γR1 gene has been studied in different populations with conflicting results. An association between microsatellite variants in this gene and susceptibility to TB was found in a Croatian population (Fraser et al., 2003) but not in a Gambian population (Awomoyi et al., 2002). Similarly, we have demonstrated (T. Lundwall-Roos, unpublished data) an association between the T allele of a single nucleotide polymorphism (SNP) in the amino terminal region of the SP-D gene and susceptibility to TB in our population, whereas Floros et al. (2000) showed association with the C allele of the same SNP in a Mexican population (Floros et al., 2000). A difference between associations found in different populations is not completely unexpected for a number of reasons. Populations differ genetically, therefore different alleles could contribute to a disease phenotype in one population but not another. There are also compounding environmental factors that could play a large role in these observed differences in association. It is possible that the polymorphisms do not influence the protein at a functional level (usually seen with microsatellites) but are in LD with the causative polymorphism leading to different results in different populations. Another important point to consider is that the original study showing association may have had little power due to a small sample size, and this false positive association is then likely to be contradicted by the reported lack of association in another population.

1.3.2b. Family based studies

In family based studies parents of affected offspring or unaffected siblings are used as internal controls thereby eliminating the variable of population stratification (Spielman et al., 1993; Schaid, 1998). In 1987 Falk and Rubinstein proposed the "haplotype relative risk" (HRR) method (Falk and Rubinstein, 1987). In this method the two alleles that were not transmitted to the affected offspring were combined to create a pseudocontrol, and the association of disease with particular alleles was assessed by traditional case-control methods. The frequencies of the particular marker alleles in cases and pseudocontrols were used to derive an odds ratio that is called the HRR when it is applied to pseudocontrols.

Spielman et al. (1993) proposed an alternative to the HRR method called the transmission disequilibrium test (TDT) to be used to test for linkage in the presence of an association (i.e. linkage disequilibrium) (Spielman et al., 1993). This test is used in order to determine whether the transmission frequency of alleles from heterozygous parents to an affected child

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differs from the expected Mendelian frequency when there is no linkage (Schaid, 1998). The TDT test can be used when there is only one parent available although this may introduce bias (Whittaker and Lewis, 1998; Strachen and Read, 2000). The associated allele may be the true susceptibility factor or it may be in linkage disequilibrium with the real susceptibility allele. The TDT cannot detect linkage if there is no disequilibrium (Strachen and Read, 2000). The TDT analysis is assumed to have a lower statistical power compared to a case-control study (Morton and Collins, 1998) and is prone to type II errors where the null hypothesis is incorrectly accepted; in other words, an association is not detected when it is present.

After identification of the area on chromosome 15q in a genome-wide linkage study (**Section 1.3.1**), Cervino et al. (2002) examined this locus on further in a study involving 136 African families and were able to identify the region 15q11-13. On closer inspection it was observed that there was an association with a 7bp deletion in the ubiquitin protein ligase 3A gene (UBE3A) in families screened from the Gambia, Guinea and South Africa suggesting that UBE3A or a gene close by may be a TB-susceptibility locus (Cervino et al., 2002).

1.4 – DISEASE PROGRESSION TO TUBERCULOSIS

M. tuberculosis enters the body via the upper respiratory tract through the inhalation of droplet nuclei, and passes through to the lower respiratory tract. Once the organisms have made their way to the lung they have four potential fates (**Figure 1.3**).

FIGURE 1.3: Chronologic events after inhalation of *M. tuberculosis* adapted from van Crevel *et al*. (2002).
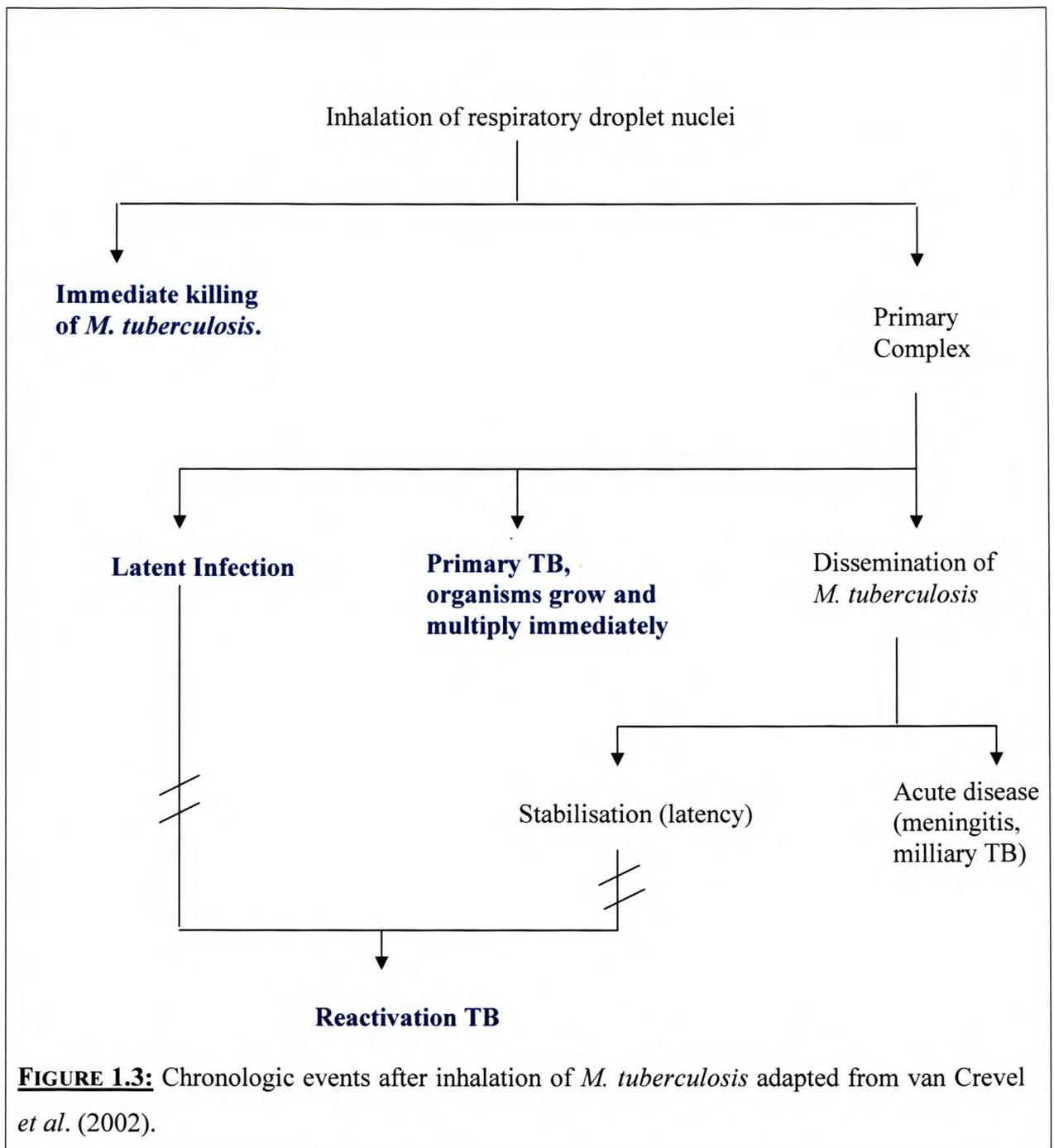
Firstly, the initial host response can completely and effectively kill all the bacilli via the alveolar macrophage. If, however, the bacilli are not killed immediately they will take up residence inside the macrophage, multiply and cause the destruction of the macrophage. At this stage other inflammatory cells and blood monocytes are recruited into the lung. The monocytes differentiate into macrophages and both cell types ingest the bacilli, which may not be killed. During this phase the bacteria are growing logarithmically, blood derived macrophages accumulate in the lungs but little tissue damage occurs during this stage.

Most often the infection will be stabilised at this point and infection becomes dormant. Bacilli can then be in a dormant non-replicating state, or actively replicating but the cytotoxic immune response is able to keep up with the replicative rate of the bacteria, conversely the bacilli could be metabolically altered having limited replicative cycles. These bacilli could remain dormant and never cause active TB, but in some cases these dormant organisms eventually begin to grow because of a breakdown of the immune responses that are required for containment of the bacilli and cause re-activation TB, with necrosis and lung damage. Infection with HIV, treatment with corticosteroids, alcohol or drug abuse as well as ageing can all increase an individual's risk of latent TB reactivation (Selwyn et al., 1989; Spencer et al., 1990; Rajagopalan and Yoshikawa, 2000a ; Rajagopalan and Yoshikawa, 2000b).

In a few individuals active TB will develop immediately once the organisms have entered the respiratory tract (primary TB) and in some cases dissemination leads to acute disease. Dissemination may occur after primary infection but could also take place many months or years later (post-primary TB) in the case of a depleted or inefficient immune system. Cavity formation in the lung could lead to rupture of the bronchi which allows the spread of *M. tuberculosis* through the airways to other parts of the lung as well as to the outside environment. Thus the final outcome of the disease is dependent on the interaction of the bacillus and the host defence mechanisms that it encountered.

1.5 – INNATE IMMUNITY

Host defences in the lung involve both innate and acquired immune responses. The acquired immune response is effective when the host has been previously exposed to the invading pathogens. Clonal expansion of T and B lymphocytes takes approximately 24 – 72 hours to develop and it is suggested that this gap in host immunity is filled by the molecules of the innate immune system.

The innate immune response consists of structural defenses, antimicrobial molecules generated in the airways and phagocytic defences (alveolar macrophages and polymorphonuclear leukocytes) that protect the host from pathogens before the generation of acquired immunity. These molecules are recruited into the lung in response to pulmonary infection. They are responsible for the removal of microbes found on the surface of the airways and for the elimination of bacterial pathogens from the alveolus. The innate immune

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response is responsible for limiting infection during the first few hours of exposure and it plays a role in modifying the manner in which the acquired immune system develops.

There are numerous cells that fulfill important functions in the innate immune response in the lung, including epithelial cells, alveolar macrophages and polymorphonuclear leukocytes. Lung epithelial cells line the respiratory tract and provide a physical barrier against invading pathogens. *In vitro* studies have shown that pulmonary epithelial cells and mononuclear phagocytes have the capacity to produce components of the complement cascade (Cole et al., 1983; Strunk et al., 1988; McPhaden and Whaley, 1993; Johnson and Hetland, 1988) which contributes to their bactericidal function.

Alveolar macrophages are the most abundant cells that are recovered by lung lavage and Green and Kass (1964) provided evidence that the alveolar macrophage is a primary defender of the alveolus. They are highly phagocytic, motile cells that are able to release a variety of inflammatory mediators and cytokines (Takemura and Werb, 1984).

Polymorphonuclear leukocytes enter the lungs in response to severe infection and inflammation. Neutrophils are implicated in numerous noninfectious diseases such as idiopathic pulmonary fibrosis (IPF), asthma and emphysema. They are capable of secreting proteases, glycosidases and acid hydrolases as well as numerous other antimicrobial substances (Wright, 1997).

Besides the cells discussed above, the upper airway of the lung has evolved an intricate system involving mechanical, reflux and cellular mechanisms as well as locally synthesized defense molecules (Crouch, 1998), with a delicate balance between pro- and anti-inflammatory responses to avoid damage to the lung (Wright, 1997). These defences include: serum leukocytes, molecules such as reactive oxygen and nitrogen species, lysozyme, complement, immunoglobulins A and G, fibronectins, lactoferrin, defensins and pulmonary surfactant (LeVine and Whitsett, 2001; Crouch, 1999).

1.6 – SURFACTANT AND THE COLLECTIN MOLECULES

Pulmonary surfactant is a complex mixture of lipids and proteins (85 – 90% lipids, approximately 10% proteins and 2% carbohydrates) which are important for normal respiratory function (Rooney et al., 1994). It plays a distinct role in the regulation of alveolar macrophage function and inflammation and contributes to the innate immune defence of the

lung. Pulmonary surfactant is involved in the stabilization of alveolar walls in order to prevent the collapse of the alveoli during exhalation (van Golde et al., 1988). In order to maintain surfactant homeostasis, surfactant needs to be continually degraded. Alveolar macrophages have been shown to actively degrade surfactant thus playing an important role in its turnover and clearance (Wright and Youmans, 1995; Grabner and Meerbach, 1991). The proteins present in pulmonary surfactant are designated surfactant proteins (SP) -A, -B, -C and -D. The major surfactant proteins A and -D are hydrophilic whereas SP-B and -C are hydrophobic. Their classification is based on the fact that they are found in lavage fluid, are synthesized by alveolar type II cells and are present in large amounts specifically in the lung (**Figure 1.4**). Clara cells, which are non-ciliated cells that are found in the epithelium of the respiratory and terminal bronchioles, synthesize all the surfactant proteins except SP-C.

SP-B and -C confer surface-tension lowering properties and are important for the adsorption and spreading of surfactant (Whitsett and Weaver, 2002).

SP-A and D belong to the group of mammalian lectins designated collectins, and are an important part of the innate immune response to infectious agents. Other members of this family include; MBL (Turner, 1996) which activates the serum complement system, and at least two bovine serum lectins, conglutinin, bovine serum lectin 43 (CL-43) and a collectin discovered in liver cells, collectin – liver 1 (CL-1) (Holmskov et al., 1995; Ohtani et al., 1999).
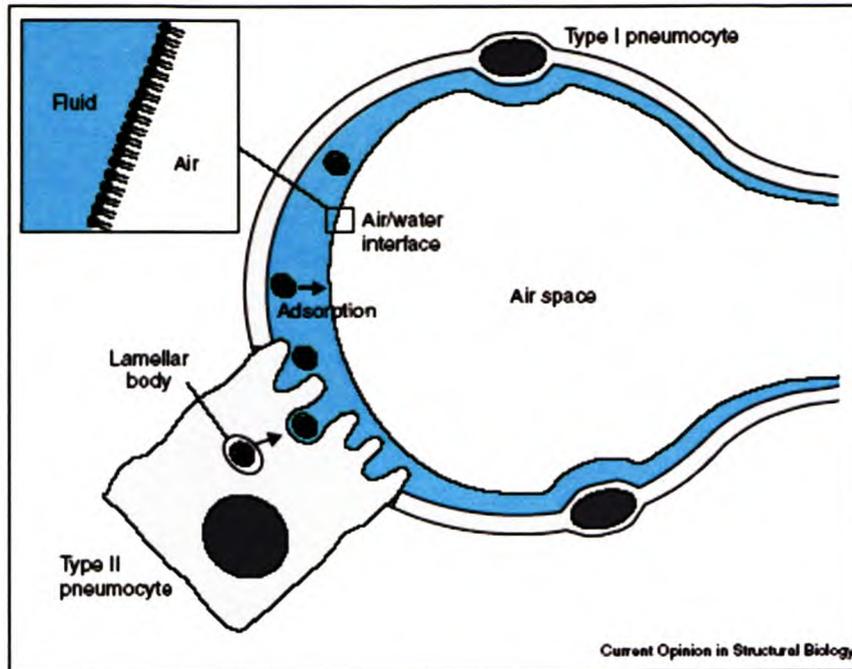
FIGURE 1.4: Schematic of pulmonary surfactant in an alveolus of the lung. Surfactant constituents are synthesized by type II pneumocytes. The surfactant components are then packaged into the concentric bilayers of lamellar bodies. After secretion, the bilayers unravel and adsorb to the air/water interface (see inset). During exhalation, the decreasing alveolar surface area compresses the interfacial film. Points of controversy simplified by this diagram include the following: at least part of the interfacial film consists of a multilayer (Schurch et al., 1995), the lateral extent of which is uncertain; although continuous (Bastacky et al., 1995), the aqueous layer may at some points be too thin to be considered liquid (Dorrington and Young, 2001); and although strong physiological evidence either for or against such a process is lacking, respreading of collapsed material excluded from the interface by over compression could contribute during re-expansion to the maintenance of a functional film (Notter et al., 1980). Taken from Piknova *et. al.* (2002).

The overall structure of collectins is highly conserved between species. They have common characteristics, being a short amino terminal, disulphide cross linking region and a triple helical collagen-like domain connected to a calcium dependent carbohydrate recognition

domain (CRD) by a short, trimeric coiled-coil linking domain or "neck" region (Hoppe et al., 1994; Lu, 1997). The word collectin is composed of "coll" for the collagen-like domain and "lectin" for the lectin-like CRD. The amino terminal domain is held together as a trimer by interchain covalent interactions that are important in the formation of higher order oligomers. The collagen-like sequence forms the collagen-like triple helix (20 - 46nm long). The neck region bridges the collagen-like arm and the globular CRD. This CRD forms the carboxy-terminal domain of the collectins. The binding affinity of monomeric lectin domains for carbohydrates is very low (Weis and Drickamer, 1996), but once they have associated into their trimeric forms their binding affinity is significantly increased.

SP-A and MBL share a common "bouquet" arrangement made up of octadecamers consisting of six trimeric subunits each composed of 26-35 kDa monomers **(Figure 1.5)**. SP-D is expressed as dodecamers consisting of four homotrimeric subunits composed of 43kDa monomers (Crouch et al., 1994b) **(Figure 1.6)**. SP-D and conglutinin have characteristic "cruciform" structures. The collagen-like region of SP-D is highly conserved and lacks interruptions of the Gly-X-Y repeats (where X and Y represent different amino acids) unlike SP-A and MBL, which have a break in the collagen-like region causing the bouquet-like formation.

FIGURE 1.5: Schematic representation of the structure of Surfactant Protein A. SP-A has a bouquet-like structure like that of MBL.

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C-terminal globular head region containing the carbohydrate recognition domains

Triple helical collagen -like region

Cysteine containing N-terminal cross-linking domain

Trimeric alpha helical coiled-coil neck region

FIGURE 1.6: Schematic representation of the dodecameric form of Surfactant Protein D.

1.6.1 – Chromosomal location of SP-A, -D and MBL

MBL, SP-A and SP-D are found in close proximity on the long arm of chromosome 10 (**Figure 1.7**). The SP-A locus has been placed at 10q21 - 24 and the MBL locus at 10q11.2 – q21 (Bruns et al., 1987; Kolble et al., 1993; Fisher et al., 1987; Sastry et al., 1989). SP-A is composed of the product of two very similar, but non-identical genes SP-A1 (White et al., 1985) and SP-A2 (Katyal et al., 1992) each being approximately 5kb in length. These genes are found in opposite transcriptional orientation with a pseudogene situated in between the two (Korfhagen et al., 1991; Floros and Hoover, 1998; Hoover and Floros, 1998; Bruns et al., 1987). The pseudogene is nonfunctional and contains sequences highly homologous to exons encoding the α-helical neck region and the CRD of SP-A (Korfhagen et al., 1991). SP-D has been placed at 10q22.2 – 23.1 (Crouch et al., 1993b). A single gene encodes the human SP-D protein with a transcribed region spanning more than 11kb (Crouch et al., 1993b).
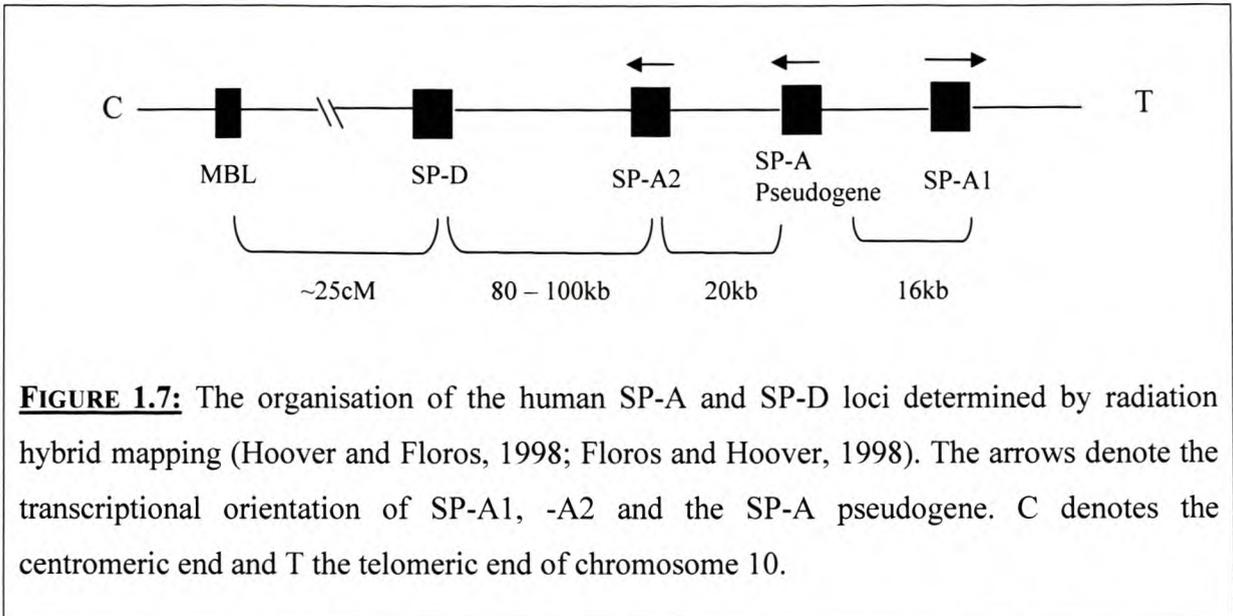
FIGURE 1.7: The organisation of the human SP-A and SP-D loci determined by radiation hybrid mapping (Hoover and Floros, 1998; Floros and Hoover, 1998). The arrows denote the transcriptional orientation of SP-A1, -A2 and the SP-A pseudogene. C denotes the centromeric end and T the telomeric end of chromosome 10.

1.7 – PULMONARY SURFACTANT PROTEIN A

SP-A exists as a single copy gene in mice (Korfhagen et al., 1992), rabbits (Boggaram et al., 1988; Chen et al., 1992) and rats (Fisher et al., 1988) however humans possess two copies of this gene, SP-A1 and SP-A2. The genes have a high degree of structural homology (>95%). They are 94% identical at the nucleotide level and 96% identical at the amino acid level. The most significant difference between the SP-A1 and -A2 transcripts is the presence of an extra cysteine residue in the SP-A1 gene at position 85, between the interruption of the collagen-like region and the alpha helical neck region (Voss et al., 1991). SP-A1 and -A2 are in opposite transcriptional orientation with SP-A2 being closer to SP-D. The SP-A1 and -A2 genes consist of 4 coding exons (**Figures 1.8a and b**). Both SP-A gene products are required to form a functional and stable mature SP-A protein. Each functional SP-A gene is extremely complicated. This complexity includes the numerous untranslated exons at the 5' end that generate several alternately spliced transcripts, allelic variability which is based on polymorphisms in the coding regions as well as sequence variability in the 3' untranslated region (UTR) (**Figure 1.9**).
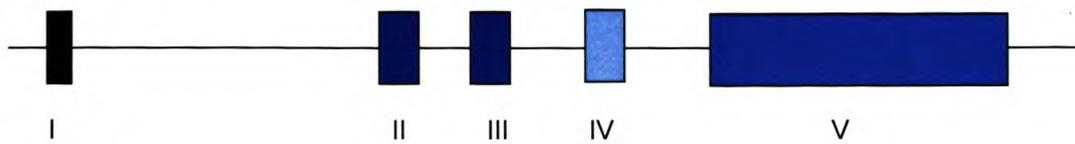
FIGURE: 1.8a The SP-A1 gene showing exons I – V. Exon 1 is the first untranslated exon, exon II encodes a portion of the untranslated region, the signal peptide, the N-terminus and the first part of the collagen-like region. Exon III encodes the remainder of the collagen-like region, exon IV encodes the alpha helical neck region and exon V encodes the carbohydrate recognition domain and the 3' untranslated region.
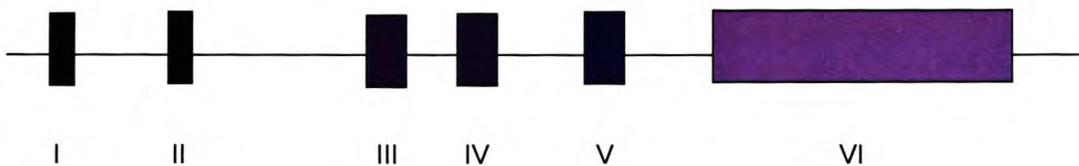
FIGURE: 1.8b The SP-A2 gene showing exons I – VI. Exon 1 is the first untranslated exon. Exon II is unique to SP-A2 encoding an additional 5' untranslated sequence that is 30bp in length and is absent from SP-A1. Exon III encodes a portion of the untranslated region, the signal peptide, the N-terminus and the first part of the collagen-like region. Exon IV encodes the remainder of the collagen-like region, exon V encodes the alpha helical neck region and exon VI encodes the carbohydrate recognition domain and the 3' untranslated region.
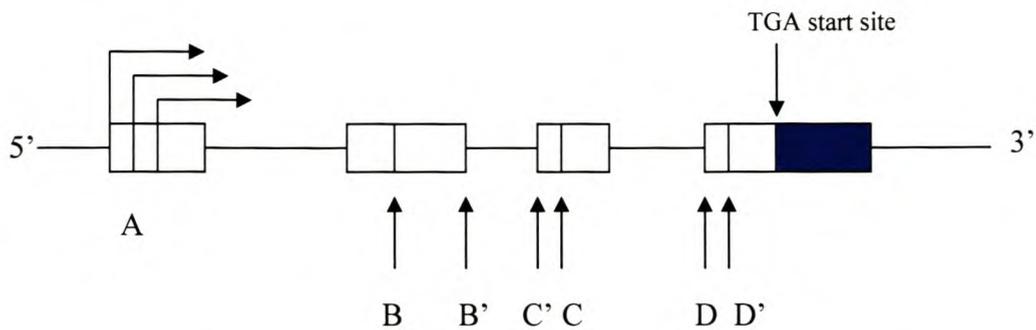
FIGURE 1.9: 5' splice variants for the SP-A1 and -A2 genes. There are numerous 5' untranslated exons (A, B/B', C/C' and D/D') in both SP-A1 and SP-A2. These exons splice in configurations specific for SP-A1 (AD' – major transcrpit, ACD' and AB'D' – minor transcripts) and for SP-A2 (ABD and ABD' – major transcripts). The arrows in exon A represent different transcription start sites (Karinch et al., 1997). The blue box represents the first coding exon 1 of both SP-A genes, with TGA denoting the translation start site.

The most common splice variant for SP-A1 is AD' which is found at a frequency of approximately 0.8 (Karinch and Floros, 1995). The most common splice variant for SP-A2 is the ABD and ABD' transcripts. These transcripts are seen at different frequencies among humans depending on the SP-A2 genotype of the individual (Karinch et al., 1997; Karinch and Floros, 1995).

Five alleles have been characterised for SP-A1 and six for SP-A2 based on differences within the coding regions of these genes (**Table 1.2**). The most common SP-A1 allele is $6A^2$ and the most common SP-A2 allele is $1A^0$ (Floros et al., 1996). One SP-A genotype in particular $(6A^26A^21A^01A^0)$ has been shown to be associated with a low to moderate level of SP-A mRNA (Karinch et al., 1997).

TABLE 1.2: Alleles identified for SP-A1 and –A2. Adapted from Floros and Hoover (1998). The positions of the relative amino acids are: 9, 19, 50, 66, 73, 81, 85, 91, 219 and 223 for both SP-A1 and –A2. The amino acids that distinguish SP-A1 form SP-A2 are in bold.

Gene	Allele	Amino Acid Differences
SP-A1	6A	NAL**MDIC**PRQ
	$6A^2$	NVV**MDIC**PRQ
	$6A^3$	NVL**MDIC**PRQ
	$6A^4$	NVL**MDIC**PWQ
	$6A^5$	NAL**MDIC**PWQ
SP-A2	1A	TA**VTNVR**PRQ
	$1A^0$	NA**VTNVR**ARQ
	$1A^1$	TA**VTNVR**ARK
	$1A^2$	TA**VTNVR**ARQ
	$1A^3$	NA**VTNVR**ARK
	$1A^4$	TA**VTNVR**ARK

The collagen-like region of the SP-A protein, encoded by exons II and III of SP-A1 and III and IV of SP-A2, is similar to collagen and contains 23 Gly-X-Y (where X and Y represent different amino acids, usually proline in the X position and hydroxyproline in the Y position) repeats with a break between the 13th and 14th repeat. The carboxy terminal region of the protein encodes the CRD and is characteristic of other C-type lectins. This region is capable of binding maltose (Drickamer et al., 1986; Haagsman et al., 1987). The collagen-like domains consisting of three SP-A monomers (two SP-A1 polypeptides and one SP-A2 polypeptide) form a triple helix with a bend where the Gly-X-Y repeats are interrupted **(Figure 1.10)** (Hoover and Floros, 1998). Six of these triple helices oligomerise to form the characteristic 'bundle of tulips' formation (King et al., 1989) (Voss et al., 1988). The predominant molecular form of SP-A is a hexamer of trimers, however, smaller oligomeric forms have been observed (Hickling et al., 1998) as well as multi-molecular complexes (Hattori et al., 1996a; Hattori et al., 1996b).
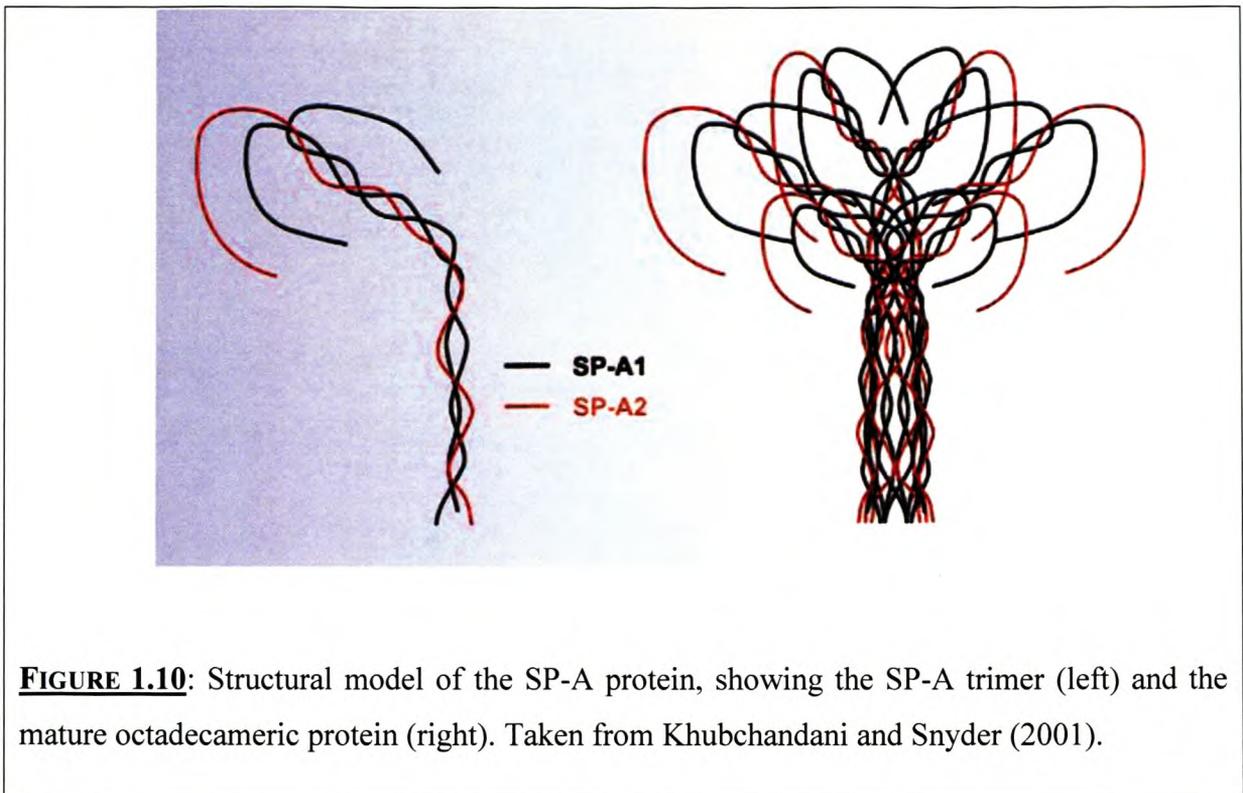
FIGURE 1.10: Structural model of the SP-A protein, showing the SP-A trimer (left) and the mature octadecameric protein (right). Taken from Khubchandani and Snyder (2001).

1.8– PULMONARY SURFACTANT PROTEIN D

The SP-D gene is made up of 8 exons (the first being untranslated) and spans more than 11kb. The first translated exon (exon 1) encodes the signal peptide, the amino terminal domain, and the first seven Gly-X-Y repeats of the collagen-like region. The remainder of the collagen-like region is encoded by exons 2-5. The alpha helical coiled-coil neck region is encoded on exon 6 and the entire lectin domain is encoded by exon 7 (**Figure 1.11**).
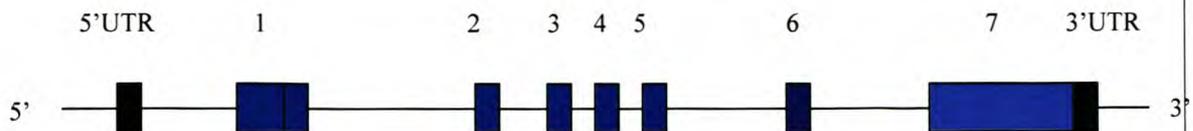
FIGURE: 1.11 Diagrammatic representation of the SP-D gene. 1-7 represents the seven exons encoding the gene. These include the exon encoding the signal peptide, amino terminus and a hydrophilic amino terminal collagen-like sequence (1); the remainder of the collagen-like domain (2-5); a C-type lectin carbohydrate-binding domain (7); and a linking peptide (the neck region) (6) between the collagen-like domain and the carbohydrate-binding domain. The 5'UTR is interrupted by at least one intron (Crouch et al., 1993b).

The amino terminal region is essential for the overall assembly of SP-D. It contains two conserved cysteine (Cys) residues (Cys 15 and Cys 20), which are important in the formation of interchain disulphide bonds that stabilize the dodecameric structure of SP-D (Crouch et al., 1994b). It has been shown that if these two cysteine residues are substituted with serine, trimeric SP-D is still secreted, but dodecameric forms of SP-D are not assembled thus demonstrating the importance of the two cysteine residues (Brown-Augsburger et al., 1996). Trimeric SP-D molecules are less effective at agglutinating microorganisms as has been shown by Eda *et al.* (1997) who investigated the binding and agglutinating ability of truncated SP-D molecules. They observed that these truncated molecules were not able to agglutinate influenza A virus as efficiently as native, dodecameric SP-D molecules (Eda et al., 1997). It stands to reason that if a polymorphism disrupts either of these cysteine residues a mature, dodecameric protein may not be secreted, alternately an increased number of trimeric molecules may be secreted leading to an individual who is more susceptible to infection as SP-D is unable to efficiently agglutinate the pathogens. Zhang *et al.* (2001) generated transgenic mice that expressed 'mutant' SP-D, which had cysteine 15 and 20 substituted with serine in these positions (Zhang et al., 2001). They showed that an increased expression of these mutant SP-D molecules decreased the amount of the normal disulphide cross-linked SP-D molecules and lead to the accumulation of foamy macrophages and emphysema in the mice without altering the alveolar phospholipid concentration.

The long collagen-like domain accounts for the pronounced linear characteristic of this molecule. The collagen-like domain of SP-D determines the maximum spatial separation of

the trimeric CRDs and it is possible that it has an impact on the correct molecular assembly and secretion of the protein. Ogasawara *et al.* (1995) demonstrated that if the entire collagen-like region of rat SP-D was deleted SP-D trimers were secreted instead of dodecamers (Ogasawara and Voelker, 1995). There is an asparagine (asn) -linked oligosaccharide at asn[70] in the collagen-like domain. Asn-linked sugars present in the collagen helix have been implicated in maintaining the conformation of the triple helical domains during the assembly of Type IV collagen tetramers (Langeveld et al., 1991). It has also been proposed that these asn-linked sugars are important in intracellular targeting and secretion of SP-A (Alcorn et al., 1992; O'Reilly et al., 1988).

The CRD is involved in pathogen binding by binding to carbohratyes on the surface of bacteria (Sastry and Ezekowitz, 1993; Botas et al., 1998). A trimeric cluster of CRDs is required for high-affinity binding to carbohydrates present on pathogens and it has been reported that monomeric CRDs have an approximately 10-fold lower binding affinity for multivalent ligands than trimeric CRDs (Hakansson et al., 1999). The neck domain is important for correct assembly and trimerisation of the CRDs, and interactions in the C-terminal region of the neck stabilise the CRD trimer (Hakansson et al., 1999) (Hoppe et al., 1994). The neck region appears to be highly conserved as no polymorphisms have been reported in this region.

Each member of the collectin family has a different degree of higher order oligomerisation (Lawson and Reid, 2000). A monomeric SP-D molecule is 43kDa in the reduced state. Each molecule is made up of three trimeric subunits that associate by their amino termini forming docecameric structures (~520kDa). The majority of SP-D is purified in the dodecameric form, but the proportions of various oligomers may vary between species. This can be seen in alveolar proteinosis patients where the majority of SP-D molecules are of higher order oligomerisation containing up to or more than 32 trimeric subunits (Crouch et al., 1993a). Hartshorn *et al.* (1998) were able to show that the degree of multimerisation of SP-D was a critical determinant of both aggregating activity and potency in enhancing bacterial uptake, and Zhang *et al.* (2001) demonstrated that the activity of SP-D *in vivo* is dependent on the oligomeric structure of the SP-D molecule. SP-D is able to bind to and agglutinate a wide variety of microorganisms and it is thought that the agglutination activity is dependent on the oligomerisation and the ability of SP-D to bridge large distances between organisms (Eda et

al., 1997). SP-D and conglutinin have a characteristic "cruciform" structure. It has been shown that SP-D dodecamers are able to form highly ordered stellate multimers sometimes referred to as astral bodies **(Figure 1.12)** (Crouch et al., 1994a; Crouch et al., 1994b). The final multivalent molecules have diameters of 25 to 100nm and this probably allows for efficient cross-linking or agglutination of bound pathogens, which is a characteristic of collectins.

a)   b)   c)
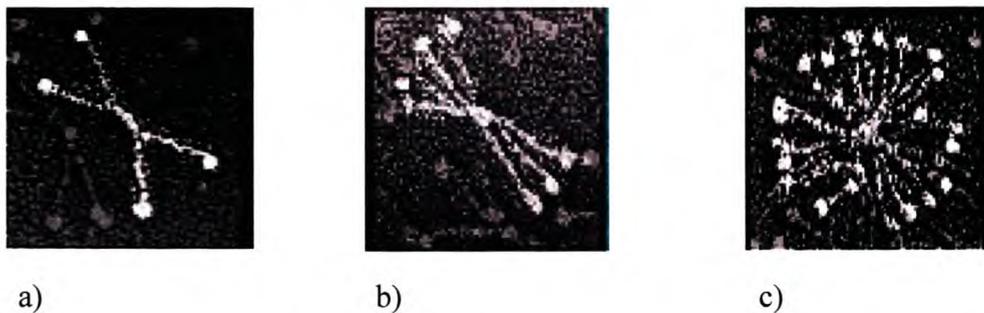
FIGURE 1.12: Transmission electron micrographs of SP-D showing differing degrees of oligomerisation. a) SP-D dodecamer, b) intermediary molecule, c) astral body (Crouch et al., 1994a; Crouch et al., 1994b).

SP-D is expressed primarily in the respiratory epithelium, specifically by alveolar type II cells and Clara cells (Korfhagen et al., 1998; Crouch, 1998; Voorhout et al., 1992). Expression of SP-D has also been detected in the gastrointestinal tract, genitourinary tract, the skin and soft tissue as well as in organs such as the heart, brain, testis, pancreas (endocrine) and the placenta (Leth-Larsen et al., 2004; Murray et al., 2002; Bourbon and Chailley-Heu, 2001). A SP-D like gene has been identified in rodents, birds as well as amphibians (Lawson et al., 1999), however as yet there has not been a related gene identified in fish.

1.9– SP-A AND –D KNOCKOUT STUDIES

Transgenic mice studies (SP-A and -D knockouts) have been conducted using SP-A and -D. These studies have provided some information about the function of these collectin molecules in agglutination, phagocytosis and killing of numerous microorganisms. LeVine et al. (2000) showed that SP-D deficient (SP-D$^{-/-}$) mice, infected intratracheally with group B

streptococcus (GBS), were able to kill the bacteria as efficiently as the wild-type mice and the number of bacteria associated with macrophages was lower than observed in the wild-type mice. In contrast to this SP-A deficient (SP-A$^{-/-}$) mice infected with the same microorganism did not kill the bacterium as efficiently as the wild type or SP-D$^{-/-}$ mice, however the number of bacteria associated with macrophages was also decreased in the SP-A$^{-/-}$ mice compared to wild type. In the presence of SP-D there was increased association of GBS with alveolar macrophages but phagocytosis was not altered. The same group also infected SP-A$^{-/-}$ and D$^{-/-}$ mice with *Haemophilus influenzae* and observed a similar situation in that these mice were able to kill this bacterium as efficiently as the wild-type. Also the number of bacteria associated with and internalised by alveolar macrophages was significantly less in SP-A$^{-/-}$ and D$^{-/-}$ mice compared to the wild-type mice. This suggests that there is a defect in opsonisation and/or phagocytosis. In both cases SP-A$^{-/-}$ and D$^{-/-}$ mice showed increased inflammation and inflammatory cell recruitment into the lung after infection. LeVine *et al.* (2000) showed that SP-D$^{-/-}$ mice were susceptible to respiratory syncytial viral pneumonia. The uptake of viral particles by alveolar macrophages and the clearance of the virus from the lung were deficient in SP-D$^{-/-}$ mice. These results suggest that the collectin molecules play distinct roles in the innate immune response to different microorganisms. Studies of this nature have not yet been conducted in connection with *M. tuberculosis* but would be useful in elucidating the function of SP-D in the progression of TB.

1.10– SP-A AND D IN BRONCHO ALVEOLAR LAVAGE FLUID AND SERUM

Alterations of the levels of pulmonary surfactant may contribute to the pathogenesis of numerous lung diseases including, respiratory distress syndrome (RDS), acute respiratory distress syndrome (ARDS), interstitial lung diseases, lung cancer and perhaps TB.

The abundant expression of SP-A and -D in the lung makes these collectins possible markers for lung diseases. The use of enzyme-linked immunosorbent assays (ELISA) for these collectins has enabled the determination of the absolute amount of proteins in bronco alveolar lavage fluid (BALF) (Kuroki et al., 1985; Inoue et al., 1994), and although lung surfactant has previously been believed to exist solely in the lung, both SP-A and -D have been detected in serum (Nagae et al., 1997; Greene et al., 1999). Since the detection of surfactant proteins in serum, it was speculated that this could be a useful and non-invasive diagnostic tool for specific lung diseases.

1.11 – INTERACTIONS OF SP-A AND –D AND MICROORGANISMS

The collectins are able to recognise and bind a wide variety of bacteria, fungi and viruses. **Table 1.3** summarises some microbial targets of SP-A and SP-D.

There are numerous overlaps between microbes recognised by both SP-A and -D. However, the overlaps of the individual collectins are only partial and the actual interaction between the specific collectin molecule and a microorganism, and outcome, can vary greatly **(Figure 1.13)**. For example it has been shown that SP-A and -D are able to bind *M. tuberculosis*, however, SP-A enhances the adherence to macrophages and phagocytosis of *M. tuberculosis* whereas SP-D binding to *M. tuberculosis* decreases the adherence of bacteria to human macrophages (Gaynor et al., 1995; Ferguson et al., 1999).

SP-D elicits different responses to various microorganisms. It is able to cause agglutination, allowing more efficient removal of pathogens from the lung (Lawson and Reid, 2000). SP-D is able to act as an opsonin although this does not always promote phagocytosis. For example, SP-D is able to enhance the binding of *Pneumocystis carinii* to alveolar macrophages but this does not result in phagocytosis (O'Riordan et al., 1995). Surfactant proteins act as chemotactic agents by recruiting or curtailing phagocytic cells at the sites of inflammation and immune reaction. SP-D acts as a potent chemoattractant for phagocytes, neutrophils and monocytes (Madan et al., 1997; Crouch et al., 1995; Cai et al., 1999). The mechanism and receptors behind this process are not fully characterised, but a central feature of chemotaxis is actin breakdown and repolymerisation (Tino and Wright, 1999).

TABLE 1.3: Microbial Targets of SP-A and SP-D.

Collecitn	Microorganism	Reference
SP-A	*Escherichia coli* J5	(Pikaar et al., 1995)
	Group A *Streptococcus*	(Tino and Wright, 1996)
	Group B *Streptococcus*	(LeVine et al., 1997; LeVine et al., 1999b)
	Haempohilus influenza type a	(McNeely and Coonrod, 1994; Tino and Wright, 1996)
	Herpes simplex	(van Iwaarden et al., 1991)
	Mycoplasma pulmonis	(Hickman-Davis et al., 1998)
SP-D	Certain strains of *Escherichia coli*	(Hartshorn et al., 1998)
	Salmonella minnesota	(Lim et al., 1994)
	Candida albicans	(van Rozendaal et al., 2000)
SP-A and D	*Staphylococcus aureus*	(Hartshorn et al., 1998; Geertsma et al., 1994)
	Streptococcus pneumoniae	(Hartshorn et al., 1998; McNeely and Coonrod, 1993; Jounblat et al., 2004)
	Klebsiella pneumoniae	(Kabha et al., 1997)
	Pseudomonas aeruginosa	(LeVine et al., 1998)
	Mycobacterium tuberculosis	(Gaynor et al., 1995; Ferguson et al., 1999)
	Influenza A	(Hartshorn et al., 1997; Hartshorn et al., 1994; Malhotra et al., 1994)
	Respiratory syncytial virus	(LeVine et al., 1999a; Hickling et al., 1999)
	Aspergillus fumigatus	(Madan et al., 1997)
	Pneumocystis carinii	(Phelps and Rose, 1991; O'Riordan et al., 1995)
	Cryptococcus neoformans	(Schelenz et al., 1995)
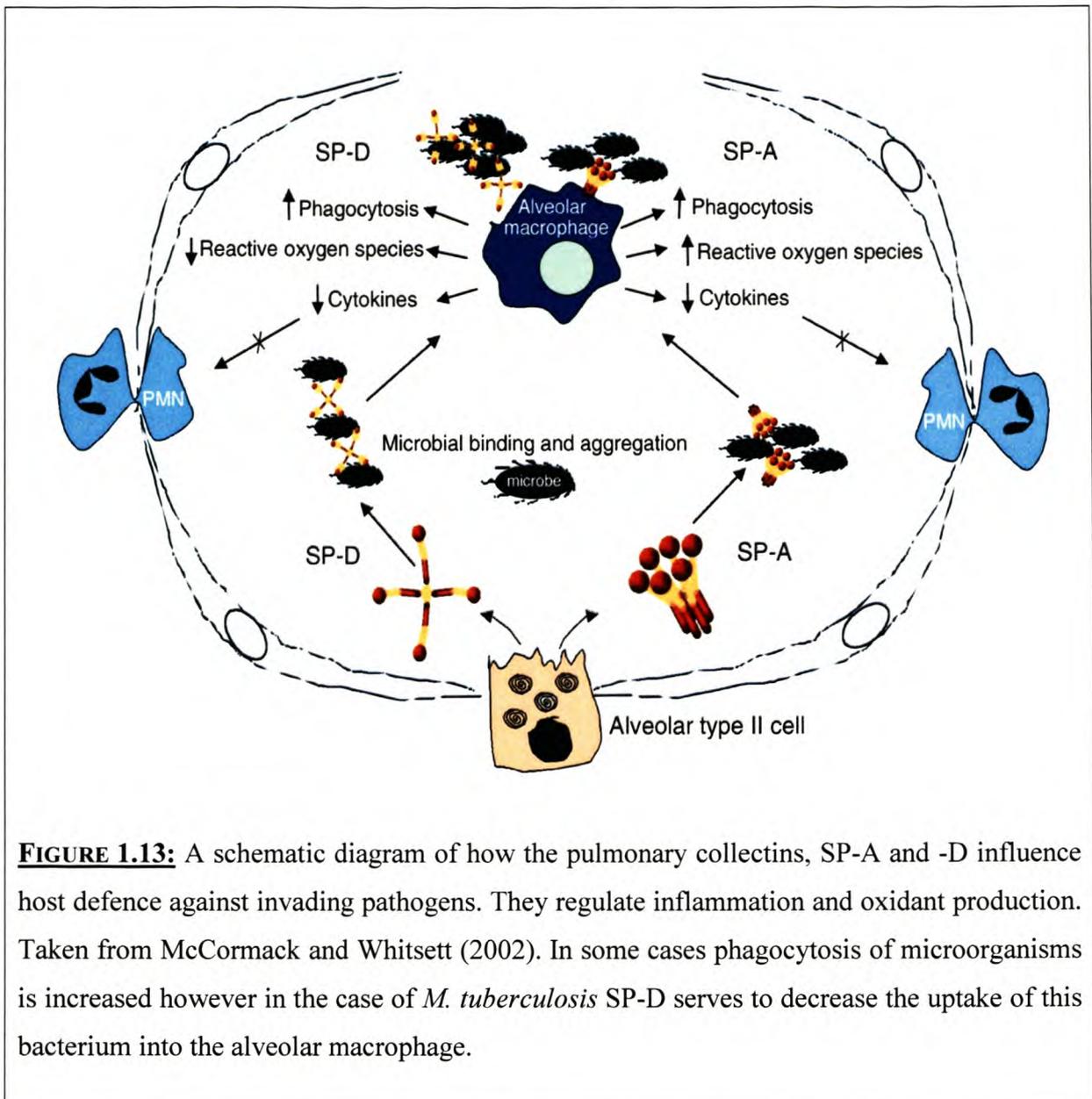
FIGURE 1.13: A schematic diagram of how the pulmonary collectins, SP-A and -D influence host defence against invading pathogens. They regulate inflammation and oxidant production. Taken from McCormack and Whitsett (2002). In some cases phagocytosis of microorganisms is increased however in the case of *M. tuberculosis* SP-D serves to decrease the uptake of this bacterium into the alveolar macrophage.

SP-D is able to mediate changes in the architecture of alveolar macrophages, unlike C1q and MBL (Tino and Wright, 1999). The surfactant collectins have been shown to enhance phagocytosis in some cases. SP-D is able to bind and enhance macrophage phagocytosis of mucoid and non-mucoid srains of *Pseudomonas aeruginosa* but it is unable to aggregate either strain, which suggests that SP-D enhances phagocytosis through receptor mediated opsonisation and killing (Restrepo et al., 1999). The mucoid strains of *Pseudomonas aeruginosa* are important in the pathogenesis of chronic lung disease associated with cystic fibrosis and this could be related to the decreased levels of both SP-A and D that have been reported in lung lavages of at least some patients with cystic fibrosis (Postle et al., 1999).

1.12 – SP-A AND –D AND *Mycobacterium tuberculosis*

The initial immunologic response to *M. tuberculosis* in the lung is important, as entry of this inhaled pathogen into the alveolar macrophage is the defining event in disease pathogenesis (Ferguson et al., 1999). The clearance of pathogenic organisms from the lung is dependent on a rapid and directed response that includes migration of macrophages to the site of infection, extension of filipodia and pseudopodia to capture and surround the particles, followed by retraction of the plasma membrane to internalize the bacteria (Warheit and Hartsky, 1993). It is important that an immune cell is able to control its cytoskeletal redistribution in an effective manner. In this respect Tino and Wright (1999) were able to demonstrate that the collectins SP-A and -D, induced a directional actin-based response in alveolar macrophages but not in peripheral blood monocytes. It is hypothesized that these effects are mediated by a receptor(s) that is expressed on alveolar macrophages only. This result contrasts with those that were found for the other members of the collectin family, C1q and MBL, which induced a non-directional actin response in alveolar macrophages. These results show that the pulmonary collectins have specific effects on alveolar macrophages, which are the primary immune cells present in the lungs. SP-D is able to bind directly to alveolar macrophages (Miyamura et al., 1994; Voorhout et al., 1992) and a putative receptor, glycoprotein-340 (gp-340), has been identified on alveolar macrophages. Gp-340 appears to play a role in the internalization of SP-D by macrophages (Holmskov et al., 1997).

M. tuberculosis is likely to come into contact with surfactant when inhaled into the alveolus and it is proposed that the interaction between *M. tuberculosis* and surfactant components alters the ultimate fate of the bacterium (Ferguson et al., 1999). Ferguson *et al.* (1999) investigated the binding of SP-D to *M. tuberculosis* and the effect of this binding on the adherence of *M. tuberculosis* to human macrophages. They demonstrated that specific binding of SP-D to *M. tuberculosis* is saturable, calcium dependent and carbohydrate inhibitable. On incubation with SP-D, the virulent Erdman strain of *M. tuberculosis* agglutinated but there was minimal binding to the avirulent strain of *M. smegmatis*. SP-D was found to bind predominantly to the LAM of *M. tuberculosis* Erdman, but was unable to bind to the AraLAM of *M. smegmatis*. It was observed that SP-D was able to agglutinate *M. tuberculosis*, but the adherence of the bacteria to human macrophages was decreased. In contrast, Gaynor *et. al.* (1995) found that SP-A acts to enhance *M. tuberculosis* adherence to macrophages as well as phagocytosis of the bacteria. Within this context, mechanisms that enhance entry of

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M. tuberculosis into the alveolar macrophage (its natural host cell) may be detrimental to the host, whereas mechanisms that reduce the entry of *M. tuberculosis* may be protective.

Sumiya *et al.* (1991) showed that children carrying MBL mutations were more susceptible to recurrent infections, which supports the concept that collectins are part of the innate immune response against microbial pathogens. In support of this Matsushita *et al.* (1995), found that polymorphisms in the nucleotide sequences of the collagen domain of the MBL gene altered oligomerisation and were associated with defects in host defence function. It is therefore thought that increased levels of variant SP-D that are unable to form higher orders of multimerisation, might influence the function of SP-D. This, in turn, could contribute to the pathogenesis of various lung diseases including TB (Zhang et al., 2001).

CHAPTER 2

Study Overview

Every now and again take a good look at something not made with hands - a mountain, a star,

the turn of a stream. There will come to you wisdom and patience and solace and, above all,

the assurance that you are not alone in the world.

Sidney Lovett

2.1. OVERVIEW

Surfactant protein (SP) -A and -D play major roles in the first line defence, or innate immune response to a number of pathogens, including *M. tuberculosis*. It is therefore important to investigate these proteins in order to determine and understand their role in the innate immune response to infection with *M. tuberculosis*.

A single nucleotide polymorphism (SNP) (SP-D11) within the amino terminal region of the SP-D gene was found to be associated with susceptibility to tuberculosis (TB) in a Mexican population (Floros et al., 2000) as well as in the South African Coloured population in the Western Cape (T. Lundwall-Roos, unpublished data). The amino terminal region is important in the trimerisation of SP-D monomers into trimers and subsequently into dodecameric structures. These dodecameric structures are the minimal structural requirement for efficient bacterial agglutination. Because of the location of the SNP we hypothesized that this amino acid substitution (threonine/methionine) could disrupt the usual formation of the SP-D molecule resulting in an increase in the number of monomeric and trimeric SP-D molecules being formed leading to decreased bacterial agglutination and therefore increased uptake of the bacilli into their preferred environment of the alveolar macrophage.

SP-A also plays an important role in the innate immune response to microorganisms including *M. tuberculosis*. Polymorphisms within the SP-A gene have been investigated in population based case-control association studies in order to determine whether the polymorphisms influence an individual's susceptibility to TB or not. Recently a number of polymorphisms were identified within the collagen-like region of the SP-A gene in an Indian population (Madan et al., 2002). Floros *et al*. (2000) have also studied polymorphisms in the SP-A genes and have reported significant associations with susceptibility to TB. We determined which polymorphisms were found in a local population and performed population based case-control association studies to ascertain their possible effect on susceptibility to TB.

HYPOTHESIS

Polymorphisms in SP-A and –D, which affect the level or function of these molecules, are likely to affect, directly or indirectly, the ability of the individual to control an infection with *M. tuberculosis*.

2.2. AIMS

1. Perform a TDT analysis using South African Coloured families from the same community as the cases and controls used in the study that showed an association between SP-D11 and susceptibility to TB.

2. Sequence the proximal promoter, amino terminal and neck regions of the SP-D gene to screen for novel polymorphisms in our population.

3. Determine if SP-D11 has an impact on the overall structure of the SP-D protein by partially purifying SP-D from serum using a Superose 6 HR 10/30 affinity column and detecting the presence of SP-D in the fractions collected using an ELISA assay.

4. Investigate the relationship between the SP-D11 genotype and concentration of SP-D in serum of patients and controls using an ELISA assay.

5. Purify SP-D from broncho alveolar lavage fluid of individuals with a known SP-D11 genotype and use this purified SP-D to determine whether SP-D produced by different genotypes impacts on the ability of the protein to bind to different mycobacterial strains and whether the genotype has an effect on the ability of the protein to agglutinate different mycobacterial strains.

6. Sequence approximately 500bp of the collagen-like region of the SP-A1 and -A2 genes to determine whether SNPs identified by Madan *et. al.* (2002) are present in our population.

7. Determine whether there are any novel polymorphisms within the collagen – like region of SP-A1 or -A2 genes in our population.

8. From the sequence data determine which SNPs are polymorphic in our population and use the selected SNPs to genotype TB patients and controls to be used in a case-control association study.

CHAPTER 3

MATERIALS AND METHODS

Vision is the art of seeing things invisible to others.

Jonathon Swift

A) MATERIALS

1. Buffers and Solutions

- <u>5x Tris-Borate-EDTA buffer (TBE)</u>

  54g Tris-HCL

  27.5g Boric acid

  20ml 0.5M EDTA (pH8.0)

  Make up to 1L with distilled water

- <u>1x TBE</u>

  200ml of 5x TBE made up to 1L with distilled water

- <u>TE buffer</u>

  1.21g Tris-HCl

  0.372g EDTA

  Add approximately 800ml distilled water and mix. Once dissolved adjust the pH to 8.0 with concentrated HCl and make up to a final volume of 1000ml.

  Autoclave

- <u>Ethidium Bromide stock solution</u>

  Ethidium bromide was dissolved in TE buffer to a final concentration of 10mg/ml

- <u>0.5M EDTA</u>

  93.06g EDTA.2H_2O

  Approximately 10g NaOH pellets

  Add 400ml distilled water, adjust the pH to 8.0 using the NaOH pellets.

  Make up to 500ml

Autoclave and store at room temperature

- <u>Organe G Gel loading dye</u>

  0.1% Orange G

  20% Ficoll

  10mM EDTA pH 7

Filter through a 0.22µm filter

- <u>4 x SDS Loading Buffer</u>

  60mM Tris-HCl

  800mM Urea

  30ml 10% SDS stock

  12% Glycerol

  0.1g Bromophenol blue

0.01g DTT added to 250µl of this 4 x loading buffer

- <u>5x SDS Electrophoresis Buffer</u>

  94g Glycine

  15.1g Tris-HCl

  50ml 10% SDS

  Make up to 1L with distilled water

Store at 4°C. Warm to room temperature if precipitate forms. Dilute to a 1x working solution.

- <u>1x Western Blot Transfer Buffer</u>

  3.03g Tris-HCl

  14.4g Glycine

  200ml Methanol

  Make up to 1L with distilled water

The pH should be between 8.1 and 8.4, if it is <8.0 re-make the buffer.

- <u>Maltose-Agarose Column Wash Buffer</u>

  20mM Tris-HCl, pH 7.4

  10mM $CaCl_2$

  0.02% Sodium Azide

  1M NaCl

Store at 4°C

- <u>Maltose-Agarose Column Equilibration Buffer</u>

  20mM Tris-HCl, pH 7.4

  10mM $CaCl_2$

  0.02% Sodium Azide

Store at 4°C

- <u>SP-D Column Elution Buffer</u>

  20mM Tris-HCl, pH 7.4

  100mM $MnCl_2$

Store at 4°C

- <u>Sodium Carbonate Coating Buffer</u>

  15mM Na_2CO_3

  35mM $NaHCO_3$

  pH 9.6

- <u>Coomassie Brilliant Blue R250 Stain</u>

  0.5g Brilliant Blue R250

  90ml methanol

  90ml distilled water

  20ml CH_3COOH

- <u>Protein gel destain</u>

  200ml CH_3COOH

  600ml methanol

  1.2L distilled water

- 40mM Sodium Thiosulphate

  496.37mg dissolved in 50ml distilled water

- Superose 6 Wash Buffer

  20mM Tris-HCl, pH 7.4

  0.5M EDTA, pH 8.3

  5M NaCl

  1ml 25% Tween 20

Filter with a 0.2μm filter and degas

- Dialysis Buffer

  3.45g Na_2HPO_4

  24g NaCl

  0.6g KCl

  0.6g KH_2PO_4

  30ml 0.5M EDTA

Make up to 3L with distilled water

- Superose 6 Equilibration and Running Buffer

  See dialysis buffer

- Acrylamide/bis-Acrylamide 40% stock solution mixture (19:1 Ratio)

  63ml of distilled water is added to the powdered mixture provided (Sigma, Catalogue number A-2917) and dissolved.

  Filter and store between 2°C and 8°C.

- Middlebrook 7H9 broth

  4.7g Middlebrook 7H9 broth

  0.5g Tween 80 per litre

  Make up to 900ml with distilled water

Autoclave

Once autoclaved ADC supplement (Merck, Darmstadt, Germany) is added to make up to 1 litre

- <u>Carbol-Fuchsin Stain</u>

  10g Basic fuchsin (pararosaniline chloride)

  50g Phenol

  100ml Absolute ethanol

Make up to 1 litre with distilled water.

- <u>5% Acid-Alcohol</u>

  5ml HCl

  95ml 95% ethanol

- <u>Methylene Blue</u>

  0.3% Methylene Blue in distilled water

2. Gels for electrophoresis

- 6% SDS Polyacrylamide gel

  2ml Acrylamide/bis-Acrylamide (19:1)

  5.3ml distilled water

  2.5ml 1.5M Tris-HCl pH 8.8

  100μl 10% APS

  8μl TEMED

  100μl 10% SDS

- 8% SDS Polyacrylamide gel

  2.7ml Acrylamide/bis-Acrylamide (19:1)

  4.6ml distilled water

  2.5ml 1.5M Tris-HCl pH 8.8

  100μl APS

  6μl TEMED

  100μl 10% SDS

- 3.75% Stacking Gel

  625μl Acrylamide/bis-Acrylamide (19:1)

  3.7ml distilled water

  625μl 1M Tris-HCl pH 6.8

  50μl 10% APS

  4μl TEMED

  50μl 10% SDS

- 10% non-denaturing polyacrylamide gel

  3ml 40% Acrylamide/bis-Acrylamide (19:1)

  2.4ml 5x TBE

  120μl APS

  12μl TEMED

  6.5ml distilled water

- <u>Agarose Gels</u>

1% w/v to 2% w/v were made up with 1x TBE electrophoresis buffer

3. Oligonucleotide Primers

All primers **(Tables 3.1 and 3.2)** used in this study were synthesised in the Synthetic DNA Laboratory at the University of Cape Town, South Africa using a Beckman Oligo 1000M DNA Synthesizer.

TABLE 3.1: Primers used for SP-A1 and -A2 sequencing and genotyping reactions.

	Primer Name	Orientation	Sequence 5' → 3'	T_m (°C)*	Location
SP-A1 Gene Specific Primers	SP-A1FF	Sense	ACT CCA TGA CTG ACC ACC TT	60	Intron A
	SP-A1RR	Antisense	ATG TCG AAG GCC AAG GCT AA	60	Intron E
SP-A1 nested primers for sequencing	SP-A1Fnest	Sense	GAT GGG CTC ACG GCC ATC CC	68	Intron A
	SP-A1Rnest	Antisense	GAA AA GAGA CAT GGA TGT GTA GGA T	70	Intron B
SP-A2 Gene specific primers	SP-A2FF	Sense	ATC ACT GAC TGT GAG AGG GT	60	Intron 1
	SP-A2RR	Antisense	TCC ATC TCA TGC CAA AGG CC	62	Exon 6
SP-A2 nested primers for sequencing	SP-A2Fn	Sense	CCT TTT CAG GAG GCC CAT CT	62	Intron 3
	SP-A2Rn	Antisense	ACC TGC AGG GTT TGT CTG A	58	Intron 4
Genotyping C1382G	SP-A2alleleC 1382	Sense	GGA CTT CAG TCT GCA GCGC	62	Intron 3
	SP-A2alleleG 1382	Sense	GGA CTT CAG TCT GCA GCGG	62	Intron 3
	SP-A2Rn	Antisense	ACC TGC AGG GTT TGT CTG A	58	Intron 4
	SP-A2F	Sense	TCC AGG CTG TGG GCC CTA T	62	Intron 1
Genotyping G1649C	SP-A2alleleC 1649	Antisense	TGG AGG GCC TCT CTC GCC GGG	74	Exon 4
	SP-A2alleleG 1649	Antisense	TGG AGG GCC TCT CTC GCC GGC	74	Exon 4
	SP-A2Fn	Sense	CCT TTT CAG GAG GCC CAT CT	62	Intron 3
	SP-A2R	Antisense	CGC ATT CAC CCT TCA GAC TGC	66	Intron 4

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Genotyping SP-A21691	SP-A2alleleA 1691	Antisense	TGC CCA TGT TTC CAC TGT CC	62	Intron 4
	SP-A2alleleG 1691	Antisense	TGC CCA TGT TTC CAC TGT CT	60	Intron 4
	SP-A2Fn	Sense	CCT TTT CAG GAG GCC CAT CT	62	Intron 3
	SP-A2R	Antisense	CGC ATT CAC CCT TCA GAC TGC	66	Intron 4

TABLE 3.2: Primers used for SP-D sequencing and genotyping reactions.

	Primer Name	Orientation	Sequence (5' → 3')	T_m (°C)*	Location in Gene
Sequencing SP-D N-terminal Region	SP-DFN-term	Sense	TTG GAT TCC TGC TGC TCT AG	60	Intron 1
	SP-DRN-term	Antisense	TGG TGG AGC AGT GTG GAA CT	62	Intron 2
Sequencing SP-D neck Region	SP-DneckF	Sense	CTG TGT CAG TAG ATG TGG GCT	64	Intron 6
	SP-DneckR	Antisense	AGA CCC AGG GCA GCT CTG CCT	70	Intron 7
Sequencing SP-D promoter Region	SP-DproF	Sense	CTG GGC AGT GCT GGT GAG AC	66	Promoter
	SP-DproR	Antisense	CCC ATG CCT CCT AGA CCA GA	64	Intron 1
Genotyping SP-D11 SNP	SP-D2F	Sense	CTG GAA GCA GAA ATG AAG AC	58	Exon 1
	SP-D2R	Antisense	ACC AGG GTG CAA GCA CTG CG	66	Exon 1

*Theoretical $T_m = 4 (G + C) + 2 (A + T)$

B) **METHODS**

1. Patients and Controls

This study was conducted in the Western Cape Province of South Africa where the incidence of TB is greater than 500 per 100 000 population per year (Department of National Health and Population Development).

Two classes of TB patients were used for this study: active and previous TB patients. All active TB patients had clinical as well as microbiological evidence of TB. The group of previous TB patients had received anti-TB therapy in the past, but at the time of sampling there were no clinical symptoms of TB. The control group consisted of apparently healthy people with no history of TB, living in the same high incidence community. Some of the controls were Mantoux skin tested but this was not done routinely. It is assumed that, because of the high incidence of TB in the community, the majority of the controls would have been exposed to TB. All cases and controls used in the various studies were 15 years old or older. The TB patients and controls were from the same community and from the same ethnic group, known in the Western Cape as Coloured. Ethical approval was obtained for these studies from the Stellenbosch University Institutional Review Board.

2. Genomic DNA Isolation

Genomic DNA was isolated using the Nucleon BACC3 Kit for blood and cell cultures (Amersham Biosciences, Buckinghamshire, UK) according to manufacturer's instructions. The isolated DNA was reconstituted with TE buffer and stored at -80°C until required. The purified samples were diluted to a final concentration of 100ng/μl with autoclaved distilled water and used as templates in PCR.

3. Control for Contamination

Throughout the project care was taken (especially when performing the nested PCR reactions required for the SP-A1 and -A2 sequencing and genotyping) to control for contamination during PCR amplification by including water blanks in every batch of samples that was amplified. In addition to this precaution the DNA and PCR mixture was made up and

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aliquotted in a designated DNA laboratory in which there were no amplicons. The PCR amplicons were opened for electrophoresis in an amplicon specific laboratory, so that no amplicons would come into contact with the DNA samples used in PCR reactions.

4. Genotyping the SP-D11 SNP

DNA was extracted from approximately 7ml of blood using the Nucleon BACC3 DNA extraction kit (Amersham Biosciences, Buckinghamshire, UK) according to manufacturer's instructions and genotyped as follows.

4.1. PCR Amplification

PCR reactions and oligonucleotide primers used were according to DiAngelo *et al.* (1999) (DiAngelo et al., 1999). PCR reactions were carried out in a total volume of 50μl, containing 100ng of genomic DNA, 5μl enzyme buffer (10x) provided by manufacturer, 4μl dNTPs (1.25mM stock) (Bioline, New Jersey, USA), 1.5μl of both forward and reverse primers (10μM stock), 2μl $MgCl_2$ (25mM stock) and 1U HotStarTaq DNA Polymerase (Qiagen). An Eppendorf Mastercycler Gradient PCR Machine (Eppendorf, Hamburg, Germany) was used and a touchdown PCR program was employed in order to increase the specificity of the reaction. Amplification was started by activating the HotStarTaq DNA Polymerase (Qiagen) at 95°C for 15 minutes, thereafter amplification was performed for 5 cycles of 95°C for 30 seconds, 62°C for 45 seconds and 72°C for 30 seconds, decreasing the melting temperature by 1°C every cycle until the optimum melting temperature (57°C) was reached. After 5 cycles the reaction was carried out for a further 35 cycles of 95°C for 30 seconds, 57°C for 30 seconds and 72°C for 30 seconds. After the last cycle the samples were incubated at 72°C for 5 minutes.

4.2. Polyacrylamide Gel Electrophoresis and Genotyping

The PCR amplified products (59bp) were subjected to restriction endonuclease digestion using *FspI* (New England Biolabs, Beverly, MA, USA). The digestions were carried out in a total volume of 5μl, containing 2μl of PCR amplified product, 0.5μl enzyme buffer (10x) provided by manufacturer, and 1U *FspI* enzyme. The samples were digested at 37°C

overnight and electrophoretically fractionated in 10% non-denaturing Polyacrylamide (PA) gels at 120 volts for approximately 1 hour in 1x TBE buffer, and silver stained. The expected restriction products were 40bp, 19bp and 59bp.

4.3. Silver Staining

Silver staining was carried out according to Bassam *et al.* (1991). Briefly, the PA gel was placed in 7.5% CH_3COOH for 10 minutes at 37°C in order to fix the DNA in the gel. The gel was then rinsed in distilled water three times for two minutes. Thereafter the gel was stained with 0.1% $AgNO_3$ containing 75µl 37% formaldehyde for 10 minutes at 37°C. The gel was then rinsed briefly with distilled water, (approximately 10 seconds) and then developed with an ice cold solution of 3% Na_2CO_3 containing 75µl 37% formaldehyde and 10µl 40mM sodium thiosulfate at room temperature. Once the bands had developed sufficiently the Na_2CO_3 was poured off and cold 7.5% CH_3COOH was poured onto the gel in order to stop the reaction. The gels were sealed in plastic sleeves to serve as a permanent record. The CH_3COOH solution, $AgNO_3$ and water were all heated to 37°C at the start of electrophoresis.

5. Sequencing the Amino Terminal, Neck and Promoter Regions of the SP-D Gene

In order to determine whether there were novel polymorphisms present in our population, portions of the amino terminal, neck and promoter regions were sequenced. These regions of the SP-D gene were amplified, purified and sequenced in the following manner. PCR reactions were carried out in a total volume of 25µl, containing 2.5µl of enzyme buffer (10x) (supplied with *Taq* Polymerase), 2µl of dinucleotide triphosphates (dNTPs) (2.5mM stock) (Bioline, New Jersey, USA) and 1.5U of the specified *Taq* Polymerase. PCR profiles for the individual reactions are shown in **Table 3.3**. For sequencing the amino terminal and neck regions, Promega *Taq* (Promega, Madison,WI) was used and for the promoter region, Supertherm Gold DNA Polymerase (Southern Cross Biotechnology) was used. An Eppendorf Thermal Cycler Gradient (Eppendorf, Hamburg, Germany) PCR machine was used. The concentration of each primer was always 0.3µM, and the product sizes were 490bp (amino terminal region), 351bp (neck region) and 590bp (promoter region).

TABLE 3.3: PCR profiles for sequencing portions of the SP-D gene.

Primer Set	MgCl$_2$ (mM)	T$_D$ (°C)	Time (sec)	T$_M$ (°C)	Time (sec)	T$_E$ (°C)	Time (sec)	Cycles
SP-DFN-term SP-DRN-term	2	94	30	61	30	72	45	30
SP-DneckF SP-DneckR	3	94	30	66.5	30	72	60	35
SP-DproF SP-DproR	1.5	94	30	62	45	72	30	25

(T$_D$ – denaturation temperature; T$_M$ – melting temperature; T$_E$ – extension temperature)

An initial denaturation step of 95°C for 5 minutes was used for the amino terminal and neck region PCRs but for the promoter region an initial denaturation of 95°C for 10 minutes was needed, and in each case there was a final extension time of 7 minutes at 72°C. These products were purified using the Wizard SV Gel and PCR Clean-up System (Promega, Madison, WI) according to the manufacturers' instructions.

5.1. Sequencing of the PCR Products

Sequencing was performed using an ABI Prism 3100 Avant Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) in the Department of Medical Biochemistry at the University of Stellenbosch. The sequence data was analysed using the Basic Local Alignment Search Tool (BLAST) software which is found on the National Centre for Biotechnology Information (NCBI) website (www.ncbi.nlm.nih.gov).

6. Partial Purification of SP-D from serum

6.1. Sample Collection

Blood samples were obtained from healthy adult volunteers in the Department of Medical Biochemistry, University of Stellenbosch. Approximately 20ml of blood was collected, 10ml was collected in a VACUTAINER® tube with EDTA (BD Vacutainer Systems, Plymouth, UK) and two 5ml VACUTAINER® tubes without EDTA of blood were collected in which the blood was allowed to clot at room temperature. As much serum as possible was removed from the clotted blood and centrifuged at 14 000rpm in an Eppendorf Centrifuge 5417C (Eppendorf, Hamburg, Germany). After centrifugation the serum was transferred to clean tubes and stored at -80°C until required. The EDTA blood samples were allowed to stand so that the plasma could separate. Two ml of plasma was removed, and centrifuged at 14 000rpm in an Eppendorf Centrifuge 5417C (Eppendorf, Hamburg, Germany) in order to pellet any red blood cells still present. The centrifuged plasma was transferred to a clean tube and frozen at –80°C until required. The blood remaining in the EDTA tube was transferred to a 50ml Sterilin Centrifuge tube (Bibby Sterilin Ltd, Staffs, UK) and stored at -20°C until the DNA was extracted using the Nucleon BACC3 DNA extraction kit (Amersham Biosciences, Buckinghamshire, UK) according to manufacturer's instructions. The DNA was used to genotype the individuals with respect to SP-D11 (**Method described in section 4**). The serum was partially purified by affinity column chromatography during a visit to the MRC Immunochemistry Unit, Department of Biochemistry, University of Oxford in Professor Ken Reid's lab under the supervision of Mr. Paul Townsend.

6.2. Purification of SP-D using Affinity column Chromatography

Serum samples were dialysed in phosphate buffered saline (PBS), 5mM EDTA overnight at 4°C. The column used to purify the SP-D was a Superose 6 HR 10/30 column and a guard column was also used which was 1ml Superdex 200 HP resin. The serum was filtered through a 0.22µm syringe filter before placing on the column. The column was equilibrated with 1.5 column volumes of Superose 6 equilibration buffer that had been filtered and degassed. In order to clean the loading loop 2.5ml of 20% ethanol was injected and thereafter 2.5ml PBS, 5mM EDTA. Once the column had equilibrated the filtered serum was injected into the

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loading loop and onto the column. Approximately 70 fractions were collected (0,5ml volume) into LP3 tubes. The fractions were stored at 4°C or at -20°C for long-term storage. These fractions were used in the ELISA assay (**Described in section 7 of this Chapter**) to investigate the molecular forms of SP-D present in the serum of individuals having the different SP-D11 genotypes.

7. ELISA Assay

The ELISA assay was based on a sandwich method using two polyclonal antibodies. The antibodies were rabbit anti-human SP-D IgG (primary (1°) antibody) and biotinylated rabbit anti-human SP-D IgG (secondary (2°) antibody). Both antibodies and recombinant SP-D (rSP-D) were a donation from Professor Ken Reid of the University of Oxford. The microtitre plates (Nunc Immuno plate, F96, Maxisorp) were coated with 100μl of 1° antibody (1μg/ml in sodium carbonate coating buffer) at 4°C overnight. After washing three times with 200μl of PBS containing 0.05% Tween 20 (PBST), the wells were incubated with 150μl of 3% (w/v) bovine serum albumin (BSA) in PBST at 37°C for two hours. Thereafter, the wells were washed three times with PBST and 100μl of sample was added to the wells including a positive control consisting of 500ng of rSP-D and a blank consisting of 100μl of 3% BSA/PBST, and incubated overnight at 4°C. After washing three times with PBST, 100μl of 2° antibody (1μg/ml in 3% BSA/PBST) was added to each well and incubated for two hours at 37°C. The plates were washed again three times and 100μl of streptavidin peroxidase conjugate diluted 1:10 000 with 3% BSA/PBST, was added to each well and incubated at 37°C for 30 minutes. After washing three times with PBST for the last time, 100μl of TMB/Hydrogen peroxide (Bio-Rad Laboratories, Hercules, CA, USA) was added to each well and left at room temperature for 15 minutes for the colour reaction to develop. The reaction was terminated by adding 100μl of 0.5M sulphuric acid to each well and the absorbance was measured at 450nm on a Benchmark Microplate Reader using Microplate Manager Software both from Bio-Rad Laboratories, Hercules, CA, USA. The data was exported to Microsoft Excel and analysed.

8. Purification of SP-D from bronchoalveolar lavage fluid

8.1. Affinity Chromatography

The principle of affinity chromatography is based on separating proteins using a biospecific method in which the particular biological property of the protein is exploited. In this case maltose is a ligand for SP-D. Because lectin-receptor interactions are very specific and often unique to the protein of interest, use of this method often yields highly purified proteins with a minimal number of purification steps (Coligan et al., 1996)

8.2. Lectin Affinity Chromatography

A protein is bound to an immobilised lectin through its sugar chain and the unbound proteins are washed away. The protein of interest is then eluted with the correct buffer (Coligan et al., 1996).

8.3. Purification of SP-D

Patients who were undergoing bronchoscopy were asked to be part of this study for which ethical approval was obtained and a patient information and consent form had to be completed (**Appendix 1 – 3**). Native human SP-D was purified from bronchoalveolar lavage fluid (BALF), from patients who had given consent to take part in the study, by modifications of previously described methods (Jounblat et al., 2004; Leth-Larsen et al., 1999; Madan et al., 1997). The BALF was centrifuged at 2000 x g at 4°C for 10 minutes in an Eppendorf Centrifuge 5810R (Eppendorf, Hamburg, Germany). The supernatant was transferred to a clean tube and frozen at -20°C until required. Frozen BALF was thawed at 4°C and was made 10mM with respect to EDTA. The solution was recalcified by the addition of $CaCl_2$ to a 5mM excess of calcium over the amount of EDTA and the pH was adjusted to 7.4 with sodium hydroxide. Maltose-agarose (Sigma, Missouri, USA) was packed into a column and washed with the maltose-agarose wash solution. Thereafter the column was equilibrated with maltose-agarose equilibration buffer. The recalcified BALF was applied to the maltose-agarose column. Once the BALF had passed through the column it was washed as before and the SP-D was eluted with SP-D elution buffer. The above purification was carried out at 4°C. The

purified SP-D was judged to be the correct product by SDS-PAGE (Coomassie blue stained) and Western Blotting (**Method described in section 9 of this Chapter**).

When the BALF was collected approximately 10 – 15ml of venous blood was collected from the same individual in VACUTAINER® tubes containing EDTA (BD Vacutainer Systems, Plymouth, UK) in order to extract DNA using the Nucleon BACC3 DNA extraction kit (Amersham Biosciences, Buckinghamshire, UK) according to manufacturer's instructions. The DNA was used to determine the SP-D11 genotype (**Method described in section 4 of this Chapter**). The concentration of DNA was determined using the NanoDrop® ND-1000 Spectrophotometer and the NanoDrop v3.0.1 Software (Inqaba Biotechnology, Pretoria, Gauteng, RSA).

9. Western Blotting

9.1. Sample Preparation

A 4x SDS loading buffer was used to denature the samples. 2.5µl of the 4x SDS loading buffer was added to 10µl of sample and was heated at 95°C for 5 minutes to denature the protein before being loaded onto the gel.

9.2. Polyacrylamide Gel Electrophoresis and Western Transfer

A 6% SDS – PA gel was poured, overlaid with water-saturated butanol, and allowed to set for an hour. Once the gel had set, the water-saturated butanol was poured off and the gel was rinsed thoroughly with distilled water. The glass plates were blotted dry with blotting paper and the 3.75% stacking gel was poured on top of the already set gel. The combs were inserted and the gel allowed to set for an hour. The combs were removed, the denatured samples loaded and the gel electrophoresed at 150 volts in SDS electrophoresis buffer for one hour. A prestained protein ladder ~10–160kDa (MBI Fermentas, Inqaba Biotectnology, Pretoria, Gauteng, RSA) was used to judge when the gel had electrophoresed far enough. Once electrophoresis was complete the gel was placed in western blot transfer buffer and allowed to equilibrate. After 5 minutes in transfer buffer the gel dimensions were measured and two pieces of Hybond™ Blotting Paper (Amersham Biosciences, Buckinghamshire, UK) were cut to the correct size, and at the same time the membrane (Hybond™-P, Amersham Biosciences, Buckinghamshire, UK) was also cut to the size of the gel. The membrane was placed into

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100% Methanol for approximately 10 seconds until it changed colour (white to grey) and then it was equilibrated in transfer buffer for 15 minutes. The blotting paper and the filter pads for the Mini Trans-Blot® Cell (Bio-Rad Laboratories, Hercules, CA, USA) were also soaked in the transfer buffer for 15 minutes. After equilibration had taken place the blotting apparatus was assembled, the tank filled with western blot transfer buffer and transfer took place at 150 volts for one hour. When transfer was complete the membrane was placed in blocking buffer (2% BSA/PBST) and left overnight at 4°C.

9.3. Protein Detection

The membrane was washed three times in PBST, the first and last wash for five minutes and the second wash for 10 minutes at room temperature. The membrane was then incubated with 2° antibody (biotinylated rabbit anti-human SP-D IgG) diluted 1:2000 with blocking solution (2% BSA/PBST), for one hour at 37°C. After this was complete the membrane was washed as before with PBST and incubated with Streptavidin peroxidase conjugate (Sigma, Missouri, USA), diluted 1:10 000 with blocking buffer (2% BSA/PBST), at 37°C for 30 minutes. The membrane was then washed for the last time as previously with PBST. In the dark room the western blotting ECL reagents (Amersham Biosciences, Buckinghamshire, UK) were mixed together in the ratio 1:1 and the membrane was placed, protein side down, into the mix for a few seconds and then placed, protein side up, into a light proof cassette (Hypercassette™, Amersham Biosciences, Buckinghamshire, UK) lined with cling wrap. The cling wrap was folded over the membrane and the film placed on top. The film was exposed for the desired amount of time (10 seconds to 10 minutes) and then developed using the Hyperprocessor (Amershem pharmacia biotechnology, Buckinghamshire, UK). The exposure was repeated with greater or less time of exposure if necessary.

9.4. Staining with Coomassie Brilliant Blue R-250

Once western transfer had taken place the gel was placed in approximately 100ml Coomassie Brilliant blue R-250 stain **(See page 40)** in order to check the efficiency of transfer. The following morning the gel was destained in the Protein Gel Destain solution **(See page 40)** to check that transfer was efficient. Coomassie Brilliant Blue R-250 binds non-specifically to almost all proteins allowing detection of protein bands in PA gels. Proteins that have been separated in PA gels are simultaneously fixed and stained in the Coomassie Brilliant Blue R-

250 staining solution, turning the entire gel blue. Destaining eliminates the background colour while the protein bands remain blue.

10. Ziehl-Neelsen (ZN) Staining for mycobacteria

The ZN stain was used in order to detect the presence of mycobacteria in a sample. Approximately 50µl of ZN fixing solution (purchased from the NHLS, Greenpoint, Western Cape, South Africa) was placed on a microscope slide (Labstar 1000 microscope slides, LASEC, Cape Town, South Africa) and approximately 50µl of bacterial culture was mixed with this. Thereafter the slide was placed on a 100°C heating block for two hours to dry and kill the bacteria. Once the sample had dried the slide was flooded with carbol-fuchsin stain. The slide was heated intermittently with a flame until steam came off the surface. It was important to ensure that the stain did not boil or that the sample did not dry out. The stain was left on the slide for 7 - 10 minutes. The slide was rinsed with tap water in order to remove most of the stain, flooded with 5% acid-alcohol () in order to remove the remaining carbol-fuchsin stain (), and rinsed with tap water again. The sample was counter-stained with methylene-blue () and left for 5 minutes. The slide was rinsed again with tap water and blotted dry. The sample was examined using an Olympus BX41 phase contrast microscope using the oil immersion objective (100x magnification). Under the microscope the mycobacteria appeared as red/pink stained rods (**Figure 3.1**).
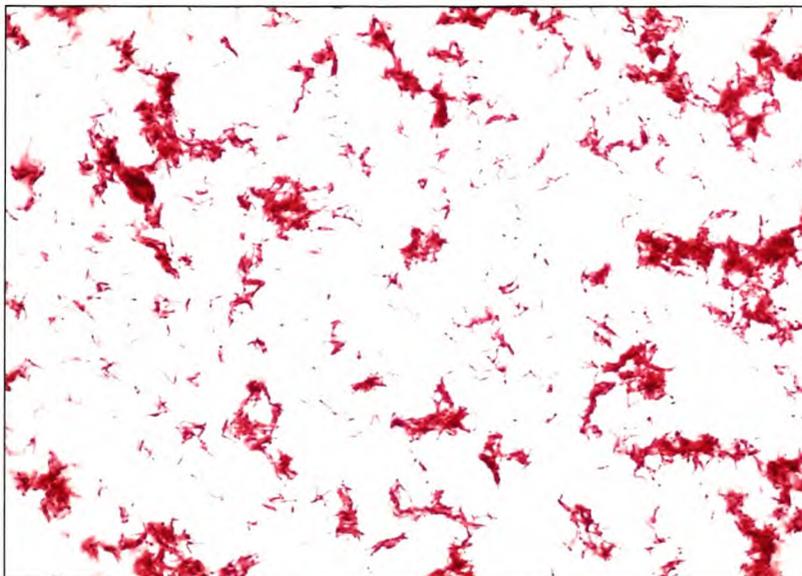
FIGURE 3.1: ZN staining of mycobacteria, appearing as pink/red rods (40x magnification).

11. Growth of mycobacteria

Three strains of mycobacteria were grown up in the Department of Medical Biochemistry, University of Stellenbosch Medical School, Tygerberg, South Africa for use in the agglutination assays. Culturing of mycobacteria was carried out in a P3 facility by Ms. Rabia Johnson, Department of Medical Biochemistry, Stellenbosch Medical School, Tygerberg, South Africa. $H_{37}Rv$ ATC 27294 was obtained from Ms. Amor Venter, Department of Medical Biochemistry, University of Stellenbosch Medical School, Tygerberg, South Africa, the Beijing/W-like R433, found in 72 clinics in the Boland/Overberg South Cape/Karoo Region, was obtained from the National Health Laboratory Services (NHLS, Greenpoint, Western Cape, South Africa) and Family 11 SAWC1925 was obtained from Prof. Robin Warren, Department of Medical Biochemistry, University of Stellenbosch Medical School, Tygerberg, South Africa. The two clinical isolates (Beijing/W-like R433 and Family 11) were cultured on Lowenstein-Jensen (LJ) slants. A scrape was taken from the LJ slants and placed in 5ml of 7H9 broth containing ADC (Merck, Darmstadt, Germany) as a supplement. A 1:5 dilution was made of an $H_{37}Rv$ liquid culture in 7H9 broth containing ADC (Merck, Darmstadt, Germany) as a supplement. These initial cultures were grown to an OD_{600} of 0.8 (approximately two weeks). A ZN stain (**see above**) was performed on these initial cultures in order to determine whether there was any contamination, and a drop of culture was placed on a blood agar plate (obtained from the NHLS). The blood agar plates were checked after two days for any growth. Growth on the blood agar plate at this stage indicated that the culture was contaminated with a mycobacterium other than tuberculosis (MOTT) and the initial culture was set up again. MOTTs are mycobacteria that can cause opportunistic infections and do not belong to the *M. tuberculosis* complex. MOTT bacteria include *M. avium* complex, *M. kansasii, M. marinum, M. scrofulaceum* and *M. ulcerans*. These bacteria are able to grow on blood agar plates but this is usually seen only after two weeks incubation of the blood agar plate.

Once the initial culture was judged to be pure a 10ml starter culture was set up by making a 1:10 dilution of the initial culture in 7H9 broth containing ADC (Merck, Darmstadt, Germany) as a supplement. This starter culture was again allowed to grow to an OD_{600} of 0.8. Once this growth has been attained, a ZN stain was once again performed on the culture and a blood agar plate was set up to determine whether or not the culture was contaminated.

Once the cultures had been shown to be pure the agglutination assay (**see below**) was carried out on the individual samples.

12. SP-D Agglutination Assay

The agglutination assay was carried out by modifications of previously described methods (Jounblat et al., 2004; Ferguson et al., 1999). SP-D purified (**method described in Section 9 of this Chapter**) from BALF of individuals with a known SP-D11 genotype (CC, CT and TT) was used to determine whether the different genotypes agglutinated mycobacterial species more or less efficiently. Bacterial cultures were grown up according to **Section 11** of this Chapter.

Purified SP-D was added at a concentration of 10μg/ml to a final volume of 50μl of mycobacterial culture and placed at room temperature for one hour to allow the SP-D to agglutinate the bacteria. At the same time a control experiment was set up in which no SP-D was added to the bacteria which was also allowed to stand at room temperature for an hour. Once the SP-D had been allowed to agglutinate the bacteria a ZN stain (**method described in Section 10 of this Chapter**) was used to assess the extent of agglutination.

13. Analysis of the Collagen-like Region of the SP-A1 and -A2 genes

A nested PCR reaction was carried out for both SP-A1 and -A2 in order to obtain smaller fragments for sequencing, as the SP-A genes are more than 80% homologous making primer design difficult. The first round of amplification amplified the entire SP-A1 or -A2 genes and the second round amplified smaller fragments (approximately 500bp) which were sent for sequencing to determine whether the SNPs identified by Madan *et al.* (2002) or others were found, and to detect any novel SNPs present in our population.

13.1. SP-A1 and -A2 Gene Amplification

PCR reactions were carried out in a total volume of 25μl containing 100ng genomic DNA, 2.5μl enzyme buffer (10x) provided by the manufacturer, 2μl dNTPs (2.5mM stock) (Bioline, New Jersey, USA), 0.2μM of each primer and 1.5U ExSel High Fidelity Taq DNA polymerase (Southern Cross Biotechnology). The PCR profiles are shown in **Table 3.4**.

TABLE 3.4: PCR conditions for amplification of the SP-A1 and -A2 genes.

Primer Set	MgCl$_2$ (mM)	T$_D$ (°C)	Time (sec)	T$_M$ (°C)	Time (sec)	T$_E$ (°C)	Time (sec)	Cycles
SP-A1FF SP-A1RR	2	94	60	60	60	72	180	35
SP-A2FF SP-A2RR	2	94	60	61	60	72	120	30

(T$_D$ – denaturation temperature; T$_M$ – melting temperature; T$_E$ – extension temperature)

A Gene Amp® 9700 PCR Machine (Applied Biosystems, Foster City, CA, USA) was used to carry out the reactions, with an initial denaturing step of 95°C for 5 minutes and a final extension at 72°C for 7 minutes. The products were purified using the Wizard SV Gel and PCR Clean-up System (Promega, Madison, WI) according to the manufactufrer's instructions. The expected size of the SP-A1 gene product was 3124bp and the SP-A2 product 3150bp.

13.2. Nested PCR Reactions

The whole gene products that were generated in the first round of amplification were diluted 1:50 with milliQ water and used as the templates for the nested reactions. Nested PCR reactions were carried out in a total volume of 25μl, containing 1μl of the diluted purified gene product, 2.5μl of the enzyme buffer (10x) provided by the manufacturer, 2μl dNTPs (2.5mM stock, Bioline, New Jersey, USA), 0.3μM of both the sense and antisense primers and 1.5U of Supertherm Gold Taq DNA Polymerase (Southern Cross Biotechnology). The PCR profiles are shown in **Table 3.5**.

TABLE 3.5: PCR conditions for the nested reactions required for sequencing.

Primer Set	MgCl$_2$ (mM)	T$_D$ (°C)	Time (sec)	T$_M$ (°C)	Time (sec)	T$_E$ (°C)	Time (sec)	Cycles
SP-A1Fn SP-A1Rn	1.5	94	30	70	45	72	30	30
SP-A2Fn SP-A2Rn	1.5	94	30	61	45	72	30	30

(T$_D$ – denaturation temperature; T$_M$ – melting temperature; T$_E$ – extension temperature)

A Gene Amp® 9700 PCR Machine (Applied Biosystems, Foster City, CA, USA) was used to carry out the reactions. An initial denaturation step was carried out at 95°C for 10 minutes and a final extension of 72°C for 5 minutes. The PCR products were purified using the Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI) according to the manufacturer's instructions and sent for sequencing. The expected size of the SP-A1 nested product was 530bp and the SP-A2 nested product 480bp.

13.3. Sequencing of the PCR Products

The collagen-like regions of SP-A1 and -A2 were sequenced using an ABI Prism 3100 Avant Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). The sequence data was analysed using the Basic Local Alignment Search tool (BLAST) software which is found on the National Centre for Biotechnology Information (NCBI) website (www.ncbi.nlm.nih.gov).

14. Amplification-Refractory Mutation System (ARMS)

In order to genotype the SNPs present in the SP-A genes an ARMS method was used (Brightwell et al., 2002; Newton et al., 1989). This method is based on using sequence specific PCR primers that will amplify only the target allele. An internal control primer is also added to the reaction mixture that amplifies in every case in order to confirm that the PCR is working. The allele specific primers were designed such that the last base at the 3' end was complementary to the SNP and therefore would only amplify should the particular allele be present in the sample. In order to make the allele specific primers more refractory to incorrect

amplification an additional mutation of a single nucleotide was made three bases from the 3' end of the primers. Therefore if there is no amplification of a specific allele band it can be concluded that allele is not present provided that the internal control band has amplified. For each sample two reaction tubes are set up with each tube containing a different allele specific primer. **Figure 3.2** explains the ARMS method. This is a simple method for genotyping SNPs as it only requires one round of amplification and electrophoresis in an agarose gel.

Expected results observed after electrophoresis of samples. a) an AA homozygote, b) an AG heterozygote and c) a GG homozygote.

FIGURE 3.2: Diagrammatic representation of the ARMS PCR and the products obtained in genotyping reactions.
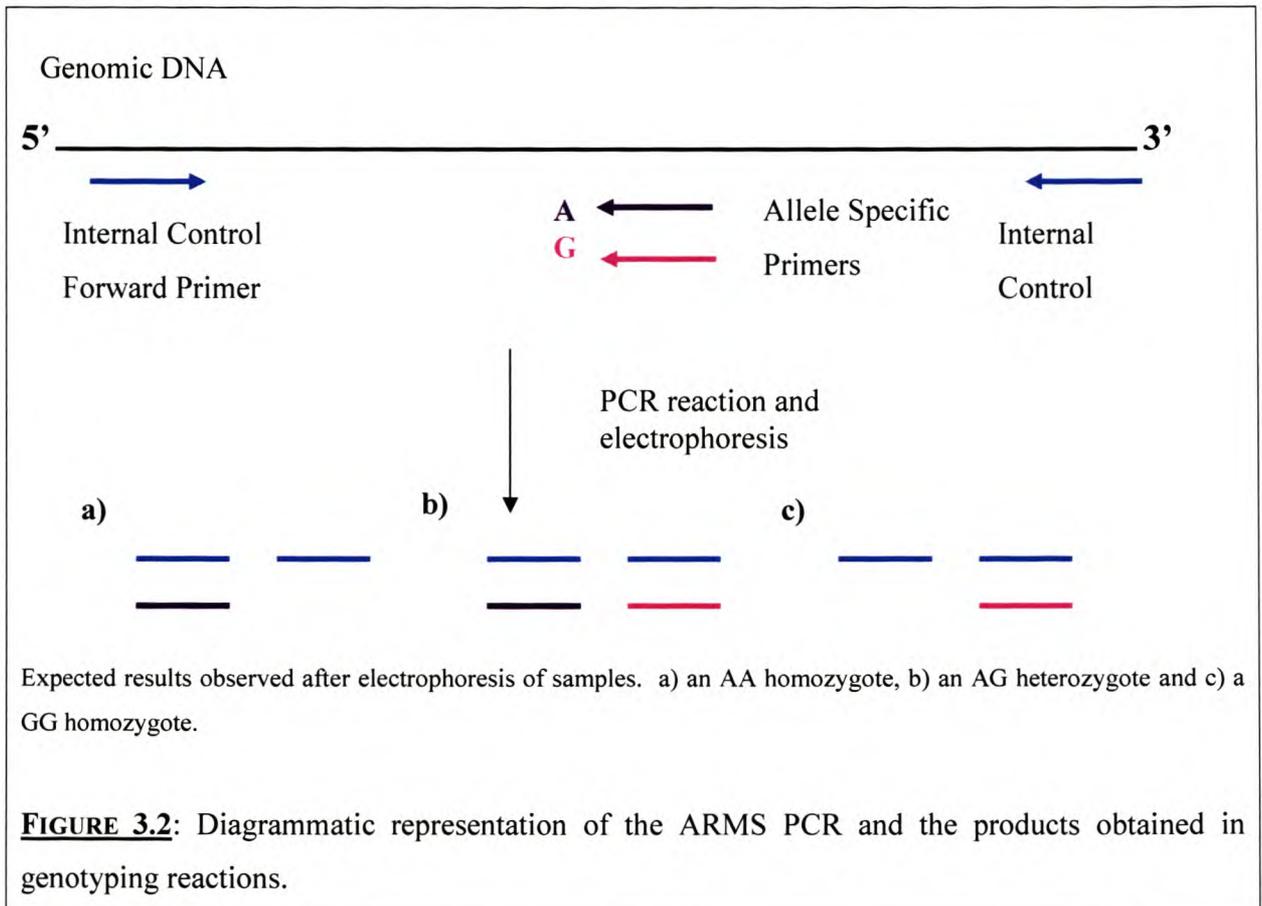
15. Genotyping SNPs in the SP-A1 and -A2 genes

SNPs in the collagen-like regions of SP-A1 and -A2 were genotyped using the ARMS method described in **section 12**. The PCR profiles for genotyping are shown in **Table 3.6**. PCR reactions were carried out in a total volume of 25μl containing 2.5μl of enzyme buffer (10x) provided by the manufacturer, 2μl of dNTPs (2.5mM stock) (Bioline, New Jersey, USA) and 1.5U of Supertherm Gold Taq Polymerase (Southern Cross Biotechnology). The concentration of primers used in each individual genotyping reaction is shown in **Table 3.7**.

TABLE 3.6: Primers and PCR conditions used to genotype SNPs in the SP-A1 and -A2 genes.

Primer Set	MgCl$_2$ (mM)	T$_D$ (°C)	Time (sec)	T$_M$ (°C)	Time (sec)	T$_E$ (°C)	Time (sec)	Cycles
SP-A1alleleC 1193 SP-A1alleleG 1193 SP-A1Fn SP-A1Rn	2	94	30	67	45	72	45	30
SP-A2alleleC 1382 SP-A2alleleG 1382 SP-A2Rn SP-A2F	1.5	94	45	61.5	45	72	45	30
SP-A2alleleC 1649 SP-A2alleleG 1649 SP-A2Fn SP-A2R	1.5	94	30	61	45	72	30	30
SP-A2alleleA 1691 SP-A2alleleG 1691 SP-A2Fn SP-A2R	1.5	94	30	61	45	72	45	30

(T$_D$ – denaturation temperature; T$_M$ – melting temperature; T$_E$ – extension temperature)

In each reaction an intital denaturation step of 95°C for 10 minutes was carried out and a final extension at 72°C for 7 minutes. An Eppendorf Mastercycler Gradient Machine (Eppendorf, Hamburg, Germany) was used to amplify the SP-A2 SNPs and a Gene Amp® 9700 PCR Machine (Applied Biosystems, Foster City, CA, USA) was used for the SP-A1 SNP.

TABLE 3.7: Concentration of primers used in the SP-A genotyping reactions.

SNP	Allele Specific Primer	Forward/ Reverse Primer	Internal Control Primer	Allele Specific Product	Internal Control Product
SP-A1 C1193G	1.4μM	0.6μM (SP-A1Rn)	0.04μM (SP-A1Fn)	347bp	530bp
SP-A2 C1382G	1.2μM	0.6μM (SP-A2Rn)	0.04μM (SP-A2F)	404bp	1249bp
SP-A2 G1649C	0.8μM	0.8μM (SP-A2Fn)	0.4μM (SP-A2R)	383bp	716bp
SP-A2 G1691A	0.8μM	0.6μM (SP-A2Fn)	0.08μM (SP-A2R)	432bp	716bp

16. Examining the efficiency of amplification

In order to determine the efficiency of amplification as well as to genotype the SP-A1 and -A2 SNPs, the PCR products were electrophoresed in the appropriate percentage TBE agarose gel containing 0.5μg/ml ethidium bromide (**Table 3.8**). The products were visualised under ultra violet (UV) light using the Kodak Digital Science Electrophoresis Documentation and Analysis System 120.

TABLE 3.8: Percentage agarose gels used in for different PCR products for this work.

PCR Product	Percentage Agarose
SP-D Amino Terminal Region	2%
SP-D Neck Region	2%
SP-D Promoter Region	2%
SP-D11 PCR Product	2%
SP-A1 Gene	1%
SP-A2 Gene	1%
SP-A1 Nested Products	1.5%
SP-A2 Nested Products	1.5%
SP-A1 1193 C/G	1.5%
SP-A2 1382 C/G	1.5%
SP-A2 1649 C/G	1.5%
SP-A2 1691 A/G	1.5%

17. Statistical Analysis

17.1. Hardy-Weinberg Equilibrium

The Hardy-Weinberg Equilibrium (HWE) relates allele frequencies to the genotype frequencies in a randomly mating population. The law is an application of the following binomial equation: $1 = p^2 + 2pq + p^2$, where p and q are the proportions of alleles p and q. The proportions of the homozygotes pp and qq, and the heterozygotes, pq are represented by p^2, q^2 and 2pq respectively (Crow, 2001; Mange and Mange, 1990).

This equation ($1 = p^2 + 2pq + p^2$) is used to calculate the expected genotype frequencies from the known allele frequencies. Therefore, to calculate the expected number of pp genotypes, the equation p^2 x n, would be used, where p refers to the allele frequency of allele p, and n is the total number of samples in that population (i.e. the control population or the TB population). Similarly, to calculate the expected number of heterozygotes, the equation 2pq x n would be used, where p is the allele frequency of the p allele and q the allele frequency of the q allele. The equation to calculate the expected number of qq homozygotes would therefore be q^2 x n. Once the expected values have been calculated the χ^2 is calculated by using the equation $\chi^2 = \Sigma$(observed number – expected number)^2/expected number. This value (χ^2) must be less than 3.84 for the population not to be significantly out of HWE. The sample sizes for the genotyping of the SP-A SNPs were chosen in order to provide 95% confidence and 80% power to detect an allele resulting in an odds ratio of 1.7 or higher.

17.2. Association of Genotype with Cases or Controls

2 x 3 contingency tables were drawn up to determine whether there was a significant difference in the distribution of genotypes between cases and controls, and 2 x 2 contingency tables were drawn up to determine if there was a significant difference in the distribution of alleles between cases and controls and a χ^2 test was performed. Epi-Info 2000 version 1.1 was used to calculate these values for the SP-A1 and -A2 genotyping. The χ^2 statistic was accepted as showing a significant difference in genotype or allele distribution when the probability (p) of chance variation was <0.05. As mentioned in the Introduction (**Section 1.3.2a**) there are numerous tests to correct for multiple testing. In this study the stringent Bonferroni test was used to correct p-values obtained for SP-A SNPs. In this test the p-value of each SNP is multiplied by the number of SNPs that have been studied in that gene.

17.3. Association of SP-D serum levels and TB

GraphPad Prism 4.01 was used to determine whether there was a significant association between the levels of SP-D present in serum and the disease status of individuals or their SP-D11 genotype.

In order to compare data from two independent groups such as the serum SP-D concentration and the SP-D11 genotype there is a non-parametric alternative to the t test, known as the Mann-Whitney test (Altman, 1991).

When there are three or more independent groups of observations it is better to use a test that enables one to examine all the data sets at one time. This method is known as a one-way analysis of variance (ANOVA) (Altman, 1991). The Kruskall-Wallis test is a non-parametric one-way ANOVA and was used to calculate whether there were significant differences between the SP-D present in the serum from individuals with different disease states, as this involves more than two groups of data.

17.4. Haplotyping

Haplotypes were constructed using the SP-A2 SNP genotype data with PHASE version 2.1 (Stephens et al., 2001). PHASE uses a Bayesian statistical method to reconstruct haplotypes from population genotype data. The software is able to construct haplotypes from SNP, microsatellite or other multi-allelic loci data in any combination. The programme is also able to deal with missing data and will construct the most likely haplotype using the allele frequency observed in the population. The PHASE programme was obtained from the Internet at the following address http://www.stat.washington.edu/stephens/software.html.

17.5. Linkage Disequilibrium Analysis

Linkage disequilibrium (LD) analysis was performed using LDMAX within the Graphical Overview of Linkage Disequilibrium (GOLD) software package (Abecasis and Cookson, 2000). This package is available on the Internet at http://www.well.ox.ac.uk/asthma/GOLD. This programme provides a graphical summary of LD, and allows for the use of case-control as well as family data. A disequilibrium coefficient (D'), p-value and the χ^2 statistic are calculated. All cases and controls that were genotyped with respect to the three polymorphisms in the SP-A2 gene were used in the analysis.

Various statistical measures can be used to summarize LD between two markers (Hedrick, 1987; Devlin and Risch, 1995), but only two are widely used: D' and r^2. Both these statistics are based on the basic pairwise-disequilibrium coefficient, D, being the difference between

the probability of observing two marker alleles on the same haplotype and observing them independently in the population. D' is favoured in medical genetics and has the scale 0 to 1. A value of 0 implies independence and a value of 1 means that all copies of the rare allele occur exclusively with one of the two possible alleles at the other marker i.e. total LD. Estimates of D' are heavily influenced by the sample size. r^2 (also denoted as Δ^2) is in some ways complementary to D', and is thought of as the measure of choice for quantifying and comparing LD in the context of mapping (Weiss and Clark, 2002; Frisse et al., 2001). This measure is the correlation of alleles at the two sites. If r^2 equals 1 this is an indication that the markers have not been separated by recombination and have the same allele frequency.

LDMAX gives a p-value when a LD analysis is performed. The p-value depends strongly on the sample size and cannot be used to compare LD between studies with different sample sizes. A statistically significant result can be obtained even when there are low levels if LD if the sample size is sufficiently large (Ardlie et al., 2002).

In this study the D' statistic was used as an indication of whether there was LD between markers or not. D' values that were >0.5 were considered to indicate positive LD between SNPs.

17.6. Analysis of Family Data and the TDT test

The TDT was conducted in order to control for any possible population stratification or other environmental factors that may have affected the population based case-control association study that was performed previously. Spielman *et al.* (1993) introduced this test and it tests for linkage where an association already exists. It tests for the unequal transmission of alleles from heterozygous parents to affected offspring and does not produce a spurious association due to population stratification (Ewens and Spielman, 1995). The program TRANSMIT 2.5.2 (Clayton, 1999) was used in this study and it compares the observed and expected rates of transmission of marker alleles from parents to offspring. This programme is available at www.hgmp.mrc.ac.uk.

CHAPTER 4

TRANSMISSION DISEQUILIBRIUM TEST

Obstacles are those frightful things you see when you take your eyes off your goal.

Henry Ford

1. INTRODUCTION

Previously a single nucleotide polymorphism (SNP) within the amino terminal region of the SP-D gene (at amino acid residue 11) was found, in a case-control association study, to be associated with susceptibility to TB in a South African Coloured population (p = 0.008, T. Lundwall-Roos, unpublished data). This SNP resulted in an amino acid transition from a threonine (C allele) to a methionine (T allele), and in our population we found that the T allele was associated with susceptibility to TB. Population based case-control association studies can produce false positive results due to population stratification and environmental factors. Spielman *et al.* (1993) devised a family-based statistical test for linkage called the transmission disequilibrium test (TDT) that focuses on linkage between a specific marker and disease allele (Spielman et al., 1993; Spielman and Ewens, 1996). Originally the TDT was intended to test for linkage with a marker located near a candidate gene, in cases where disease association had already been detected between the specific marker and the disease status, however, if there is no prior evidence of association the TDT test is still a valid test for linkage. The TDT therefore measures linkage in the presence of association (Spielman et al., 1993; Spielman and Ewens, 1998). The test uses parental data, not unrelated controls, and therefore eliminates population stratification as an explanation for association (Ewens and Spielman, 1995). Families used in a TDT study can be simplex families (affected child and two parents) or multiplex families (having two parents and multiple affected children). The affected status of the parents does not influence the TDT analysis. There are few limitations to this test as there are many extensions to the test allowing for the evaluation of multiple alleles and multiple linked loci. The TRANSMIT programme version 2.5 (www.hgmp.mrc.ac.uk) was used in this analysis and is able to analyse incomplete families i.e. missing parental information can be assessed (Clayton, 1999).

In this study 135 families from the Ravensmead and Uitsig areas of Cape Town, Western Cape, South Africa were used. The families used in this analysis were both simplex and multiplex families; however in some families, genotype data was only available from one parent. In these cases unaffected siblings of the affected child were genotyped in order for the programme to estimate a parental genotype, for the missing parent(s).

2. RESULTS

Family members were genotyped with respect to the SP-D11 SNP as described in **Chapter 3, section 4**. The TRANSMIT programme was used to determine whether a particular allele was preferentially transmitted to affected children. **Table 4.1** shows the results obtained for four different scenarios. Two analyses were conducted in each case: the first using the robust estimator which includes all families, and the second using only one affected sibling randomly selected by TRANSMIT.

In the first analysis all families were used, i.e. families having both parents, families with only one parent as well as related families. Related families usually consisted of the affected child, or a sibling, who had subsequently become a parent of another affected child. In other cases, members of different families were half-sibs of each other. No association was found with the SP-D11 T allele and TB susceptibility using either the robust estimator or when there was only one affected sibling per family selected. In the second scenario, related families were excluded and again there was no association found between a specific allele and TB susceptibility. In the third and fourth scenarios we wished to determine whether the results varied between families having both parents available and families with only one parent available. In both scenarios related families were excluded. When families having two parents were analysed and only one affected sibling was used there was a borderline significant excess in transmission of the T allele to affected individuals (p = 0.047). In the fourth scenario families that had only one parent were analysed and related families were again excluded as mentioned above. The results show that there were fewer T alleles transmitted to affected siblings (p = 0.033 using the robust estimator). When the analysis was repeated using only one affected sibling there were again fewer transmissions of the T allele to affected siblings than expected (p-value = 0.038). This contradicts the results in the third scenario and could be an indication the there is not sufficient statistical power to detect the expected association.

TABLE 4.1: TDT association of SP-D11 with TB.

	Affected sibs used (n)	Allele	Observed	Expected	Chi square	p-value
1. All Families						
All siblings	214	C	180	171	2.00	0.157
		T	248	257		
One per family	135	C	111	110	0.02	0.890
		T	159	159		
2. Related families excluded*						
All siblings	172	C	147	139	1.56	0.212
		T	197	205		
One per family	114	C	93	92	0.08	0.777
		T	135	136		
3. Families having two parents, related families excluded*						
All siblings	82	C	64	65	0.05	0.828
		T	100	99		
One per family	60	C	40	48	3.95	0.047
		T	80	73		
4. Families having only one parent, related families excluded*						
All siblings	102	C	99	90	4.57	0.033
		T	105	114		
One per family	60	C	60	53	4.31	0.038
		T	60	67		

* In certain families, a member was related to a member of another family, either as a half sibling, or where a sibling was a parent in another family. These families were designated related families.

3. DISCUSSION

The results of this study show that in the first two scenarios neither the C nor T allele was observed at a level that was significantly different to what was expected, implying that SP-D may not be associated with susceptibility to TB in the South African Coloured population. The fact that a non-significant result was obtained does not necessarily mean that the result of the case-control association study was due to population stratification as a TDT test does not have the same statistical power as a case-control association study (Morton and Collins, 1998). In scenario three the T allele appeared to be preferentially transmitted to affected siblings supporting the case-control association study in which the T allele was found associated with susceptibility to TB. The association in the TDT was found only in families with two parents and related families excluded (scenario three in **Table 4.1**). This result contrasts with that of scenario four in which there were fewer T alleles transmitted to affected siblings than expected. Out of the four scenarios the analysis that one would expect to give the most accurate result would be that of scenario three where related families are excluded, and families having two parents available for genotyping are used, i.e. TRANSMIT is not relied upon to estimate the genotype of the missing parent. This is the TDT sample that also produces a result which agreed with the previous case-control association test, when one child per family was chosen randomly by the TRANSMIT programme.

The different result obtained in scenario four in **Table 4.1** could indicate that the study did not have the power to detect the association previously observed in the case-control association study, and possibly if a larger number of families were investigated the association would be detected. It would be advisable to repeat the case-control association study using a new sample set to determine whether the first association detected was due to chance or was a real association. However it is always possible that the case-control association study was a false positive result and there is no association between the T allele and disease.

In a case-control association study in a Mexican population, Floros *et. al.* (2000) found that the C allele was associated with TB, but only when the active TB cases (n = 107) were compared with skin-test positive controls (n = 71). When the same cases were compared with general controls

i.e. a group of healthy individuals whose skin-test status was unknown (n = 101), no association was detected (Floros et al., 2000). Due to the numbers involved, the above association studies had less power than ours.

All the above confounding results indicate that SP-D is likely to be involved in susceptibility to TB, but the dissection of the actual polymorphism involved is complex, and may be different in populations with different histories.

CHAPTER 5

SEQUENCING OF THE AMINO TERMINAL, NECK AND

PROXIMAL PROMOTER REGIONS OF THE SP-D GENE

Not everything that can be counted counts, and not everything that counts can be counted.

Albert Einstein

1. INTRODUCTION

A single nucleotide polymorphism (SNP) within the amino terminal region of SP-D involving a threonine → methionine amino acid change has been associated with susceptibility to TB as well as with susceptibility to respiratory syncitial virus (RSV) (Floros et al., 2000; Lahti et al., 2002). This polymorphism was also associated with TB in the South African Coloured population of the Western Cape (T. Lundwall-Roos, unpublished data). However, the allele over represented in the TB population was methionine in the Western Cape, but threonine in the Mexican population (Floros et al., 2000). It is possible for associations to differ between populations, with different genetic history. It may also be that this polymorphism is in linkage disequilibrium (LD) with the causative polymorphism.

Nine SNPs in total have been reported in the SP-D gene, including the SNP at amino acid 11. There are two other reported SNPs in the amino terminal region of the SP-D gene, located at amino acid positions 25 and 26, neither involving an amino acid change. Two SNPs are located in the collagen-like region, one at amino acid residue 160 in exon 4 involving an alanine → threonine amino acid change, and one in exon 2, which does not involve an amino acid change. The SNP at amino acid residue 160 in exon 4 was studied by Floros *et al.* (2000) as well as by our group and was not associated with susceptibility to TB in either population (Floros et al., 2000). There are four other SNPs present in the carbohydrate recognition domain (CRD). Lahti *et al.* (2002) identified a SNP at amino acid position 270 involving a serine → threonine amino acid change, but this was not associated with susceptibility to RSV (Lahti et al., 2002). This SNP has not been investigated in a study involving pulmonary TB. No SNPs have been reported in the neck region or the promoter of SP-D and since only three SNPs have been identified in the amino terminal region, which is important in the overall assembly of the SP-D molecule, we investigated whether there were any novel SNPs within these regions of the gene.

The regions of SP-D that were selected were amplified using standard PCR, purified and sequenced (**Chapter 3, section 5**). The sequence data obtained was analysed using BLAST on the NCBI website (www.ncbi.nlm.nih.gov) in order to determine whether there were any novel polymorphisms within our population.

2. RESULTS

2.1. Sequencing of the Amino Terminal Region

The whole of the first translated exon (exon 1) that encodes the amino terminal region of the SP-D gene was sequenced. Samples from a total of 26 individuals were sequenced (13 TB patients and 13 controls) and no novel polymorphisms were identified in our population. In the SNP database on the NCBI web site three SNPs have been reported; one being the SNP at amino acid position 11 (SP-D11) which has been previously investigated and found to be associated with TB in our population. There are two other SNPs described at amino acid positions 25 and 26. Neither involves an amino acid change and were not detected in our population.

2.2. Sequencing of the Neck Region

The entire neck region (exon 7) of the SP-D gene (351 bp) was sequenced. A total of 23 samples (10 TB patients and 13 controls) were sequenced and revealed no novel polymorphisms. It appears that this region is highly conserved, as there are no SNPs reported in the SNP database. The neck region of SP-D is vital in the trimerisation of the CRD, and a polymorphism in this region could cause inefficient or lack of trimerisation of SP-D monomers into trimers and subsequently dodecamers.

2.3. Sequencing of the Proximal Promoter Region

A 421bp fragment located upstream of the first untranslated exon, the untranslated exon (39bp) and the first 130bp of intron 1 were sequenced in a total of 12 samples (6 TB patients and 6 controls) in order to detect any novel polymorphisms. This fragment included the AP-1 site, TATA Box and E Box motifs, as well as 2 glucocorticoid response element (GRE ½) sites and 4 CCAAT/enhancer-binding protein (C/EBP) binding elements (Brown-Augsburger et al., 1996; He et al., 2000; He and Crouch, 2002). A reported SNP present in the first intron was not polymorphic in our population. From the sequences obtained no novel polymorphisms were identified.

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3. DISCUSSION

In this investigation the amino terminal, neck and proximal promoter regions of SP-D were sequenced to identify novel polymorphisms within our population. It was decided to perform direct sequencing in order to discover the actual polymorphism in the first pass screening. These regions all play important roles in the molecular assembly and function of the SP-D molecule.

The amino terminal region was investigated due to its importance in the overall assembly of SP-D (See **Chapter 1, section 1.8**) and because Floros *et. al.* (2000) as well as our group (T Lundwall-Roos, unpublished data) found a SNP within this region to be associated with susceptibility to TB. However on further investigation we were unable to identify any new polymorphisms. Similarly, no novel polymorphisms were found in the neck region.

In the present study only the proximal promoter region that included the first untranslated exon was investigated. No polymorphisms within the promoter region have been reported in the literature and we were not able to identify any in this investigation. A large portion of the promoter was not sequenced and it is not known whether there are enhancer elements further upstream that could have an effect on the transcription of SP-D. Promoter polymorphisms have been associated with numerous diseases and have also been linked to phenotypes. Yamada *et al.* (2001) investigated a SNP in the promoter region of interleukin 3 (IL-3) and showed a significant association (p = 0.002) with susceptibility to rheumatoid arthritis (RA) (Yamada et al., 2001). Heesen *et al.* (2003) investigated two polymorphisms within the tumor necrosis factor alpha (TNF-α) promoter region to determine whether they were in LD and if either had an influence on the TNF-α response. This group demonstrated that the two polymorphisms were in LD and that heterozygosity for both was associated with an increased TNF-α response (Heesen et al., 2003). These and other results (Minagawa et al., 2002; Nakayama et al., 2000) show that promoter polymorphisms do play important roles in diseases however not all promoter polymorphisms influence disease outcomes or associations (Spurdle et al., 2000; Khoo et al., 2004). For these reasons it is important to study the promoter in more depth to gain further insights into the regulation of SP-D secretion.

One of the reasons for no polymorphisms being detected is probably the small sample size used in this study. Only 26 (13 TB patients and 13 controls) and 23 (10 TB patients and 13 controls) individuals were screened for the amino terminal and neck regions respectively, and we would therefore expect only alleles with a frequency of at least 7% to be detected. Similarly in the case of the proximal promoter region (6 TB patients and 6 controls) only alleles having a frequency of at least 16% would be detected. Therefore this was not a conclusive study and there is a possibility that a rare allele within the TB cases may not have been detected.

Neither the collagen-like region nor the CRD were sequenced. A polymorphism in the collagen-like region of the SP-D gene could disrupt the Gly-X-Y repeat of this region leading to an interruption of the cruciform structure of SP-D. It is possible that if the collagen-like region is influenced by an amino acid changing polymorphism it could lead to incorrect assembly of the protein and even interfere with SP-D secretion thereby possibly altering the host defence properties. The CRD needs to be investigated further because of its role in pathogen binding. A polymorphism within this region that disrupts the binding sites of the pathogens could result in an individual with heightened susceptibility to disease.

The intronic regions were not investigated. Polymorphisms in the intronic regions of genes could alter splice sites as well as protein translation and therefore these regions should not be overlooked.

Once polymorphisms have been identified and associated with a certain disease it is important to investigate the SNP further in order to determine whether it has an impact on the overall structure and function of the protein such as with the SP-D11 polymorphism that has been associated with TB in two populations and associated with susceptibility to RSV (Floros et al., 2000; Lahti et al., 2002) (T. Lundwall-Roos, unpublished data). It is not known whether this SNP has an influence on the overall structure of the SP-D molecule and should be investigated further.

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CHAPTER 6

THE IMPACT OF SP-D11 ON THE OVERALL STRUCTURE

AND SERUM LEVELS OF SP-D

Science is organized knowledge. Wisdom is organized life.

Immanuel Kant (1724 - 1804)

1. INTRODUCTION

The impact of polymorphisms on the overall structure of proteins is important to confirm especially when the polymorphism has been associated with disease susceptibility. The SP-D11 polymorphism discussed in **Chapters 4 and 5** may affect the overall structure of the SP-D molecule because of its position within the amino terminal region of the gene. There is no report in the literature linking a polymorphism in the SP-D gene to SP-D structure or protein concentration. In this investigation we tried to determine whether SP-D11 had an impact on the overall structure of the SP-D molecule and whether it affected serum levels of SP-D.

2. RESULTS

2.1. Impact of SP-D11 on the structure of SP-D

A sandwich ELISA was used to detect differences in the molecular forms of SP-D within the different genotypic categories of the polymorphism at SP-D11 (See **Chapter 3 section 7**). Serum was purified through the Superose 6 HR 10/30 column and the fractions were used to detect the presence of SP-D (See **Chapter 3 section 6.2**). **Figures 6.1 – 6.3** show the results for the three genotypic groups. Each line on the graphs represents a different individual. The results show that there are a number of different molecular forms of SP-D present in the serum of all individuals. It was expected that the SP-D astral bodies would be eluted first, approximately within the first 10 fractions, thereafter the dodecamers would follow up to approximately fraction 20. Up to about fraction 30 one would expect the trimeric SP-D to be eluted and then the monomeric SP-D. However, no clear consistency could be seen between the column profiles of serum from individuals with the same genotype.

Comparison of profiles for CC genotypes
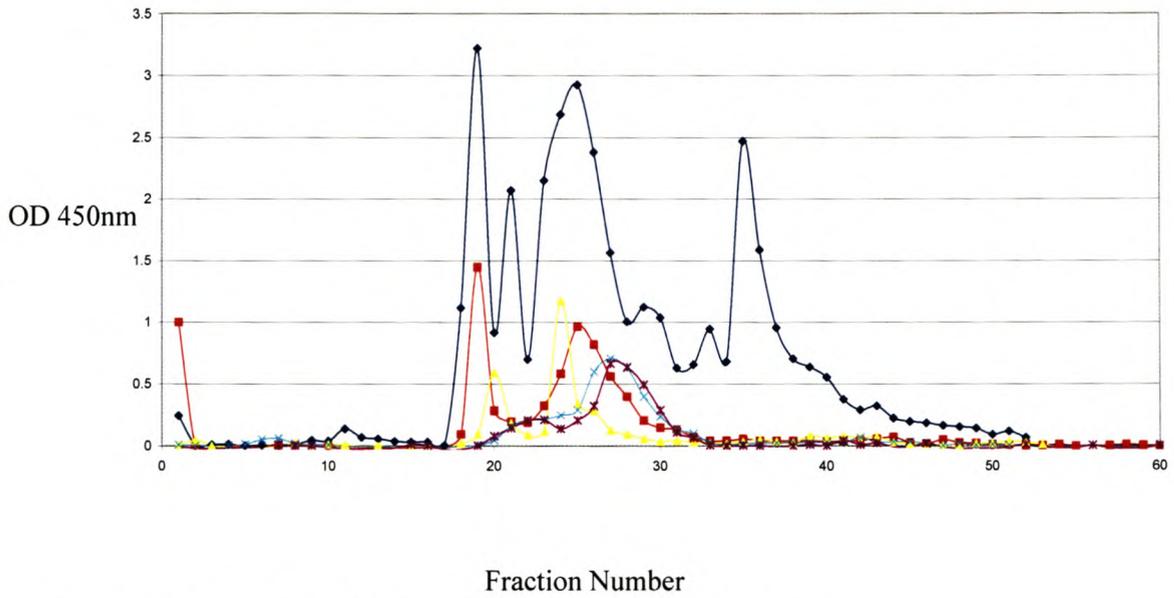
FIGURE 6.1: Profiles of individuals having a CC genotype (n = 5).

Comparison of profiles for CT genotypes
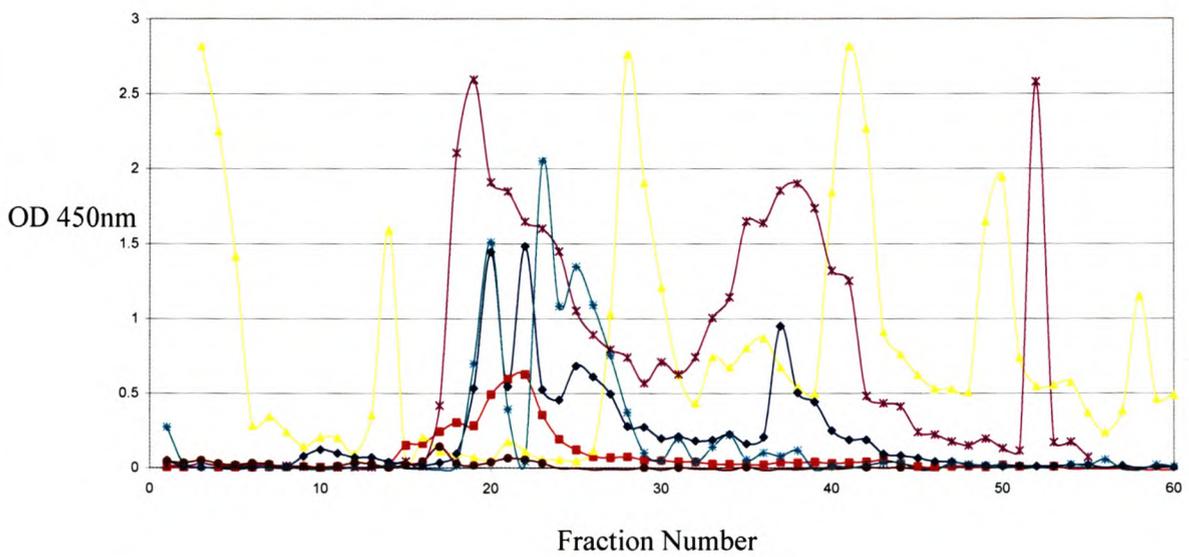
FIGURE 6.2: Profiles of individuals having a CT genotype (n = 7).

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FIGURE 6.3: Profiles of individuals having a TT genotype (n = 7).
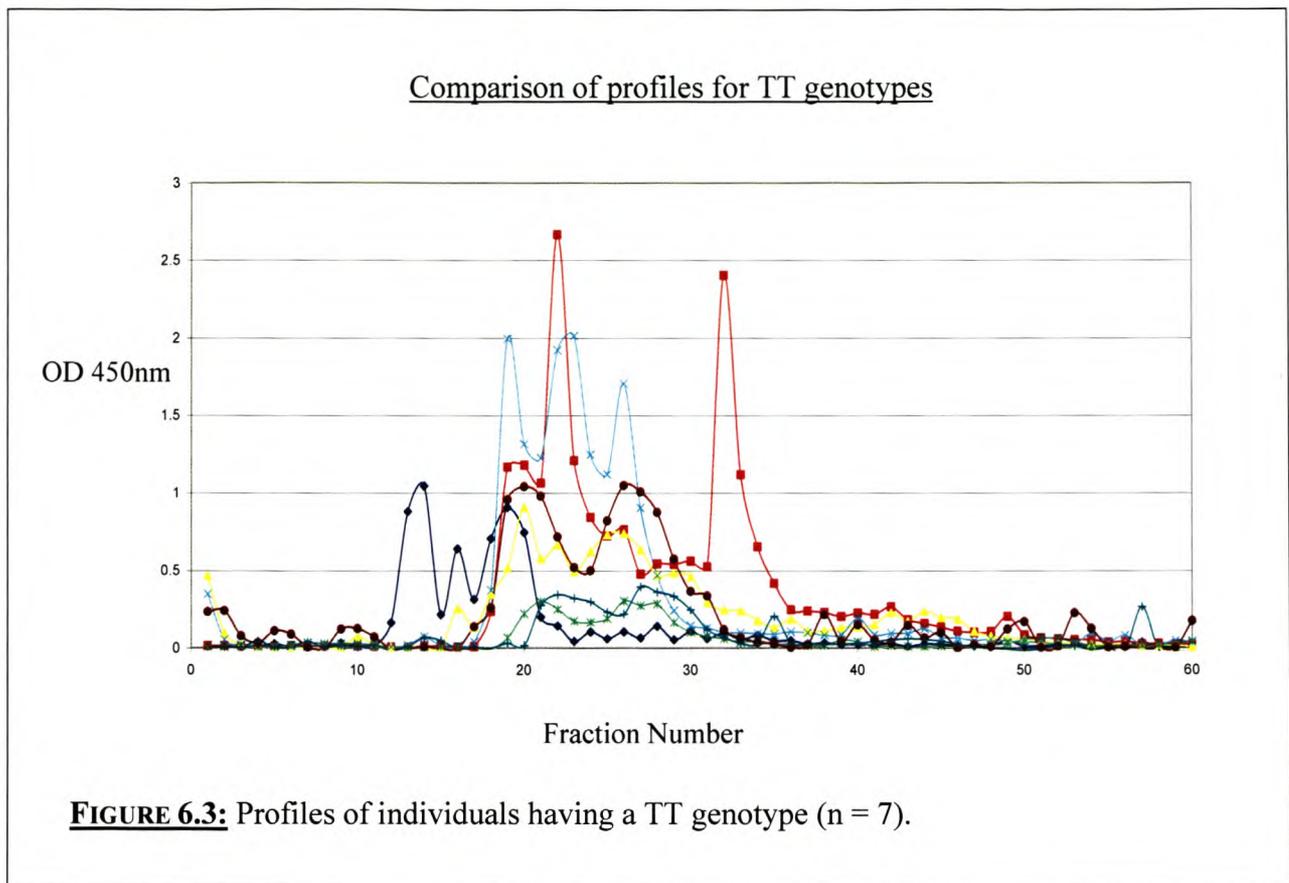
2.2. Measurement of SP-D in serum

The concentration of SP-D in serum was measured using the same sandwich ELISA as above (See **Chapter 3 section 7**). SP-D concentration was measured in active TB patients, confirmed previous TB patients i.e. patients who had recovered from TB and were healthy at the time of sampling, and controls. A total of 115 active TB patients, 32 confirmed previous TB patients and 164 control individuals were analysed (**Figure 6.4**). The results showed that the level of SP-D in the active TB group was significantly higher when compared to either the controls or the previous TB group (p < 0.0001, Mann-Whitney Test). There was no statistical difference between the control group and the previous TB group (p = 0.216, Mann-Whitney Test).
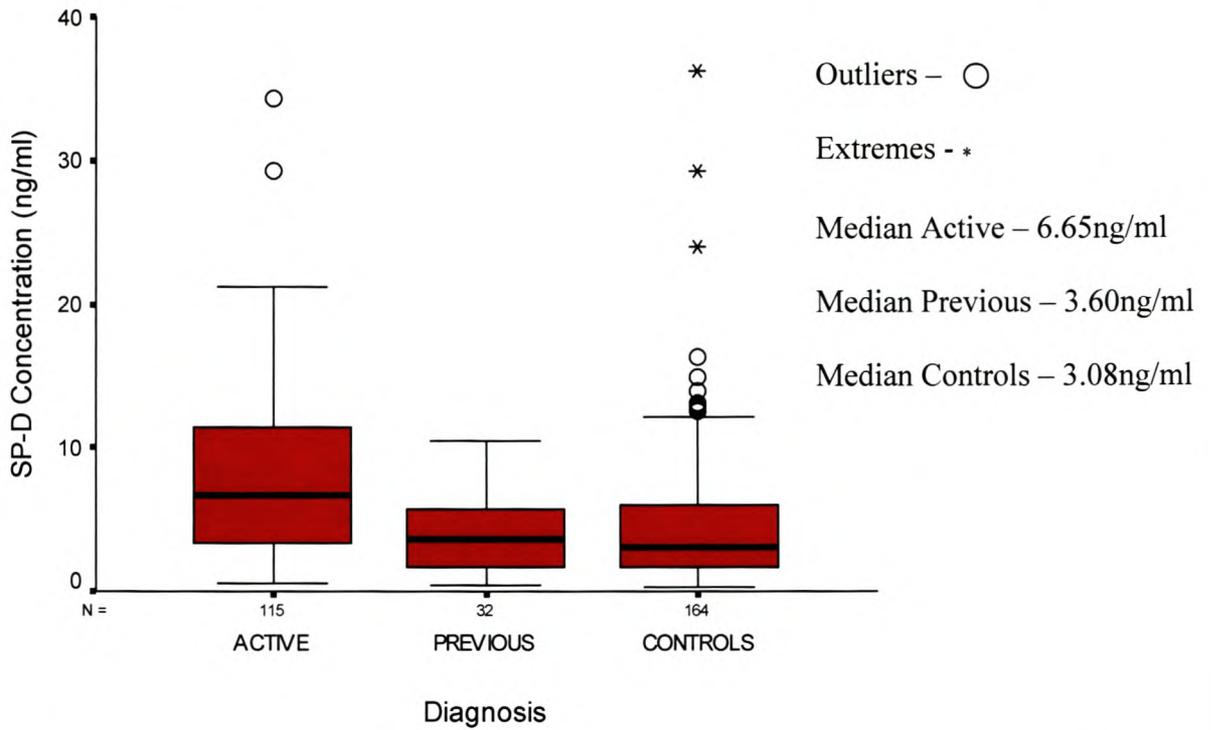
FIGURE 6.4: SP-D concentration (ng/ml) in the different disease states. Active TB Cases; Previous TB Cases and Controls. N is the total number of individuals in each diagnosis category.

These groups were further subdivided according to SP-D11 genotype (**Figure 6.5**). The results showed that within the active TB group, individuals with a TT genotype had significantly higher SP-D serum levels than those with a CC genotype (CC vs TT; $p < 0.0001$, Mann-Whitney Test), while individuals with a CT genotype had intermediate levels. There are no significant differences within the previous TB or control groups with respect to genotype.
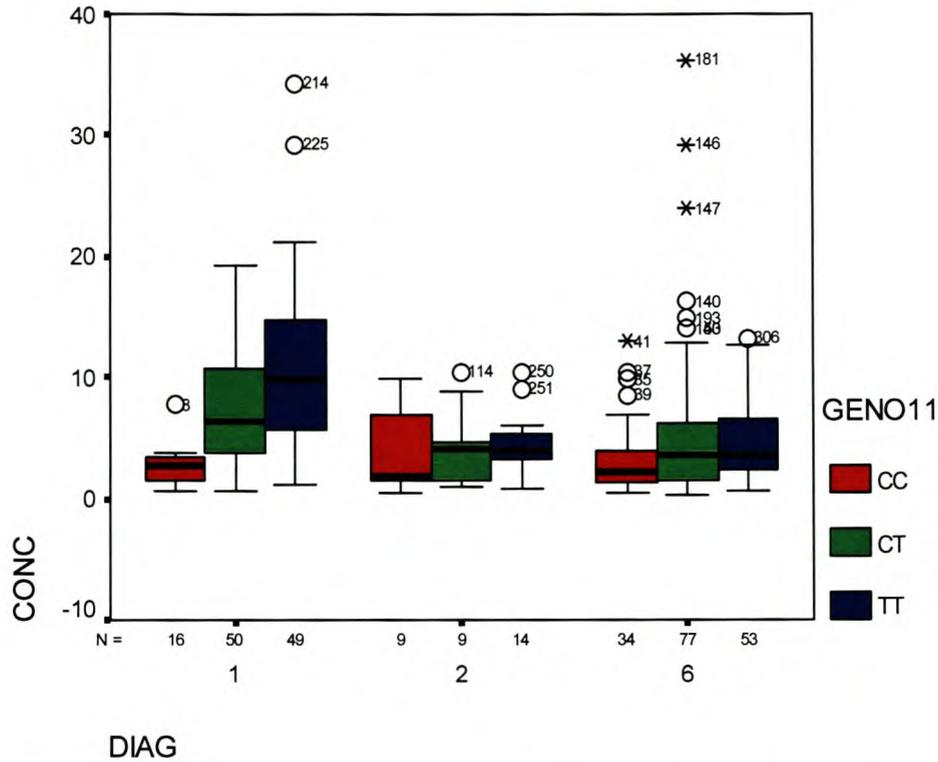
FIGURE 6.5: SP-D concentration (ng/ml) in the different diagnosis groups according to SP-D11 genotype. Concentration (ng/ml) is on the Y axis with Diagnosis on the X axis. Diagnosis 1 – Active TB Cases; Diagnosis 2 – Previous TB Cases and Diagnosis 6 – Controls. N is the number of individuals in each genotypic category.

3. DISCUSSION

The results obtained give no indication whether individuals having a TT genotype produce altered molecular forms of SP-D. We hypothesised that since the SNP is located near two highly conserved cysteine residues that are important in the trimerisation of monomeric SP-D, it may interfere with correct assembly of the trimeric and hence the dodecameric structures of SP-D. The threonine → methionine amino acid change is also a significant one in that threonine is a hydrophilic amino acid and methionine is hydrophobic and the amino terminal region of SP-D is predominantly hydrophilic which may also influence the overall assembly of SP-D. However, serum is a notoriously complex material and it is possible that the column could not efficiently separate the different forms of SP-D and therefore the technology is not

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sensitive enough to detect the different forms of SP-D, an alternate method should be investigated in the future.

Levels of SP-D have been measured in broncho alveolar lavage fluid (BALF) and serum of patients with a variety of pulmonary diseases, including TB. SP-D was found to be decreased in the BALF of patients with idiopathic pulmonary fibrosis (IPF) and interstitial pneumonia with collagen vascular disease (IPCD) compared to controls, however the serum concentration of SP-D was found to be significantly increased in pulmonary alveolar proteinosis (PAP) patients compared to controls (Nagae et al., 1997; Honda et al., 1995; Kuroki et al., 1998). Greene *et al.* (1999) have suggested that a major epithelial injury is required in patients with respiratory distress syndrome (RDS) before the SP-A and D concentrations increase significantly in serum (Greene et al., 1999). These results seem to indicate that serum SP-D concentration increase is due to spillover of the protein into the circulation because of the destruction of the barrier between the alveolar epithelium and endothelium. Thus when this barrier is destroyed surfactant proteins that are produced by alveolar type II cells may leak into the bloodstream and appear at elevated concentrations in the serum of patients with severe lung damage (Honda et al., 1995; Kuroki et al., 1998).

Kondo *et al.* (1998) investigated levels of serum SP-D in patients with TB (Kondo et al., 1998). They found that SP-D levels in patients with active pulmonary TB (smear and/or culture positive) were significantly higher than in the healthy controls ($p<0.05$) and levels of SP-D were significantly higher in patients with cavity formation than in those without ($p<0.05$). There was a significant positive correlation between the serum SP-D level and the number of tubercle bacilli in the sputum ($r = 0.416$, $p = 0.0017$) as well as with the C-reactive protein (CRP) (a measure of the acute phase response) level ($r = 0.383$, $p = 0.003$). They concluded that the level of SP-D in serum might be a useful indicator of the disease activity in pulmonary TB. In our study we confirmed these results by also showing that the levels of SP-D in active TB patients were significantly higher ($p < 0.0001$) than controls.

The acute phase response (APR) is a manifestation of the innate immune response. It is an early set of inflammatory reactions, which are induced by infection or tissue injury, and pattern recognition molecules initiate these responses. The APR results in fever, leukocytosis, changes in vascular permeability, increases in metabolic responses and enhances non-specific

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host defenses (Suffredini et al., 1999; Baumann and Gauldie, 1994). Acute phase protein concentrations increase rapidly after infection and their production is primarily controlled by IL-6 and IL-1 type cytokines and provide enhanced protection against pathogens and are able to modify inflammatory responses by effecting trafficking and mediator release. The concentrations of these acute phase proteins return to normal once the infection has been contained. MBL, a pattern recognition molecule, is involved in modulating the hepatic APR and by binding to microbial cell wall saccharides it activates complement, promotes phagocytosis and modulates CD-14 induced cytokine production. Conglutinin is also recognised as an acute phase protein and has been implicated in various aspects of the systemic response to microbial challenge. In the same way the surfactant proteins are pattern recognition molecules and may be involved in a pulmonary APR as the levels of SP-D are significantly elevated in individuals with active disease and inflammation, yet the levels return approximately to those of the controls once the individuals have been cured.

CRP is an indicator of the APR. It was the first acute phase protein described and is an extremely sensitive systemic marker of inflammation and tissue damage (Pepys and Baltz, 1983). Healthy adults have a median CRP concentration of 0.8mg/l (Shine et al., 1981), but following an acute phase stimulus values of CRP can increase to more than 500mg/l. The CRP status for the individuals investigated in this study was not always known and if they were known exact concentrations of CRP were not measured and only a yes/no status was recorded. It would be worthwhile to investigate as Kondo *et al.* (1998) did whether there is a positive correlation between the serum SP-D level and CRP level (Kondo et al., 1998). Furthermore it would be interesting to determine the extent of lung damage and cavity formation present in individuals with active TB. Since it is thought that surfactant proteins leak into the blood stream due to tissue damage it is possible that individuals with a greater extent of lung damage have a higher concentration of SP-D in their serum. It may be possible to correlate the extent of lung damage with the SP-D11 genotype, which may provide an explanation as to why higher levels of SP-D are observed in patients with a TT genotype and to a lesser extent those with a CT genotype. Patients with active TB and a TT genotype may in fact be more susceptible to lung damage or experience a more severe infection and inflammation leading to severe lung damage and therefore more surfactant proteins are able to leak into the blood circulation.

When we further subdivided the groups of patients and controls according to the SP-D11 genotype we were able to show that the individuals with active TB and a TT genotype had significantly higher serum SP-D levels than the CC genotypes in the same diagnosis category. Individuals with a CT genotype demonstrated intermediate SP-D serum levels. This seems to imply that it is the T allele that plays the most important role in determining the levels of SP-D in the active TB group. It is possible that the T allele prevents formation of higher order protein structures; the smaller SP-D structures may more effectively leak out into the serum when APR sets in. Interestingly, the levels of SP-D seen in the previous TB group and the controls are for the most part the same, with no significant difference between the two groups. In a case-control association study we found that the T allele was associated with susceptibility to TB (T. Lundwall-Roos, unpublished data) and it appears that it is this allele that accounts for the higher levels of SP-D observed in the active TB group.

CHAPTER 7

PURIFICATION OF SP-D FROM BRONCHO ALVEOLAR

LAVAGE FLUID

Having once decided to achieve a certain task, achieve it at all costs of tedium and distaste.

The gain in self-confidence of having accomplished a tiresome labour is immense.

Arnold Bennett

1. INTRODUCTION

The main function of SP-D is thought to be the agglutination of microorganisms, but the degree of specificity or sensitivity involved with different bacterial strains or different SP-D structures is unclear. Jounblat *et al.* (2004) investigated the binding and agglutination of *Streptococcus pneumoniae* (*S. pneumoniae*) by SP-D that had been isolated and purified from broncho alveolar lavage fluid (BALF) (Jounblat et al., 2004). In these experiments different serotypes were incubated with full length SP-D and it was found that SP-D aggregated some serotypes more efficiently than others. Furthermore this group went on to incubate strains of *S. pneumoniae* which had different capsule types but in some cases had the same serotypes. They found that SP-D induced the aggregation of several but not all of the strains and that the extent of aggregation differed. It was observed that pneumococcal strains having the same serotype showed differences in the amount of aggregation (Jounblat et al., 2004).

The lipopolysaccharides of mycobacteria lipoarabinomannan (LAM) and lipomannan (LM) are important in host-pathogen interactions, with LAM being considered a mycobacterial virulence factor. LAMs are thought to play a major role in immunopathogenesis of TB. They regulate cytokine secretion (Sibley et al., 1988; Barnes et al., 1992; Roach et al., 1993; Zhang et al., 1995; Gilleron et al., 1997; Yoshida and Koide, 1997; Nigou et al., 1997; Riedel and Kaufmann, 1997), block the transcriptional activation of interferon gamma (IFN-γ) (Riedel and Kaufmann, 1997) and neutralise potentially cytotoxic oxygen free radicals (Chan et al., 1991). LAMs are envelope molecules which are unique lipoglycans of the *Mycobacterium* genus and are considered major mycobacterial antigens (Chatterjee and Khoo, 1998). LAM is heavily expressed on the surface of *M. tuberculosis* resembling a bacterial capsule (Hunter and Brennan, 1990).

The LAM present on the cell wall of the virulent Erdman strain is heavily mannosylated (manLAM) which is common amongst other pathogenic strains such as *Mycobacterium leprae* and the vaccine strain *Mycobacterium bovis* BCG. Compared to this the attenuated strain $H_{37}Ra$ has arabinofuranosyl-terminated LAM (araLAM) which does not have the mannose caps. It has been shown that these different LAM molecules affect the immune response differently. Moreno *et al.* (1989) showed that LAM is capable of inducing tumor necrosis factor alpha (TNF-α) production and secretion from macrophages (Moreno et al., 1989). TNF-α is rapidly induced in macrophages after exposure to *M. tuberculosis* and

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Kindler *et al.* (1989) showed that the localised production of TNF-α at the sites of *M. bovis* BCG infection was necessary for granuloma formation and subsequent bacterial containment and clearance (Kindler et al., 1989). AraLAM from the attenuated strain H37Ra was able to dramatically increase the induction of TNF-α when compared to the manLAM from the virulent Erdman strain. NF-κB is a critical component in regulating many genes central to immune function including TNF-α. Brown *et al.* (1995) demonstrated that araLAM derived from $H_{37}Ra$ is capable of rapid activation of NF-κB binding activity in bone marrow derived macrophages and murine macrophage-like cell lines, whereas manLAM is less potent at stimulating NF-κB (Brown and Taffet, 1995).

Another interesting comparison between araLAM and manLAM is that the binding of $H_{37}Ra$ (posessing araLAM) to macrophages is mediated through complement receptors, but binding of the virulent Erdman strain (posessing manLAM) to macrophages is mediated by both complement receptors and mannose receptors. It has been speculated that the mode of uptake by macrophages may affect the intracellular survival of the pathogen (Schlesinger, 1993) and is therefore possible that different LAM structures may influence whether the bacteria grow within the macrophage or are killed. Analysis has revealed that the virulent Erdman strain has a significantly faster doubling time than $H_{37}Ra$ or *M. bovis* BCG (North and Izzo, 1993). This suggests that the more mannosylated the LAM is the better the bacterium is able to evade the host defence mechanisms and persist in the macrophage.

SP-D binds to the terminal mannosyl units of *M. tuberculosis* LAM due to the fact that SP-D is able to bind to mannose. Ferguson *et al.* (1999) investigated the binding of SP-D to the avirulent *Mycobacterium smegmatis* (*M. smegmatis*) and to the virulent Erdman strain of *M. tuberculosis* (Ferguson et al., 1999). This group found that SP-D binds minimally to the avirulent *M. smegmatis*. *M. smegmatis* has araLAM which lacks the mannose capping as is found in the avirulent $H_{37}Ra$ strain. AraLAM is approximately 10% capped with inositol phosphate (Khoo et al., 1995). This is believed to be another reason why *M. tuberculosis* LAM stimulates much less TNF-α production by macrophages than araLAM (Chatterjee et al., 1992), and it is the mannose cap that mediates binding of LAM to the mannose receptor (Schlesinger et al., 1994). It is possible that other carbohydrates present on the surface of *M. tuberculosis* may also participate in the binding of SP-D. Free, complex carbohydrates such as

mannan, arabinomannan and glucan have been described on the surface of *M. tuberculosis* (Ortalo-Magne et al., 1995).

The marked differences between SP-D binding to pathogenic and non-pathogenic mycobacteria may indicate that the host immune system has developed a mechanism to distinguish virulent bacteria possessing unique carbohydrates on their surfaces, or conversely pathogenic bacteria evade the immune function of SP-D. Until the exact function of SP-D is known this will remain unclear.

To investigate this further, three strains of *M. tuberculosis* were studied; the laboratory strain $H_{37}Rv$ and two clinical isolates. The first isolate belonged to the Beijing/W-like family and the second was a Family 11 isolate. These two strains of *M. tuberculosis* are highly prevalent within the Western Cape communities of South Africa (Victor et al., 2004; Streicher et al., 2004).

Our hypothesis therefore was that SP-D purified from individuals with different SP-D11 genotypes shows variant forms that results in differential agglutination of mycobacterial strains. In addition, a given SP-D form binds to a greater or lesser extent to different strains of mycobacteria.

2. RESULTS

2.1. SP-D11 Genotyping

Ethical approval was obtained in order to collect BALF samples from consenting volunteers (**Appendix 1 to 3**). A total of nine BALF and blood samples were received. DNA was extracted and genotyped for SP-D11 (See **Chapter 3, section 4** for detailed methods). At least one representative of each SP-D11 genotype (CC, CT, TT) was obtained (**Figure 7.1**).
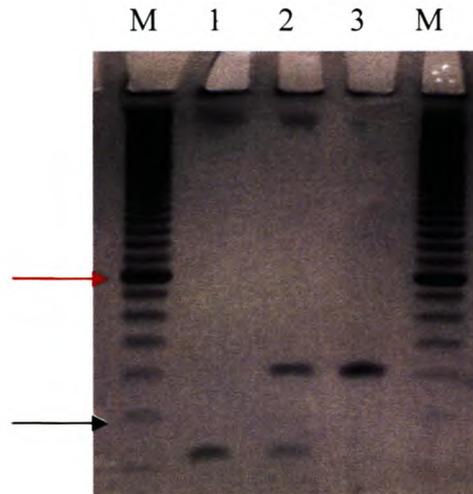
FIGURE 7.1: Restriction digestion products of SP-D11. M corresponds to the 10bp standard ladder (Promega, Madison, WI, USA) that was used to confirm that the correct restriction products were obtained. ← indicates 100bp and ← indicates 50bp band. Lanes 1 – 3 contain DNA from: 1 – TT homozygote (40bp), 2 – CT heterozygote (59bp, 40bp), 3 – CC homozygote (59bp).

2.2. Purification of SP-D from BALF

Out of the nine samples received SP-D was successfully purified from four BALF samples using a maltose-agarose affinity column (See **Chapter 3, section 9** for detailed methods) and used in the agglutination assays. Of these four samples there were two samples that had a CT genotype, one sample with a CC genotype and one sample with a TT genotype. Western blotting was used to confirm that the eluted protein from the affinity column was SP-D (**Figure 7.2**) (See **Chapter 3, section 8** for detailed methods).
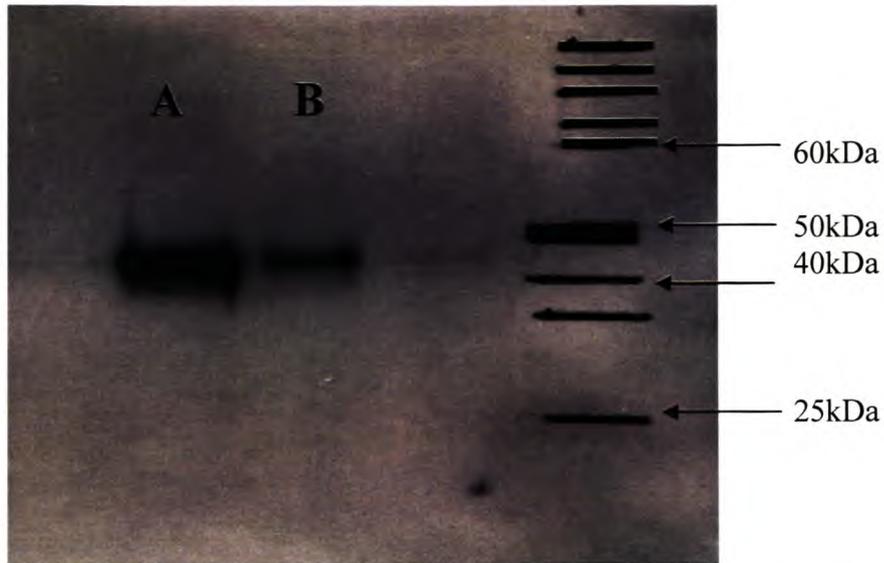
FIGURE 7.2: Autoradiograph of a BALF sample purified by affinity chromatography and detected in a western blot to determine whether the protein purified was SP-D and of the correct size. Lane A and B: Fractions collected by affinity chromatography containing purified SP-D. A PageRuler™ Protein Ladder 200 – 10kDa (MBI Fermentas, Inqaba Biotechnology, Pretoria, Gauteng, RSA), used to determine whether the purified SP-D was of the correct size (approximately 43kDa). The protein ladder was graphically manipulated in order make the bands more visible.

2.3. Agglutination Assay

The agglutination assay was carried out by modifications of the methods described by Ferguson *et al.* (1999) and Jounblat *et al.* (2004) (Ferguson et al., 1999; Jounblat et al., 2004) (See **Chapter 3**, **section 12** for detailed methods). Three mycobacterial strains were studied; H_{37}Rv ATC 27294, Beijing/W-like R433 found in 72 clinics in the Boland/Overberg South Cape/Karoo Region and an isolate from Family 11 SAWC 1925. The number of clumps was counted in at least 20 fields of view using an Olympus BX41 phase contrast microscope. The average area covered by a clump was estimated by estimating the average diameter of each clump by visual observation. GraphPad Prism version 4.01 was used for statistical analysis. Mycobacteria were identified as red/pink rods after Ziehl – Neelsen staining.

2.3.1. $H_{37}Rv$

In the analysis of the median number of clumps in $H_{37}Rv$ control (n = 20 fields of view (FOV), median number of clumps = 4) versus after the addition of SP-D11 of a specific genotype (**Figure 7.3**), there appears to be a significant difference between the groups (p < 0.0001, Kruskal–Wallis Test). Fewer clumps were observed in the CC genotype group (n = 18 FOV, median number of clumps = 1) compared to the control as well as in the CT (n = 40 FOV from two samples, median number of clumps = 3) and TT (n = 19 FOV, median number of clumps = 2) groups. These preliminary results suggest that there is a difference in the sizes of clumps between the different groups (p = 0.0073, Kruskal–Wallis Test). Median size of all clumps was $0.491 \mu m^2$ (**Figure 7.4**). It does not appear that fewer clumps are observed in the CC group because they represent larger clumps. The median size of clumps is in fact larger in the TT and CT genotype groups and the median number is higher. **Figures 7.5** to **7.9** show examples of the clumping observed in the individual samples analysed.

FIGURE 7.3. Number of clumps per field observed in $H_{37}Rv$ that was incubated with $10\mu g/ml$ of SP-D from individuals with SP-D11 genotypes CC, CT or TT or without SP-D (control). The horizontal bar indicates the median number of clumps within each category.

Area of clumps inH_{37}Rv

FIGURE 7.4. Area of clumps (μm^2) observed in H_{37}Rv that was incubated with 10μg/ml of SP-D with SP-D11 genotypes CC, CT or TT or without (control). The horizontal bar indicates the median area of clumps in each category.
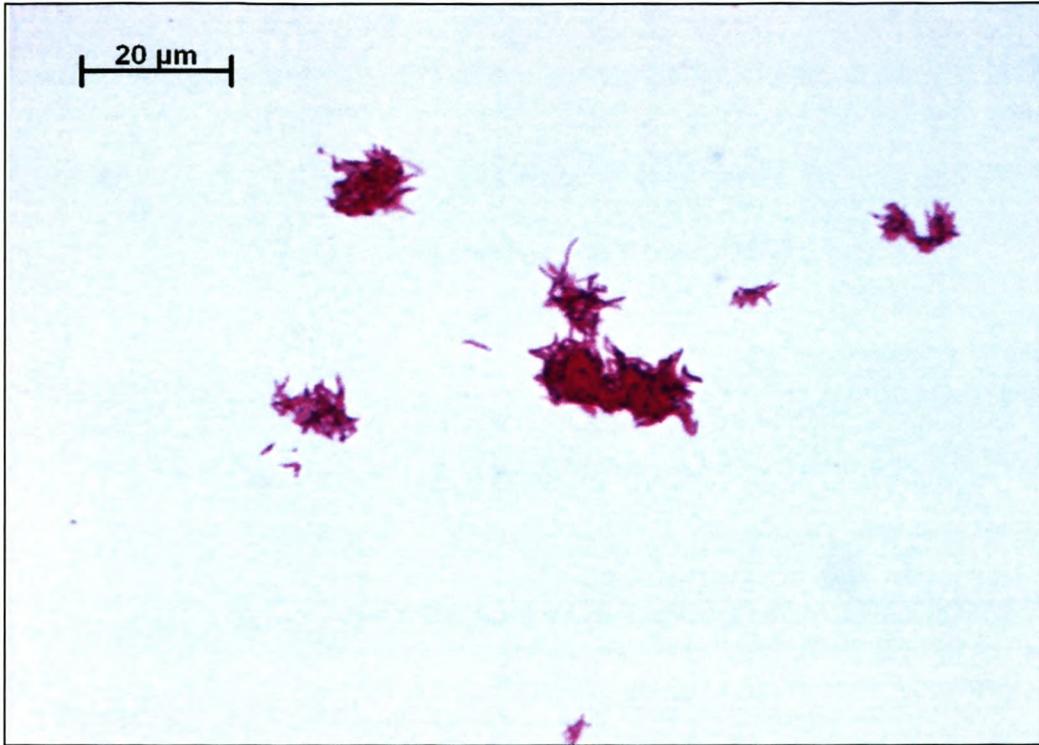
FIGURE 7.5: $H_{37}Rv$ control i.e. no SP-D or any other agglutinating agent was added (1000x, oil immersion).
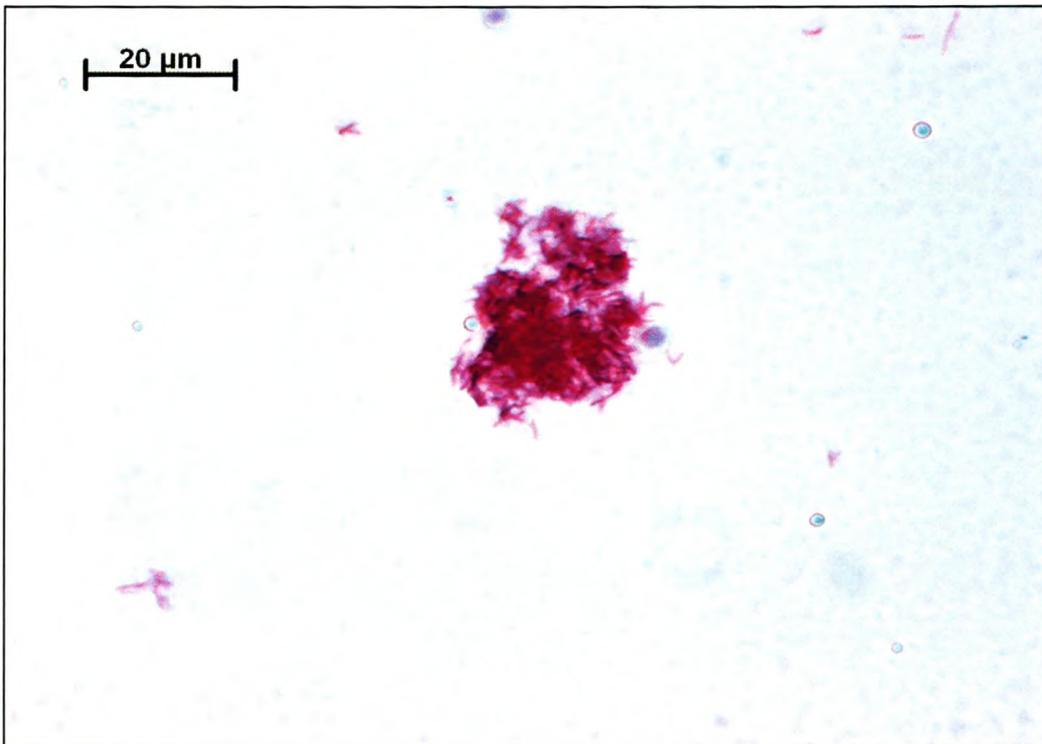
FIGURE 7.6: $H_{37}Rv$ sample 1 agglutinated with SP-D11 CT genotype (1000x, oil immersion).
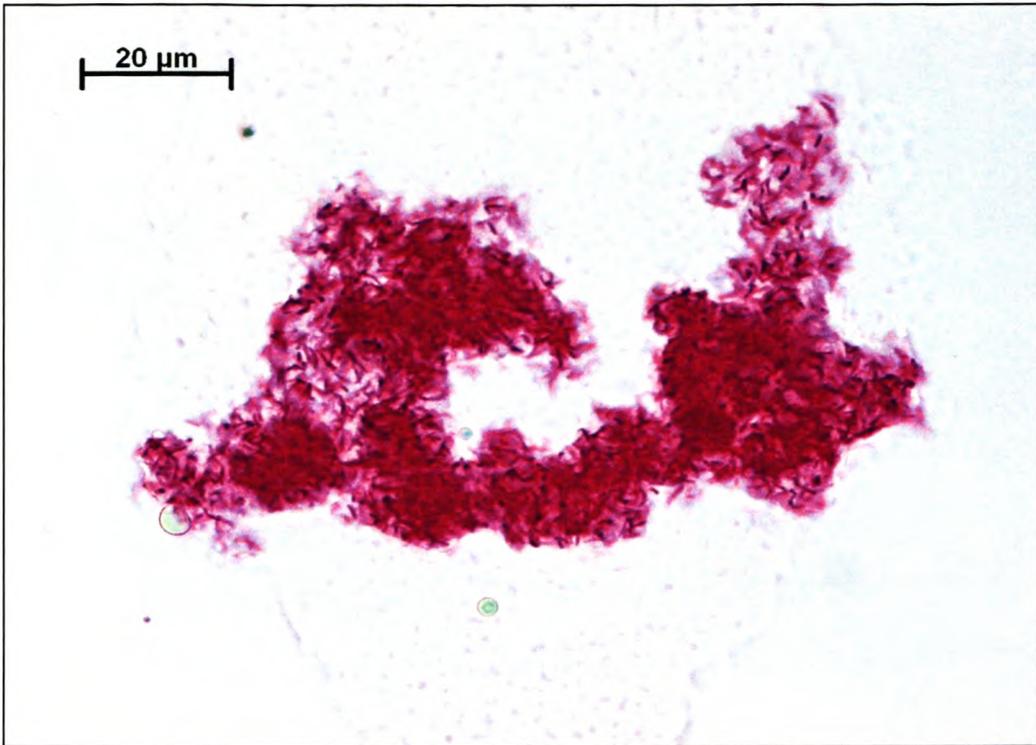
FIGURE 7.7: $H_{37}Rv$ sample 2 agglutinated with SP-D11 CT genotype (1000x, oil immersion).
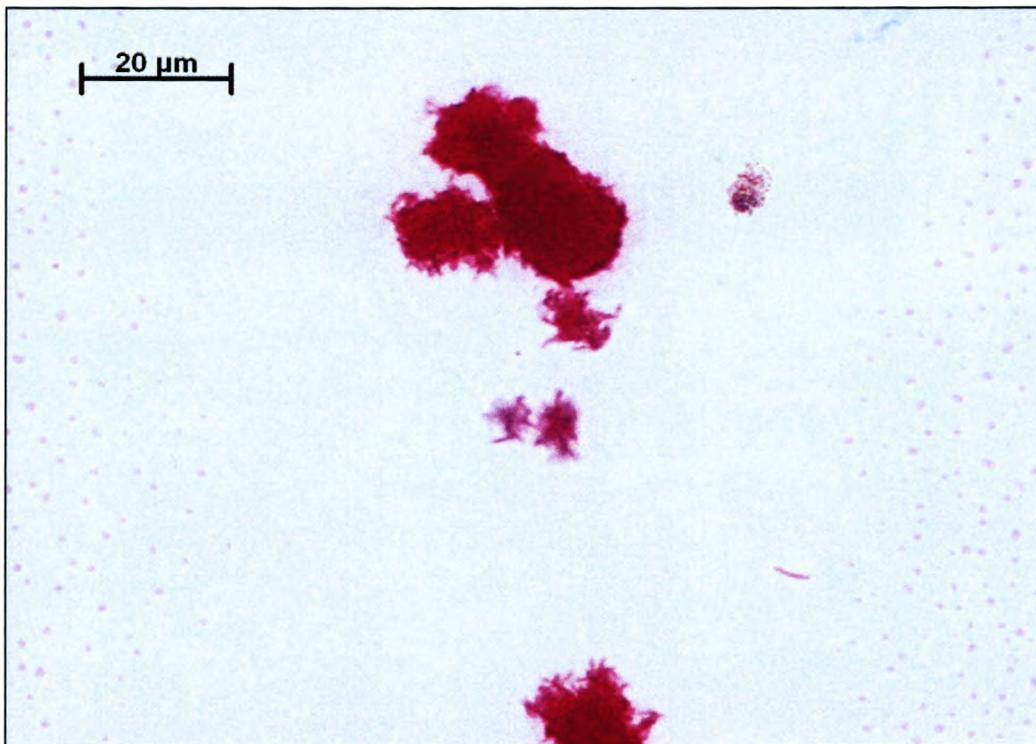
FIGURE 7.8: $H_{37}Rv$ agglutinated with SP-D11 CC genotype (1000x, oil immersion).
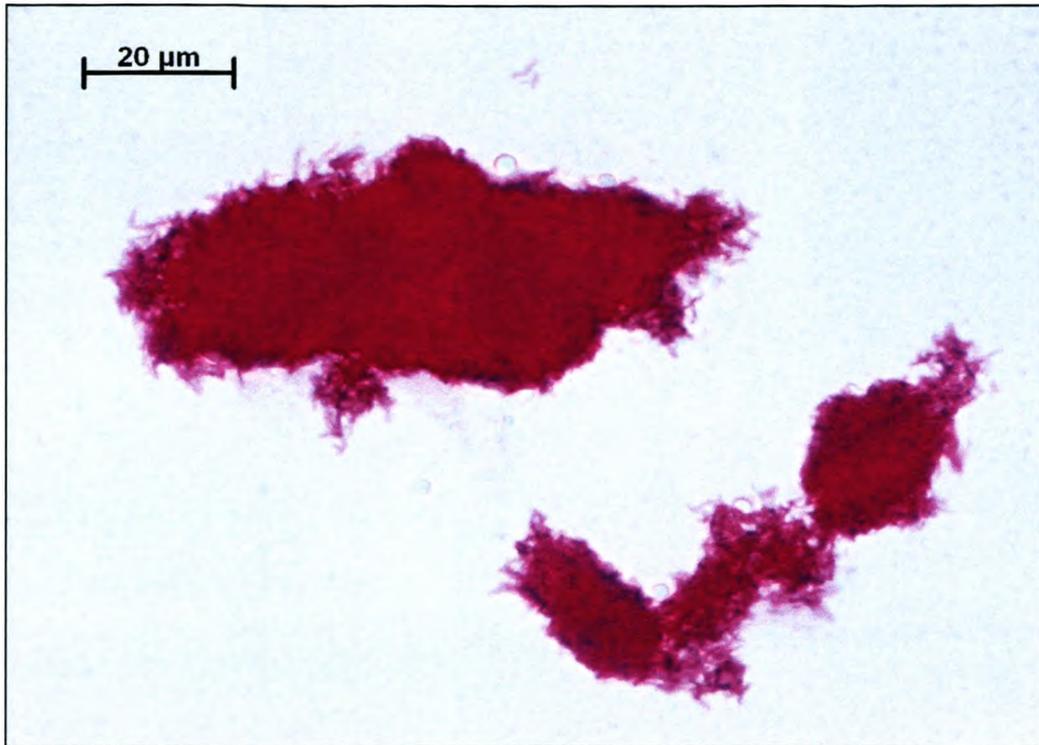
FIGURE 7.9: H$_{37}$Rv agglutinated with SP-D11 TT genotype (1000x, oil immersion).

2.3.2. Beijing/W-like strain

Control Beijing/W-like bacilli appeared dispersed with little clumping evident (**Figure 7.10**), however this did not appear to change once SP-D was added to the bacteria (**Figures 7.11 – 7.14**). In the SP-D treated samples there were a few larger clumps observed compared to the control sample however these clumps were very widely dispersed and one could not conclude that the SP-D treated samples agglutinated the Beijing/W-like strain more efficiently than when SP-D was not added. Overall there was little evidence of clumping in any of the samples.
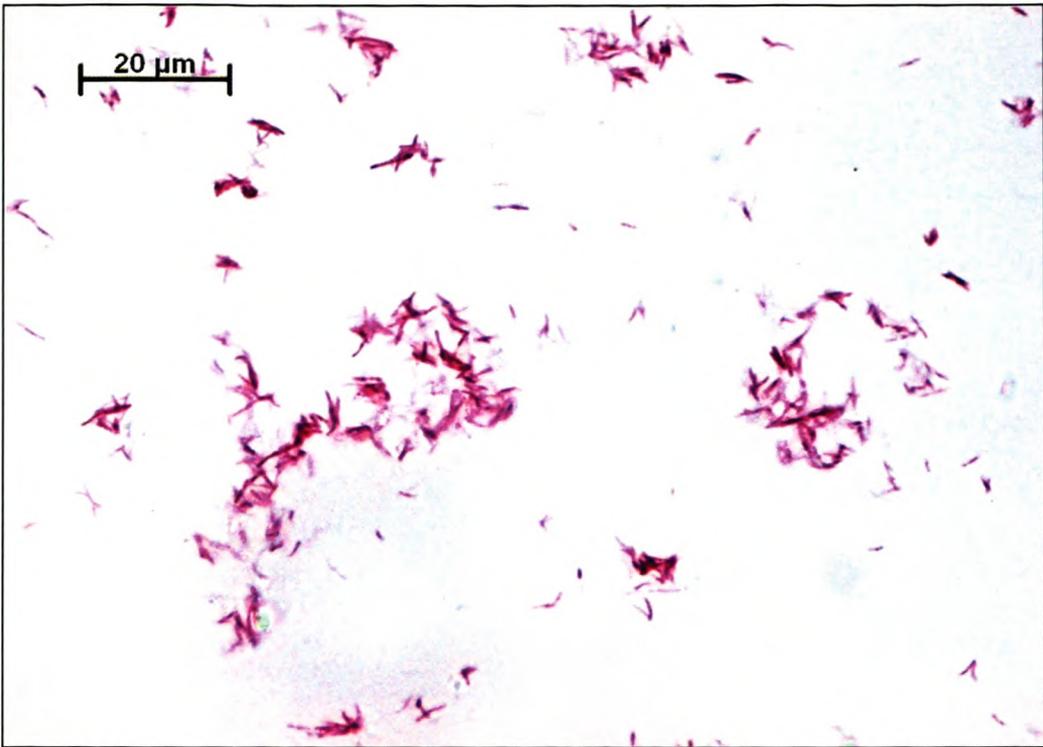
FIGURE 7.10: Beijing/W-like control i.e. no SP-D or any other agglutinating agent was added (1000x, oil immersion).
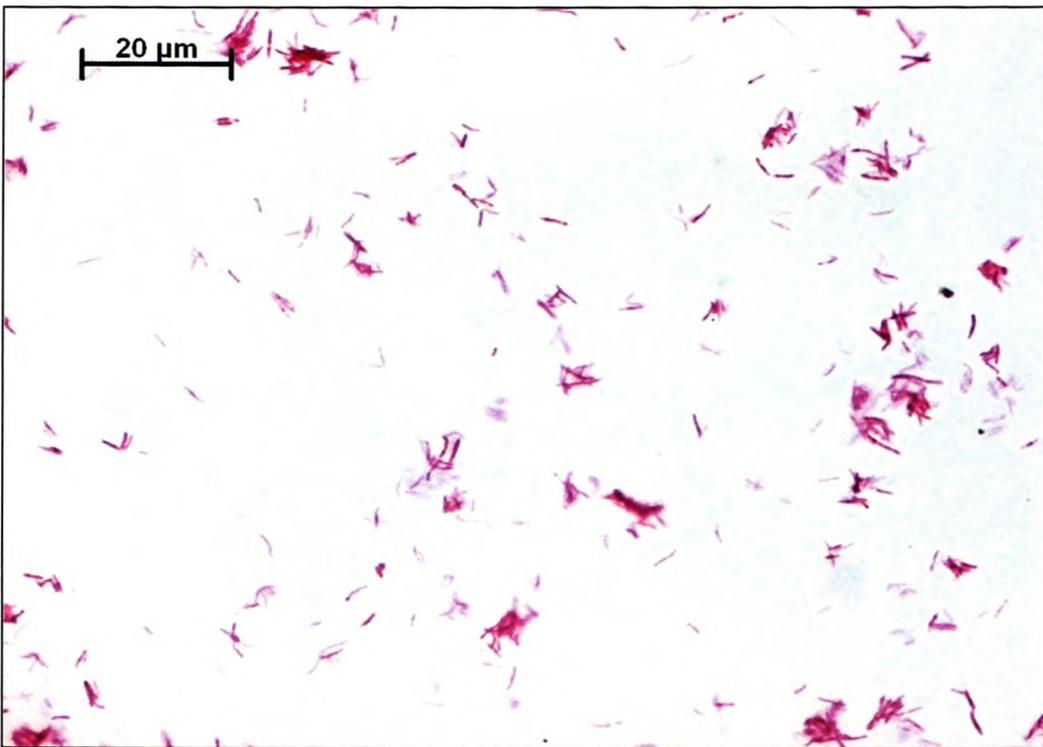
FIGURE 7.11: Beijing/W-like sample 1 agglutinated with SP-D11 CT genotype (1000x, oil immersion).
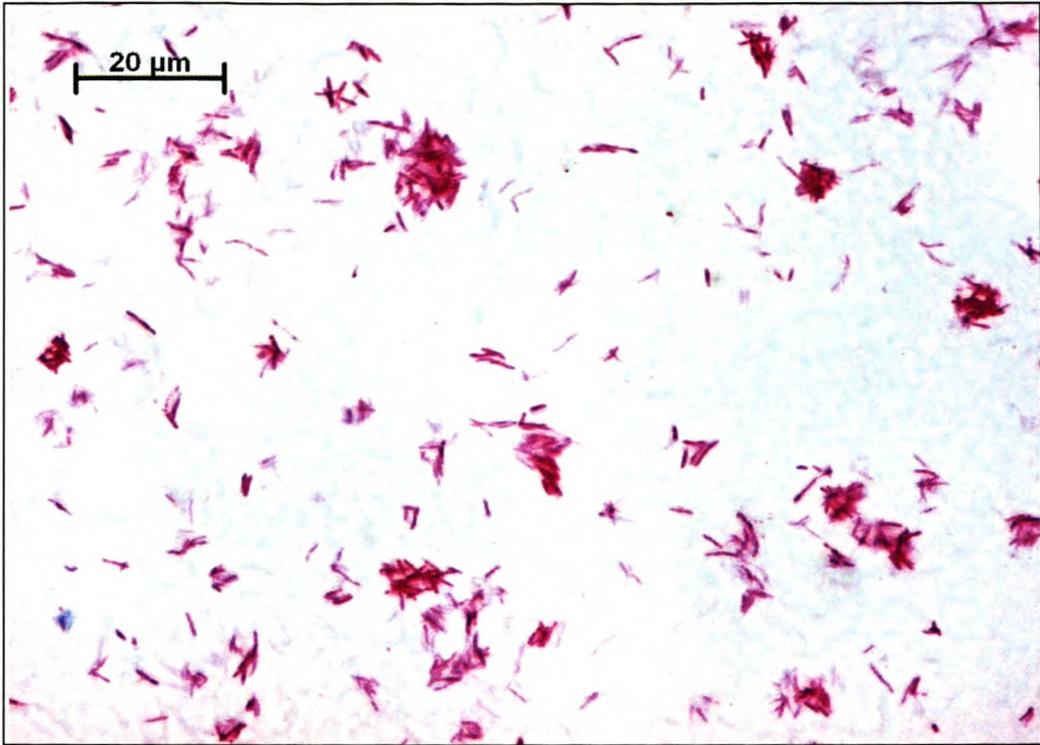
FIGURE 7.12: Beijing/W-like sample 2 agglutinated with SP-D11 CT genotype (1000x, oil immersion).

FIGURE 7.13: Beijing/W-like agglutinated with SP-D11 CC genotype (1000x, oil immersion).
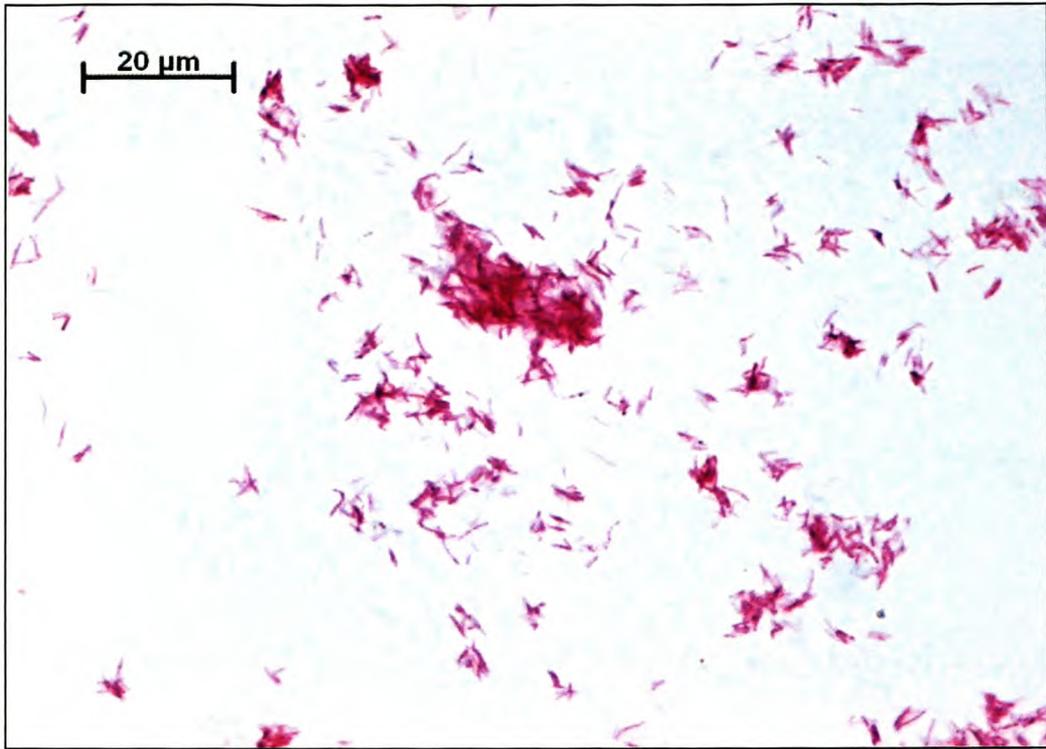
FIGURE 7.14: Beijing/W-like agglutinated with SP-D11 TT genotype (1000x, oil immersion).

2.3.3. Family 11

The slide showing agglutination of the second CT and TT samples was accidentally destroyed before it could be read. More clumps were observed in the control group (n = 20 FOV, median number = 2) compared to either of the experimental groups, CC and CT (n = 20, median number of clumps = 1 and n = 20 FOV, median number of clumps = 1 respectively) (p = 0.0064, Kruskal–Wallis Test) (**Figure 7.15**). This appears to coincide with the fact that the median size of the clumps in the CC (n = 20 FOV, median size of clumps = $1.96\mu m^2$) and CT (n = 20 FOV, median size of clumps = $1.96\mu m^2$) groups were larger than those observed in the control group (n = 20 FOV, median size of clumps = $0.177\mu m^2$) (**Figure 7.16**) (p <0.0001, Kruskal–Wallis Test). **Figures 7.17** to **7.19** show the extent of clumping observed in the individual experiments.

FIGURE 7.15 Number of clumps observed in Family 11 that was incubated with 10μg/ml of SP-D with SP-D11 genotypes CC and CT or without (control). The horizontal bar indicates the median number of clumps in each category.

FIGURE 7.16 Area of clumps (μm^2) observed in Family 11 that was incubated with $10\mu g/ml$ of SP-D with SP-D11 genotypes CC and CT or without (control). The horizontal bar indicates the median area of clumps in each category.

FIGURE 7.17: Family 11 control i.e. no SP-D or any other agglutinating agent was added (1000x, oil immersion).
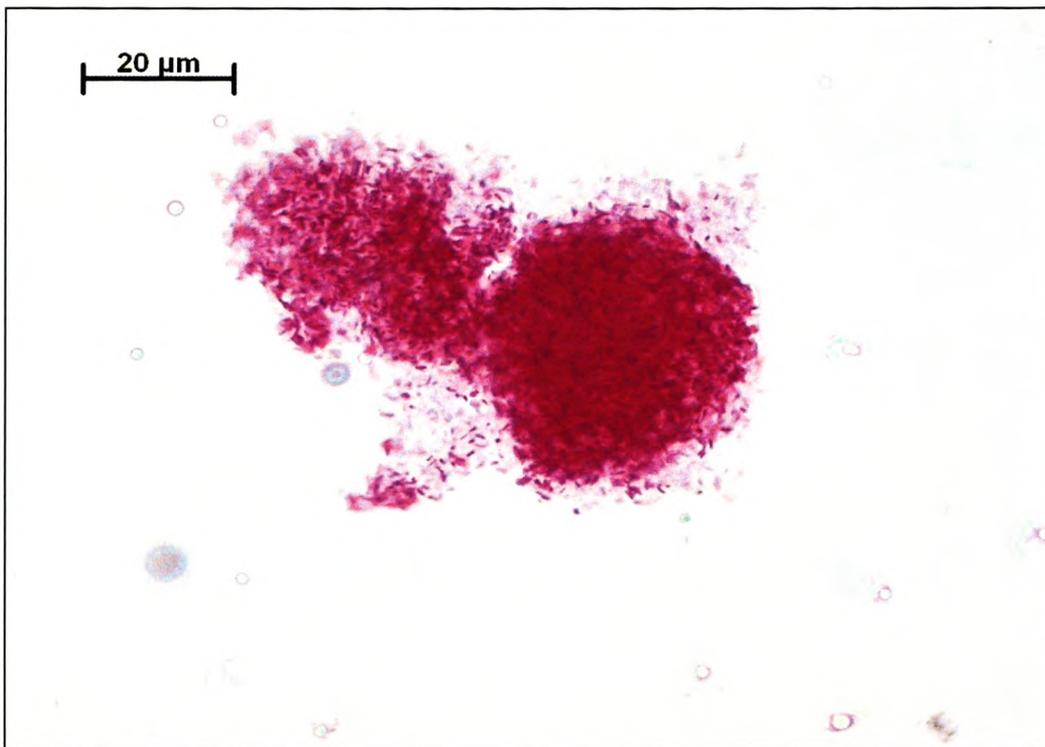
FIGURE 7.18: Family 11 agglutinated with SP-D11 CT genotype (1000x, oil immersion).
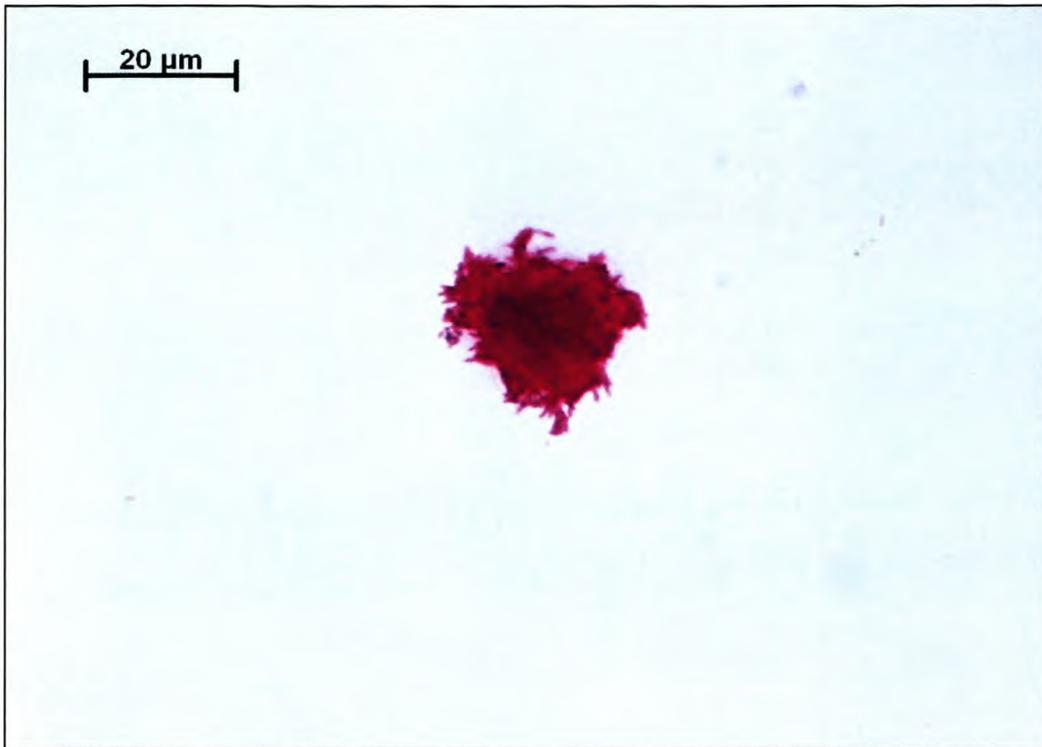
FIGURE 7.19: Family 11 agglutinated with SP-D11 CC genotype (1000x, oil immersion).

3. DISCUSSION

Only four different BAL samples were used in this study comprising at least one sample from each SP-D11 genotype. The preliminary data from this pilot study suggests that SP-D agglutinates mycobacteria differently depending on the strain investigated. The small sample size precludes any definitive conclusions, but the following agglutination characteristics were observed. In general, the Beijing/W-like SAWC1925 strain did not agglutinate notably, while the Family 11 strain from the 72 clinics (Boland/Overberg South Cape/Karoo Region) treated with SP-D of SP-D11 genotypes CC or CT formed about half the number of clumps seen with the untreated control, but these clumps covered an area about five times larger. No difference was seen between SP-D11 genotypes CC and CT as regards agglutination. Similarly, the laboratory strain $H_{37}Rv$ ATC 27294 formed fewer clumps in SP-D11 treated samples than in controls, and these clumps were slightly larger in the CT and TT groups than in the control and CC groups. The CC group produced fewer clumps per field than the CT and TT groups, which in turn produced fewer than the untreated control group. The significance of the

agglutination of mycobacteria to their future uptake and survival is not yet clear, but it is interesting to note that both the Family 11 strain from the 72 clinics (Boland/Overberg South Cape/Karoo Region) and the $H_{37}Rv$ ATC 27294 were agglutinated into fewer, but larger clumps by SP-D. This pattern was also evident in the area covered by agglutinated mycobacteria, **Table 7.1**, where, with the exception of the SP-D11 CC with $H_{37}Rv$, the area of agglutinated mycobacteria was larger with SP-D than in the untreated control mycobacteria.

TABLE 7.1: Total area (μm^2) covered by agglutinated mycobacteria.

	Beijing/W-like R433	F11 SAWC 1925	$H_{37}Rv$ ATC 27294
Control	very low	19.88	39.87
SP-D11 CC	very low	114.49	14.25
SP-D11 CT	very low	117.61	64.24
SP-D11 TT	very low	no result	50.46

Differential patterns of agglutination by SP-D purified from individuals with different SP-D11 genotypes will require larger numbers to confirm and expand the results. Preliminary results indicate that CC individuals may produce smaller and fewer clumps than CT and TT individuals. The reason for the lack of agglutination by any SP-D of the Beijing/W-likeR443 strain is not known.

CHAPTER 8

POLYMORPHISMS WITHIN THE COLLAGEN-LIKE REGION

OF THE SP-A1 AND SP-A2 GENES

The only man who never makes mistakes is the man who never does anything.

Theodore Roosevelt (26th President of The United States)

1. INTRODUCTION

Polymorphisms within the SP-A genes have been investigated in numerous populations in order to determine whether any allelic variants are associated with respiratory diseases such as TB or not. Given that SP-A is important in the lung it is a good candidate gene for investigating susceptibility to a variety of respiratory pathogens such as acute respiratory distress syndrome (ARDS), respiratory distress syndrome (RDS) and TB. Several polymorphic sites have been identified within the SP-A1 and -A2 genes. Certain SNPs within the SP-A genes have been associated with diseases including RDS and chronic obstructive pulmonary disease (COPD) and TB (Kala et al., 1998; Ramet et al., 2000; Guo et al., 2000; Floros et al., 2000). More recently Madan *et al.* (2002) reported novel polymorphisms within the collagen-like region of SP-A1 and -A2 and association with TB in an Indian population (Madan et al., 2002). This group also investigated some previously reported SNPs. Significant associations were found with one SNP in intron B of SP-A1 and with three SNPs in SP-A2, one in intron 3 and two in exon 4, one of which involved an amino acid change. However, this was a very small study and confirmation is required.

The collagen-like region of SP-A has been shown to be involved in receptor binding on macrophages, surfactant regulation and secretion and lipid uptake by type II cells (McCormack et al., 1999). Due to the importance of the collagen-like region and the associations found by Madan *et al.* (2002), we investigated these polymorphisms in a South African population in order to determine firstly whether they were present in our population and secondly whether any of them are associated with susceptibility to or protection against TB. We also wished to determine whether there were any novel polymorphisms in our population.

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2. RESULTS

2.1. SP-A1 and -A2 Whole Gene Product Amplification.

The entire coding sequence of the SP-A1 and -A2 genes were amplified (See **Chapter 3, section 13.1**) and the products electrophoresed in a 1% agarose gel to validate the product (**Figure 8.1**). These products were then purified and a nested PCR reaction was performed to obtain smaller fragments.
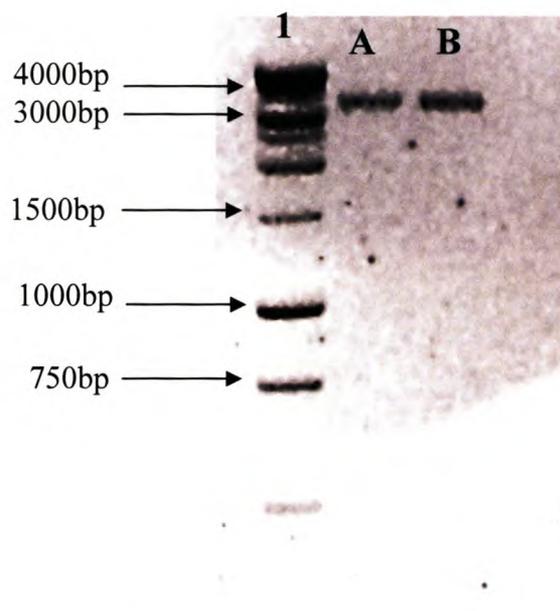
FIGURE 8.1: 1% agarose gel showing SP-A1 and -A2 whole gene products generated in the intial PCR amplification. Lane 1: 1kb DNA Ladder (Promega, Madison, WI), Lane A: SP-A1 whole gene product (3124bp), Lane B: SP-A2 whole gene product (3150bp).

2.2 SP-A1 and -A2 Nested PCR Reactions

The whole gene products that were generated in the first round of amplification were diluted 1:50 with distilled water and used as the templates for a second round of amplification (See **Chapter 3, section 13.2**). The products obtained were electrophoresed in a 1.5% agarose gel in order to determine whether the correct products were amplified (**Figure 8.2**). These

products were judged to be correct and were purified and sent for sequencing. **Table 8.1** shows a summary of the SNPs that were investigated for this work.
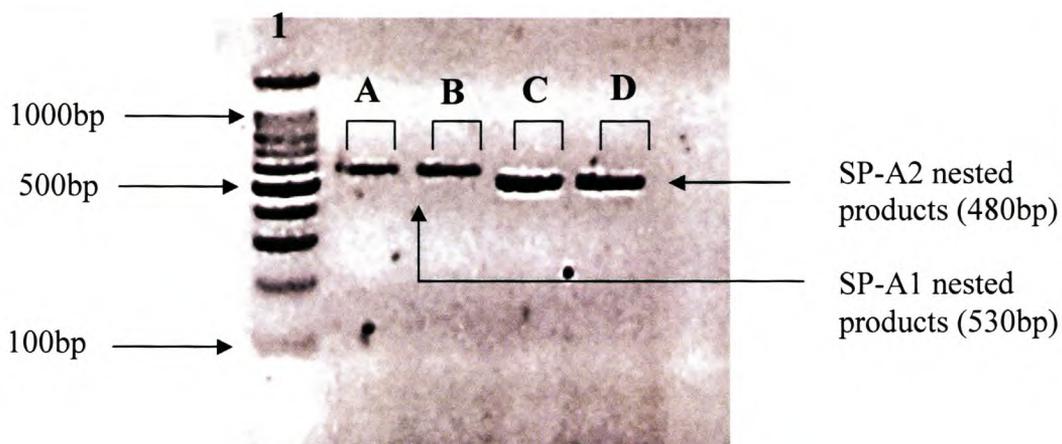
FIGURE 8.2: 1.5% agarose gel showing the SP-A1 and -A2 PCR products obtained in the nested PCR reactions. Lane 1: 100bp DNA Ladder (Promega, Madison, WI), Lanes A and B: SP-A1 nested products from two individuals, Lanes C and D: Nested SP-A2 products of two different individuals.

TABLE 8.1: Summary of the SP-A1 and –A2 SNPs investigated.

Gene Location	Nucleotides		Amino Acids	
	Position	Change	Position	Change
SP-A1				
Exon 2	1162	C/T	39	His-His
Exon 2	1193	C/G	50	Leu-Val
Intron B	1416	C/T	-	-
SP-A2				
Intron 3	1382	C/G	-	-
Intron 3	1492	T/C	-	-
Exon 4	1649	G/C	91	Ala-Pro
Exon 4	1660	A/G	94	Arg-Arg
Intron 4	1691	G/A	-	-

2.3 SP-A1 Sequencing

A total of 16 samples (7 TB cases and 9 controls) were sequenced to determine whether the three SNPs investigated by Madan *et al.* (2002) were present in our population and if so at what frequencies. **Table 8.2** shows the allele frequencies, in our South African population, of the SP-A1 SNPs reported by Madan *et al.* (2002). Of the three SNPs present, C1416T was not polymorphic in our population. Madan *et al.* (2002) found this polymorphism to be significantly associated with TB (p=0.0024) (Madan et al., 2002). The other two polymorphisms (C1162T and C1193G) were not found to be significantly associated with TB in the Indian population. After sequencing C1193G was chosen for further investigation as it appeared a more worthy candidate for further investigation.

TABLE 8.2: Polymorphisms reported by Madan *et al.* (2002) in the collagen-like region of SP-A1 and their allele frequencies in a South African population.

Polymorphism	Allele Frequencies (%)	
	Tuberculosis (n=7)	Controls (n=9)
C1162T		
C	93	83
T	7	17
C1193G		
C	57	83
T	43	17
C1416T		
C	100	100
T	0	0

2.3.1 Genotyping SP-A1 C1193G

A total of 311 TB patients and 328 controls were genotyped with respect to this intronic SNP using an Amplification-Refractory Mutation System (ARMS) technique explained in **Chapter 3 section 14 and 15**. After amplification the PCR products were electrophoresed in a 1.5% agarose gel in order to genotype the individuals. **Figure 8.3** shows an example of an agarose gel and an example of each genotype expected. Although the nested reactions produced reasonably pure products (**Figure 8.2**), some non-specific priming occurred resulting in non-specific bands, which did not affect accurate genotyping. At least 20 samples of various genotype were validated by sequencing. Genotype and allele frequencies for this SNP are shown in **Table 8.3a and b**. Statistical analysis showed that this SNP is not significantly associated with TB susceptibility in our population and that the control groups were in Hardy-Weinberg equilibrium.
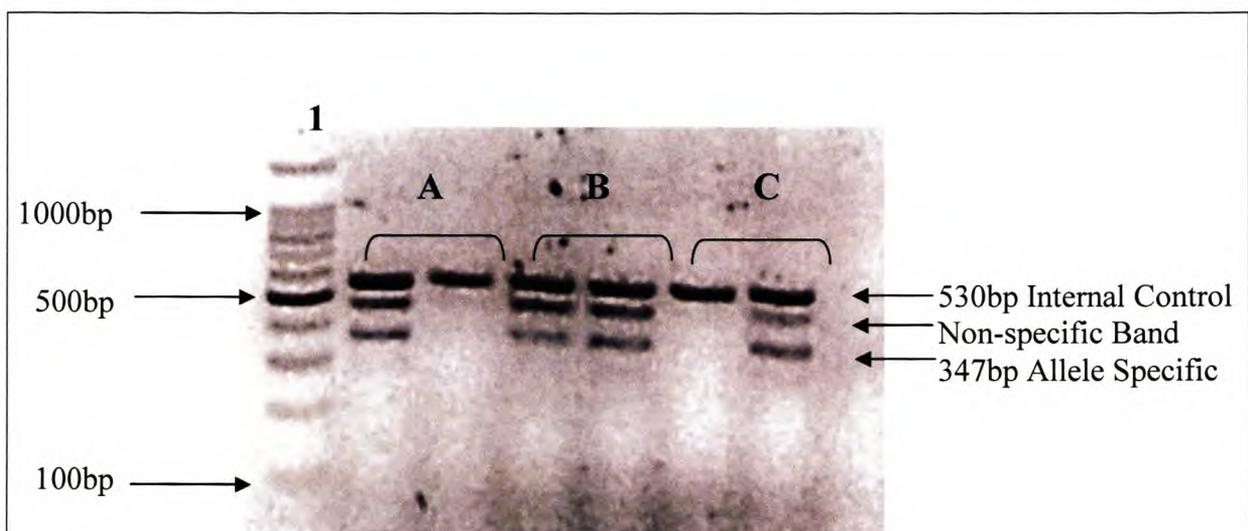
FIGURE 8.3: A 1.5% agarose gel of SP-A1 C1193G genotype patterns obtained using an ARMS PCR (See **Chapter 3, section 12**). Lane 1: 100bp DNA Ladder (Promega, Madison, WI), Lane A: CC homozygote, Lane B: CG heterozygote and Lane C: GG homozygote.

TABLE 8.3: The distribution of C1193G genotypes (a) and alleles (b) in the TB and control populations in this study

(a)

Genotype	Observed Numbers		Chi Square	p-value
	TB Patients (%)	Controls (%)		
CC	173 (56)	193 (59)	1.25	0.535
CG	120 (39)	113 (34)		
GG	18 (6)	22 (7)		
Total	311	328		

(b)

Allele	TB Patients		Controls		Chi Square	OR* (95% CI)	p-value
	Number	Frequency	Number	Frequency			
C	466	0.75	499	0.76	0.23	0.95 (0.73<OR<1.24)	0.634
G	156	0.25	157	0.24			

*OR = Odds Ratio   CI = Confidence Interval

2.4 SP-A2 Sequencing

A total of 23 samples (10 TB cases and 13 controls) were sequenced to determine whether the four SNPs investigated by Madan *et al.* (2002) were present in our population. Of the four SNPs, A1660G was not polymorphic in our population. Madan *et al.* (2002) reported this polymorphism to be significantly associated with TB in their population (Madan et al., 2002). Of the other three SNPs, the ones that appeared most promising in our population were C1382G and G1649C (**Table 8.4**). Madan *et al.* (2002) reported that both of these SNPs were associated with TB in the Indian population (p-values = 0.0054 and 0.0096 respectively), however re-analysis of the reported data by our group using Epi-Info version 1.1, shows that neither C1382G (n=16) nor G1649C (n=16) were associated with susceptibility to TB (p = 0.60 and 0.32 respectively) (Madan et al., 2002). C1492T was not associated with susceptibility to TB in the Indian population and was therefore excluded from this study.

TABLE 8.4: Polymorphisms reported by Madan *et al.* (2002) in the collagen region of SP-A2 and their allele frequencies in a South African population.

Polymorphism	Allele Frequencies (%)	
	Tuberculosis (n=10)	Controls (n=13)
C1382G		
C	90	10
G	10	35
C1492T		
C	35	55
T	65	45
G1649C		
C	20	32
G	80	68
A1660G		
A	100	100
G	0	0

Sequence analysis revealed a novel polymorphism at nucleotide position 1691 in the SP-A2 collagen-like region. This SNP is previously unreported and is present in intron 4. **Figure 8.4** shows the sequencing results allowing the identification of this polymorphism.

FIGURE 8.4: Chromatograms of a portion of the collagen-like region of SP-A2 and the SNP identified at nucleotide position 1691. **A** is a heterozygote AG and **B** and **C** show homozygotes A and G respectively.
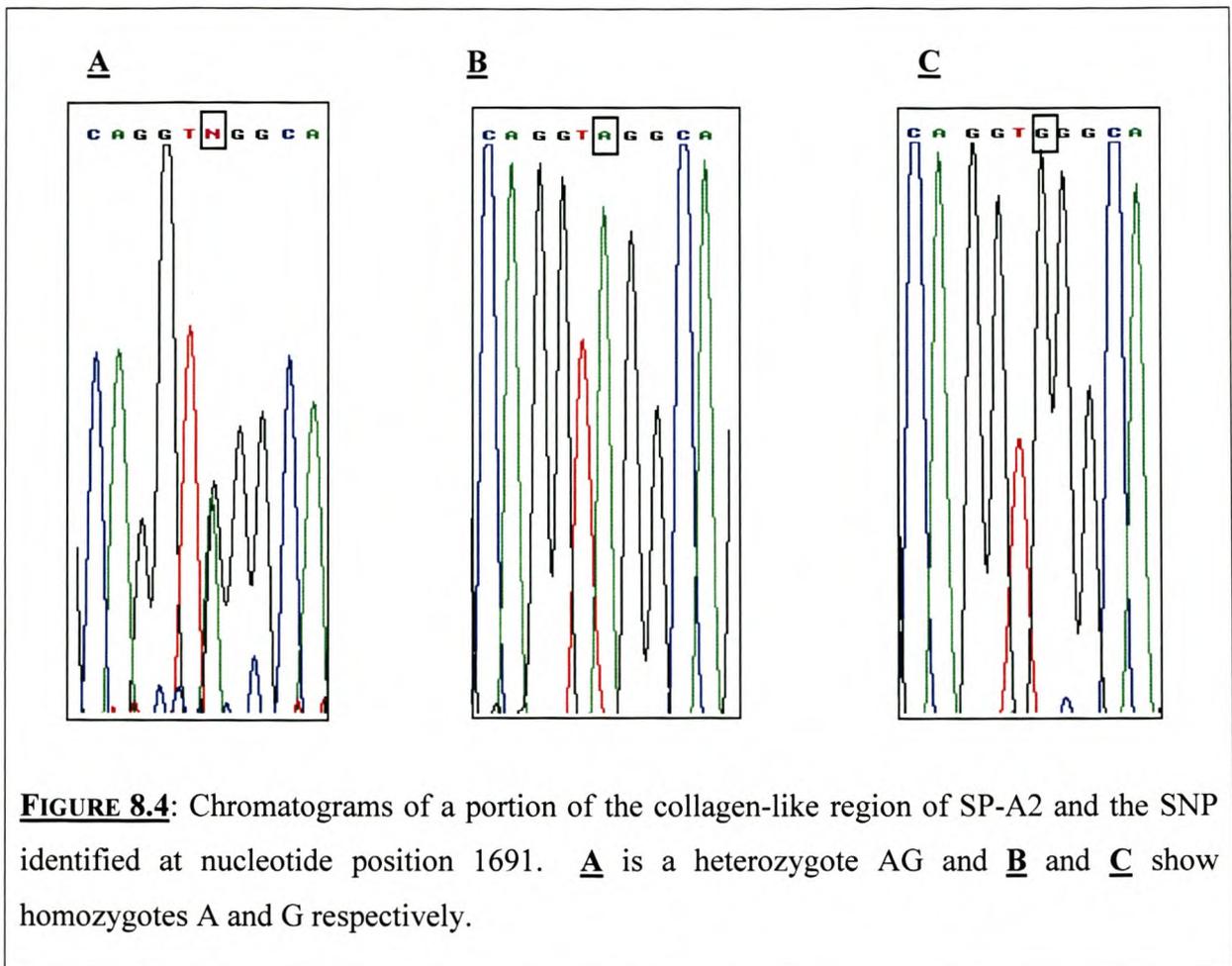
2.4.1 Genotyping SP-A2 C1382G

A total of 321 TB patients and 327 controls were genotyped using an ARMS technique with respect to this intronic SNP (See **Chapter 3, section 15**). **Figure 8.5** shows an example of the agarose gel used to genotype samples and the expected genotypes. Genotype and allele frequencies for this SNP are shown in **Table 8.5a and b**. Statistical analysis showed that this SNP is not significantly associated with TB susceptibility in our population. It was also determined that the control group was in Hardy-Weinberg equilibrium.
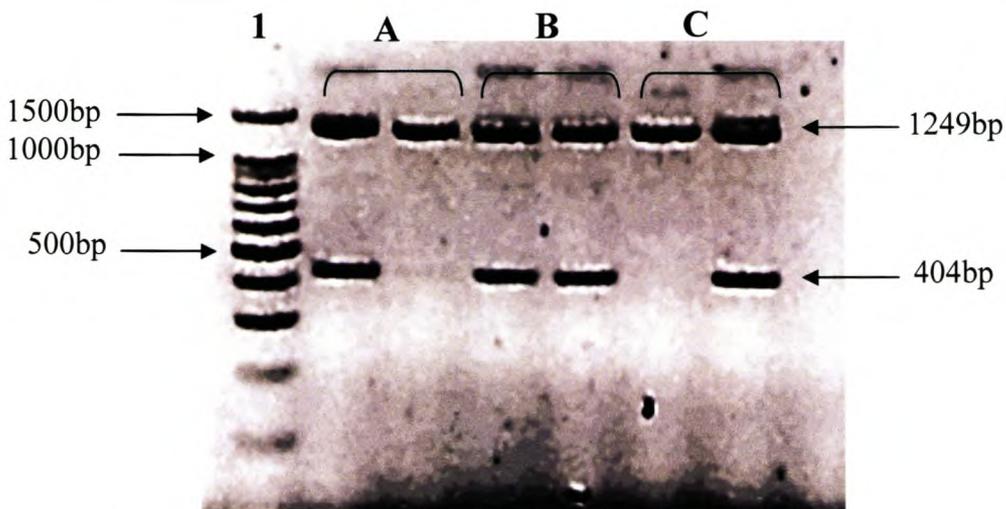
FIGURE 8.5: A 1.5% agarose gel of SP-A2 C1382G genotype patterns using an ARMS PCR (See **Chapter 3, section 12**). Lane 1: 100bp DNA Ladder (Promega, Madison, WI), Lane A: CC homozygote, Lane B: CG heterozygote and Lane C: GG homozygote.

TABLE 8.5: The distribution of C1382G genotypes **(a)** and alleles **(b)** in the TB and control populations in this study

(a)

Genotype	Observed Numbers		Chi Square	p-value
	TB Patients (%)	Controls (%)		
CC	179 (56)	193 (59)		
CG	123 (38)	112 (34)	1.21	0.547
GG	19 (6)	22 (7)		
Total	321	327		

(b)

Allele	TB Patients		Controls		Chi Square	OR* (95% CI)	p-value
	Number	Frequency	Number	Frequency			
C	481	0.75	498	0.76	0.26	0.94 (0.72<OR<1.22)	0.608
G	161	0.25	156	0.24			

*OR = Odds Ratio   CI = Confidence Interval

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2.4.2 Genotyping SP-A2 G1649C

A total of 358 TB patients and 399 controls were genotyped with respect to this exonic SNP using an ARMS method (See **Chapter 3, section 15** for a detailed method). Genotype and allele frequencies for this SNP are shown in **Table 8.6a and b**. **Figure 8.6** shows the agarose gel that was used to genotypes the individuals for this SNP and examples of the genotypes expected.
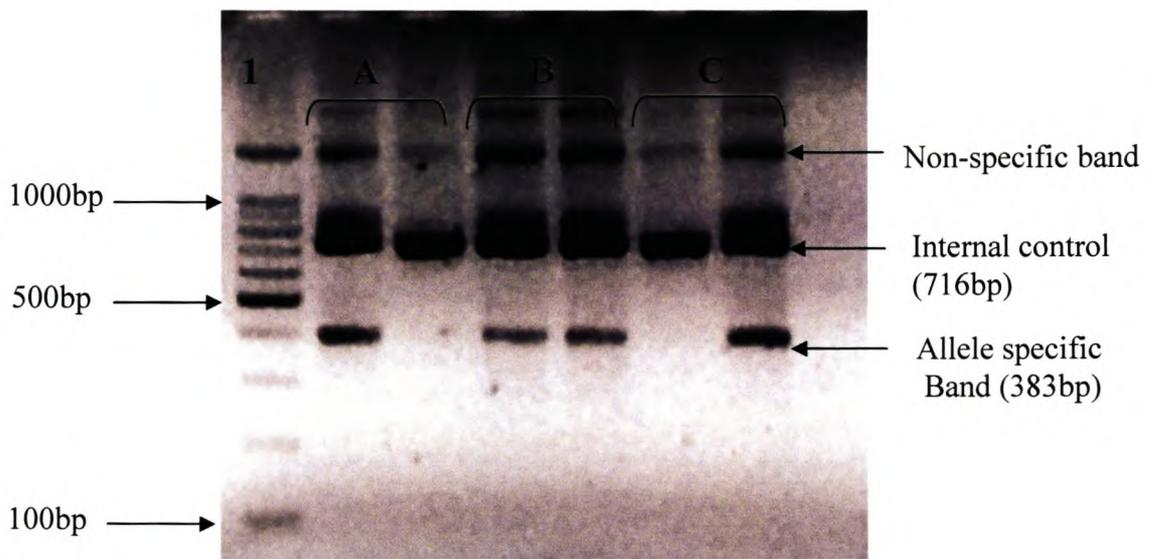

FIGURE 8.6: A 1.5% agarose gel of SP-A2 G1649C genotype patterns using an ARMS PCR (See **Chapter 3, section 12**). Lane 1: 100bp DNA Ladder (Promega, Madison, WI), Lane A: CC homozygote, Lane B: CG heterozygote and Lane C: GG homozygote.

TABLE 8.6: The distribution of G1649C genotypes **(a)** and alleles **(b)** in the TB and control populations in this study

(a)

Genotype	Observed Numbers		Chi Square	p-value
	TB Patients (%)	Controls (%)		
CC	74 (21)	98 (25)		0.014 (Uncorrected)
CG	146 (41)	187 (47)	8.49	
GG	138 (39)	114 (29)		0.042 (Corrected)
Total	358	399		

(b)

Allele	TB Patients		Controls		Chi Square	OR* (95% CI)	p-value
	Number	Frequency	Number	Frequency			
C	294	0.41	383	0.48	7.34	0.75 (0.61<OR<0.93)	0.0067 (Uncorrected)
G	422	0.59	415	0.52			0.0201 (Corrected)

*OR = Odds Ratio   CI = Confidence Interval

Statistical analysis showed that this SNP is significantly associated with susceptibility to TB in our population and that the control group was in Hardy-Weinberg equilibrium. There is a higher frequency of the GG genotype in the TB group when compared to controls. In this analysis corrections were made for multiple testing. The stringent Bonferroni method (See **Chapter 3, section 17.2**) was used to correct and after correction this polymorphism is still associated with susceptibility to TB.

2.4.3 Genotyping SP-A2 G1691A

A total of 303 TB patients and 300 controls were genotyped with respect to this intronic SNP using an ARMS technique (See **Chapter 3, section 15** for a detailed method). **Figure 8.7**

shows an example of an agarose gel that was electrophoresed in order to genotype the individuals as well as an example of the genotypes expected. Genotype and allele frequencies for this SNP are shown in **Table 8.7a and b**. Statistical analysis showed that this SNP is not significantly associated with TB susceptibility in our population and that the control group was in Hardy-Weinberg equilibrium.
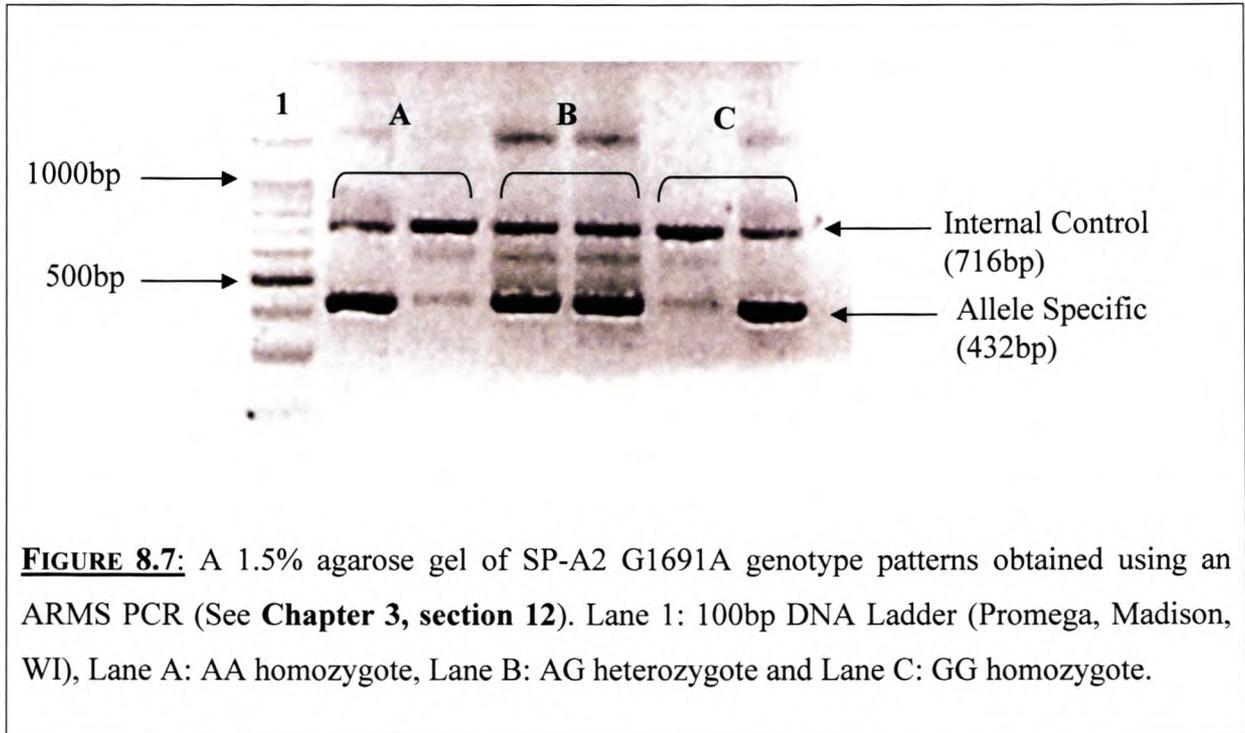
FIGURE 8.7: A 1.5% agarose gel of SP-A2 G1691A genotype patterns obtained using an ARMS PCR (See **Chapter 3, section 12**). Lane 1: 100bp DNA Ladder (Promega, Madison, WI), Lane A: AA homozygote, Lane B: AG heterozygote and Lane C: GG homozygote.

TABLE 8.7: The distribution of G1691A genotypes **(a)** and alleles **(b)** in the TB and control populations in this study

(a)

Genotype	Observed Numbers		Chi Square	p-value
	TB Patients (%)	Controls (%)		
AA	52 (17)	48 (16)	0.33	0.85
AG	158 (52)	154 (51)		
GG	93 (31)	98 (33)		
Total	303	300		

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(b)

Allele	TB Patients		Controls		Chi Square	OR* (95% CI)	p-value
	Number	Frequency	Number	Frequency			
A	262	0.43	250	0.42	0.3	1.07 (0.84<OR<1.35)	0.582
G	344	0.57	350	0.58			

*OR = Odds Ratio   CI = Confidence Interval

2.5 Haplotyping

Haplotypes using the genotypes of the three SP-A2 SNPs (C1382G, A1649C, A1691G) were constructed using PHASE version 2.1 (Stephens et al., 2001).(**Chapter 3, section 17.4**) Haplotype frequencies are shown in **Table 8.8**. Eight possible haplotypes were identified from the genotype data.

TABLE 8.8: Haplotype frequencies within the population that was studied in this investigation.

Haplotype	**Sample Frequency**	**Standard Deviation**
CCA	0.11	0.008
CCG	0.17	0.008
CGA	0.15	0.014
CGG	0.33	0.016
GCA	0.08	0.010
GCG	0.04	0.007
GGA	0.09	0.009
GGG	0.04	0.010

The PHASE program is able to determine whether there is a difference between the distribution of haplotypes amongst cases compared to controls. **Table 8.9** shows the distribution of the haplotypes constructed using this program and their frequencies in the TB and control groups.

The frequencies that are reported are estimates of the sample haplotype frequencies but can also be used as estimates of the population haplotypes frequencies. The results show that the GGG and GCG haplotypes are the least frequent in this population with the CGG haplotype being the most prevalent, having a frequency of 0.33. When the data was analysed to assess whether there was a tendency for one haplotype to be found more frequently in the cases compared to controls a p-value of 0.23 was obtained indicating that there was no significant association with a particular haplotype and the cases or controls.

TABLE 8.9: Haplotype frequencies in the case and control groups.

Haplotype	Control Haplotype Frequencies	Standard Deviation	Case Haplotype Frequencies	Standard Deviation
CCA	0.11	0.010	0.11	0.008
CCG	0.19	0.010	0.16	0.008
CGA	0.14	0.008	0.15	0.009
CGG	0.32	0.010	0.34	0.009
GCA	0.09	0.008	0.08	0.008
GCG	0.04	0.006	0.03	0.006
GGA	0.07	0.008	0.10	0.009
GGG	0.04	0.006	0.03	0.007

2.6 Linkage Disequilibrium (LD)

LD analysis for the SP-A2 data was performed using the LDMAX programme within the Graphical Overview of Linkage Disequilibrium (GOLD) software package (See **Chapter 3, section 17.5**) (Abecasis and Cookson, 2000). This package provides the disequilibrium coefficient (D') values, χ^2 statistics and p-values for all possible combinations of SP-A2 C1382G, G1649C and G1691A. The results obtained indicate that SP-A2 C1382G and G1691A are in LD with each other (D' = 0.597) **(Table 8.10). Figures 8.8a, b and c** illustrate the D', p-values and χ^2 statistics obtained as GOLD graphical LD matrices respectively. **Figure 8.9** shows a summary of the LD results obtained in this analysis.

TABLE 8.10: Analysis of LD between SP-A2 alleles using GOLD. All combinations were significant and SP-A2 1382 and SP-A2 1691 are in complete LD.

SNP 1	SNP 2	N	LD χ^2	p-value	D'
SP-A2 C1382G	SP-A2 G1649C	614	9.76	0.00178	0.202
SP-A2 C1382G	**SP-A2 G1691A**	**574**	**97.55**	**0.00000**	**0.597**
SP-A2 G164C9	SP-A2 G1691A	586	5.9	0.01517	0.105

FIGURE 8.8a: A graphical illustration of the D' values observed between SP-A2 C1382G, G1649C and G1691A.
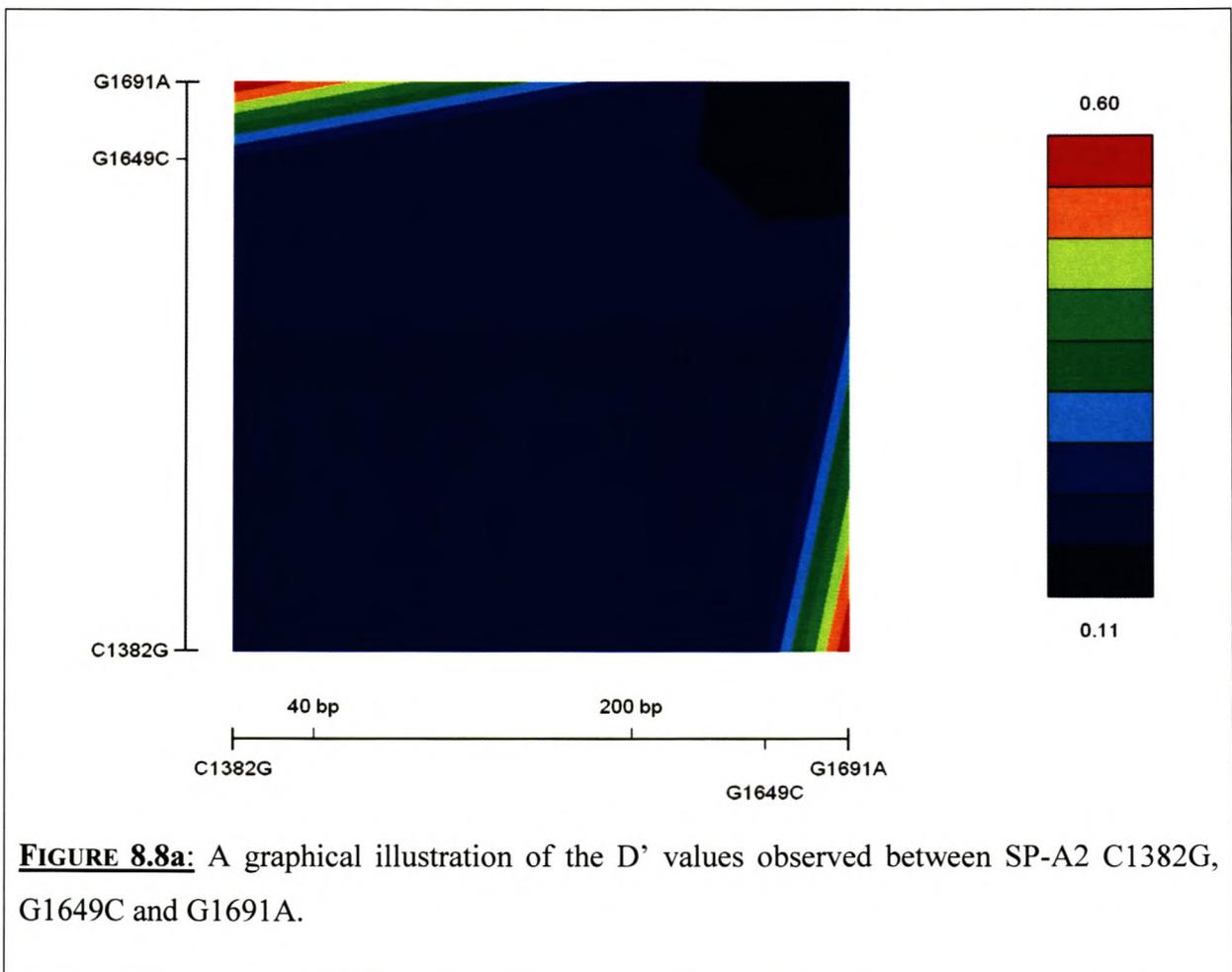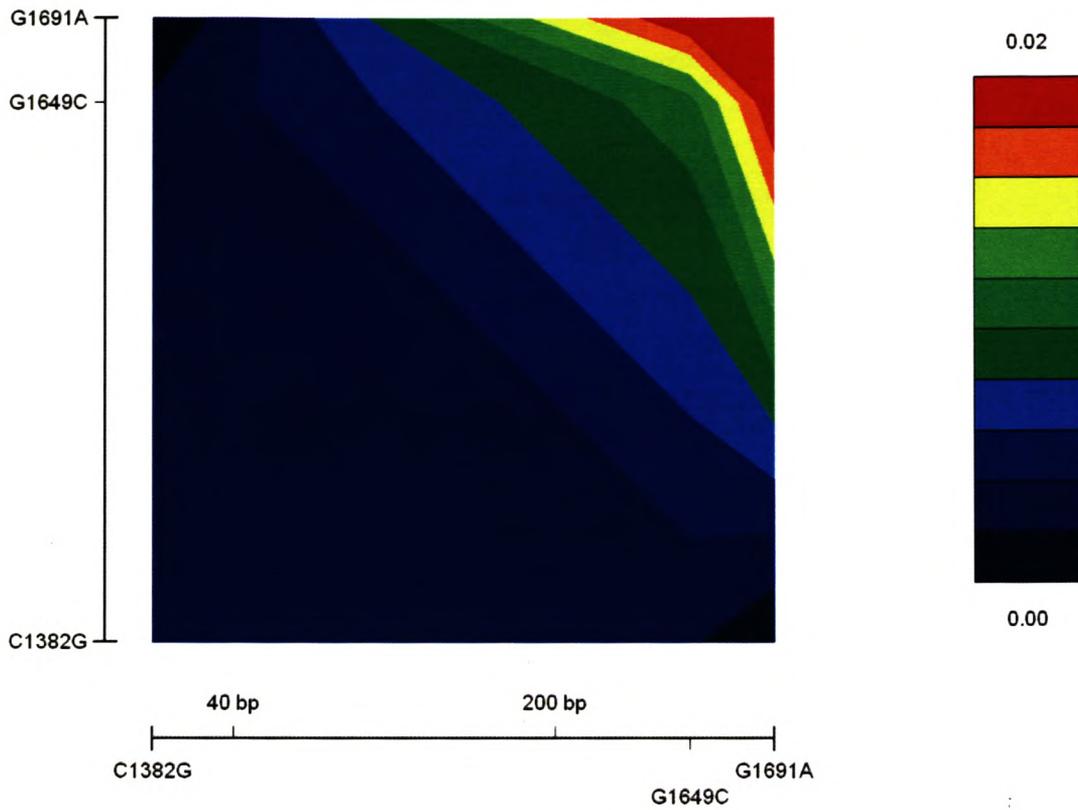
FIGURE 8.8b: A graphical illustration of the p-values obtained.
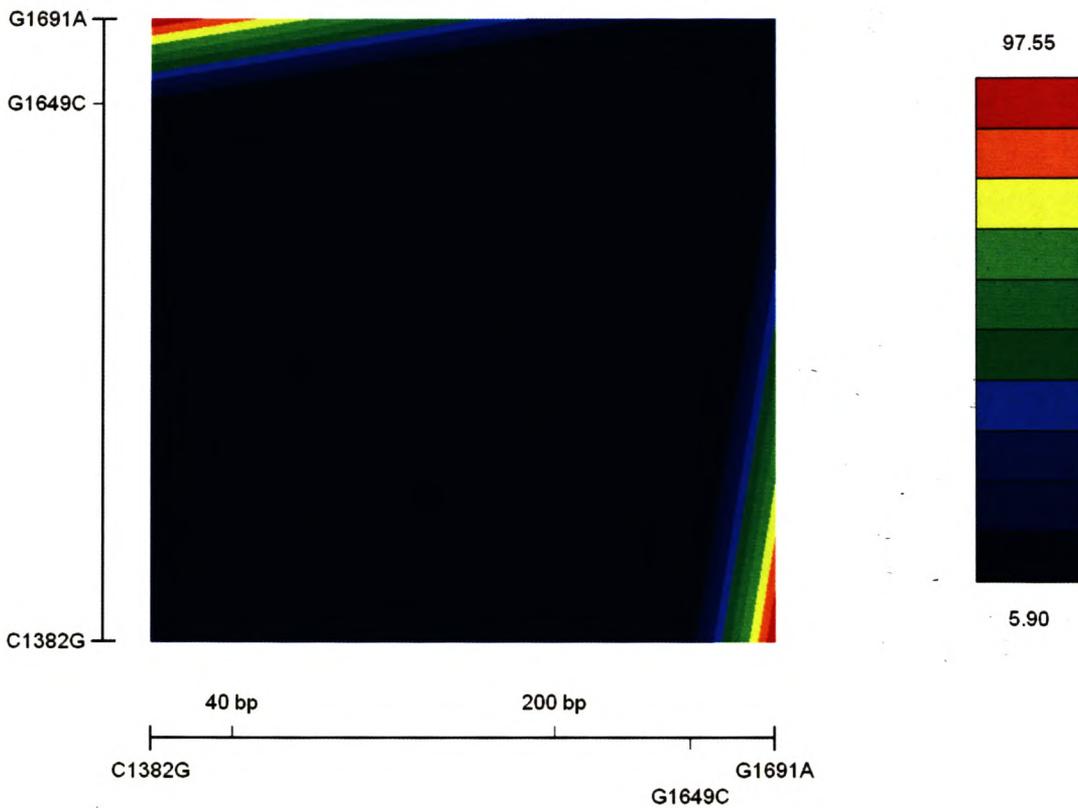
FIGURE 8.8c: A graphical representation of the χ^2 values obtained.
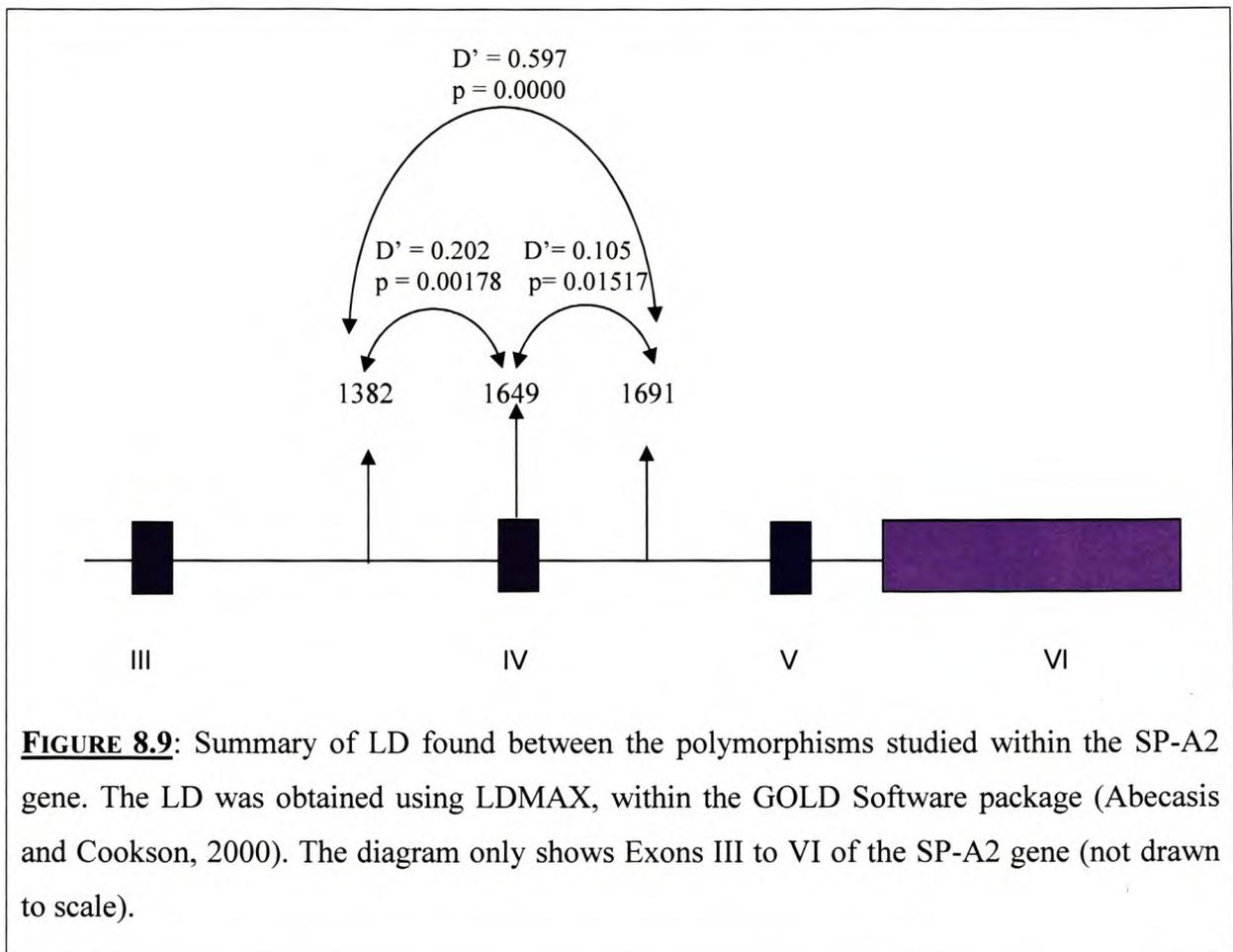
FIGURE 8.9: Summary of LD found between the polymorphisms studied within the SP-A2 gene. The LD was obtained using LDMAX, within the GOLD Software package (Abecasis and Cookson, 2000). The diagram only shows Exons III to VI of the SP-A2 gene (not drawn to scale).

3. DISCUSSION

3.1 Association Analysis

Case-control analysis revealed that there was no statistical difference between cases and controls with respect to the SP-A1 C1193G, SP-A2 C1382G and SP-A2 G1691A SNPs, however a statistically significant association was found between the GG genotype and G allele of SP-A2 G1649C and susceptibility to TB.

SP-A1 C1193G was not associated with TB in our population (p = 0.535, genotypic analysis, p = 0.634, allelic analysis), these results were supported by Madan *et al* (2002) who also did not find a significant association with the SNP and susceptibility to TB in the Indian population (Madan et al., 2002). This SNP was however reported to be associated with susceptibility to TB in a Mexican population (Floros et al., 2000). This association was found when the TB cases (n = 107) were compared to a group of skin-test positive controls (n = 71)

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(p = 0.095) as well as when the TB cases were compared to a group of general controls (n = 101) (p = 0.05) (Floros et al., 2000). These association studies conducted by Floros *et. al.* (2000) had less power than our study.

SP-A2 G1649C was found to be associated with susceptibility to TB in our population (corrected p = 0.0201). Floros *et al.* (2000) investigated the SP-A2 G1649C SNP and found no association with this SNP and susceptibility to TB (TB cases = 107, skin-test positive controls = 71 and general controls = 101) (Floros et al., 2000). Madan *et al.* (2002) also investigated this SNP in an Indian population and reported a significant association with susceptibility to TB (Madan et al., 2002), however re-analysis of the reported results by our group using Epi-Info version 1.1 indicated that this SNP is not associated with susceptibility to TB in the Indian population studies. SP-A2 G1649C results in an amino acid change from proline to alanine at amino acid residue 91, and could have an impact on the overall assembly of the SP-A molecule itself. Proline is an important component on the repetitive Gly-X-Pro subunit in the collagen-like region of SP-A and is known to provide stability to triple helical collagenous structures (Improta et al., 2001). At this stage however it is not know what effect this amino acid change would have on the protein. Karinch *et al.* (1997) showed that the levels of mRNA varied amongst the different SP-A alleles within the 5' splice regions and that the ratio of the SP-A1 and –A2 transcripts also varied suggesting that the protein level may be genetically determined (Karinch et al., 1997). One SP-A genotype in particular $(6A^26A^21A^01A^0)$ has been shown to be associated with a low to moderate level of SP-A mRNA (Karinch et al., 1997) (See **Chapter 1 section 1.7** for an explanation of SP-A alleles). These results suggest that either one or both of the alleles are low producers.

In this investigation we were able to identify a novel polymorphism (SP-A2 G1691A) within the collagen-like region of the SP-A2 gene. This polymorphism was not associated with susceptibility to TB (p = 0.85).

3.2. Haplotype Analysis

Eight haplotypes were identified using the PHASE 2.1 program of Stephens *et al.* (2001), however no particular haplotype segregated with either the cases or controls (p = 0.23) (Stephens et al., 2001).

3.3. LD Analysis

LD analysis shows that SP-A2 C1382G and SP-A2 G1691A are in LD with each other (D' = 0.597). Significant p-values were obtained for the LD analysis and therefore we are confident that the LD values that are reported are accurate.

3.4. General Discussion

The critical determinant of whether *M. tuberculosis* is able to survive within the macrophages is dependent on the organism's abitlity to evade the bactericidal mechanisms of the host. Rodent macrophages are able to kill ingested mycobacteria through the induction of inducible nitric oxide synthase (iNOS) and subsequent production of nitric oxide (Nathan 1995 p876). iNOS deficient mice are unable to produce nitric oxide and mycobacteria are able to grow well within the macrophages of these mice (Macmicking et al., 1997). Le Vine *et al.* (2000) investigated SP-A deficient mice (SP-A$^{-/-}$) as well as wild type mice that were infected with Group B streptococcus (GBS) (LeVine et al., 2000). They observed that the killing of GBS was decreased in SP-A$^{-/-}$ mice and that deficiency in SP-A was also associated with increased inflammation and inflammatory cell recruitment in the lung after infection. This group observed that macrophages isolated from SP-A$^{-/-}$ mice generated significantly less superoxide and hydrogen peroxide compared with wild type alveolar macrophages.

ELISA assays have been developed in order to detect the amount of SP-A present in broncho alveolar lavage fluid (BALF) as well as in serum of patients with respiratory diseases (Kuroki et al., 1985). The levels of SP-A have been found to be increased more than 10 fold in the BALF of patients with pulmonary alveolar proteinosis (PAP) (Honda et al., 1993), however in patients with idiopathic pulmonary fibrosis (IPF), interstitial pneumonia with collagen vascular disease (IPCD) and acute respiratory distress syndrome (ARDS) levels of SP-A were significantly decreased (McCormack et al., 1991; McCormack et al., 1995; Gregory et al., 1991). The SP-A concentration has also been investigated in serum of individuals with a variety of pulmonary diseases. It was demonstrated that serum SP-A levels were significantly increased in patients with PAP and IPF (Kuroki et al., 1993; Honda et al., 1995; Takahashi et al., 1995; Kuroki et al., 1998). The SP-A levels in patients with pneumoconiosis, TB, bacterial pneumonia and chronic pulmonary emphysema increase moderately but the levels are not significantly increased compared to controls.

During intitial infection with *M. tuberculosis* the bacilli reach to distal airways and are phagocytosed by alveolar macrophages. Gaynor *et al.* (1995) demonstrated that SP-A enhanced the uptake of *M. tuberculosis* into human macrophages and suggested that SP-A upregulates the activity of the macrophage mannose receptor (MR) (Gaynor et al., 1995). Alveolar macrophages express particularly high levels of functional MR and Beharka *et al.* (2002) were able to show that SP-A does in fact up-regulate the activity of the MR (Beharka et al., 2002) however SP-A does not alter the expression of the complement receptor (CR). Mutant SP-A molecules that lack the collagen-like region are only expressed as trimers and hexamers, and mutants lacking both the amino terminal and collagen-like regions only form trimers (McCormack, 1998). It has been shown that neither of these variants is able to stimulate an optimal increase in MR expression (McCormack, 1998). This upregulation of the MR by SP-A may provide a mechanism for enhanced phagocytosis of invading infectious organisms, such as *M. tuberculosis* whose uptake is enhanced by binding to SP-A. **Figure 8.10** represents the possible scenarios regarding the interaction of SP-A, *M. tuberculosis* and the MR.

This however would only explain part of the whole mechanism as it has been shown that SP-A enhances the uptake of bacillus Calmette-Guérin (BCG) by rat macrophages and human macrophages through a specific SP-A receptor designated SPR210 (Weikert et al., 1997). SP-A is also able to interact with complement receptors on macrophages.

SP-A plays an important role in defence functions in the host and in response to a variety of pathogens and it has been shown that SP-A enhances the uptake *M. tuberculosis* into the alveolar macrophage (Gaynor et al., 1995). It appears that it would be detrimental to the host to have a defence molecule that enhances uptake of *M. tuberculosis* into its preferred environment and that in the same way as MBL it would be advantageous to have a polymorphism within the SP-A genes that leads to decreased secretion of SP-A. A decreased serum concentration of SP-A may lead to protection against pulmonary TB, as was found with an allelic variant in the MBL gene (Hoal-van Helden et al., 1999) possibly resulting in a decreased uptake of *M. tuberculosis* into the alveolar macrophage.
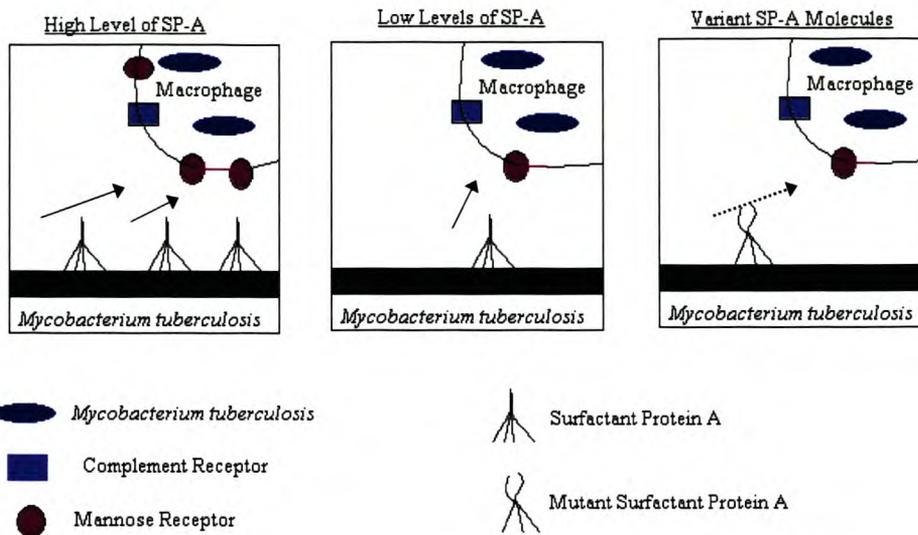
FIGURE 8.10: Possible scenarios regarding the interaction of SP-A, *M. tuberculosis* and the MR adapted from Soborg *et al.* (2003). In the first diagram, when SP-A binds to *M. tuberculosis* there is an upregualation of the MR on the surface of macrophages leading to enhanced pahgocytosis of bacilli into the macrophage. The middle diagram shows a scenario where should there be low levels of SP-A produced it is feasible to speculate that the low levels of SP-A would not stimulate increased expression of the MR to such a great extent resulting in a decreased phagocytosis of *M. tuberculosis* into the alveolar macrophage. The last diagram proposes that should a polymorphism cause a variant SP-A molecule to be produced, such as trimeric or hexameric SP-A instead of the mature octodecameric structure, it would not lead to suffucient upregulation of the MR and may contribute to a decrease in phagocytosis of *M. tuberculosis*, however lipoarabinomannan (LAM) expressed on mycobacteria is also a ligand for both the MR and CR and therefore this may lead to direct uptake and phagocytosis of *M. tuberculosis* by the receptors on the surface of the macrophage.

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CHAPTER 9

CONCLUDING REMARKS

I have learned this at least by my experiment: that if one advances confidently in the direction of his dreams, and endeavours to live the life which he has imagined, he will meet with a success unexpected in common hours.

Henry David Thoreau

There are three factors that need to be considered when investigating genetic susceptibility to infectious diseases and these are firstly, the bacterium, secondly, environmental factors and thirdly, host response linked to genetics. All play an equally important role in determining whether one individual will be susceptible to infection but another individual may not be. Once an association has been made between a polymorphism within a gene it is important to carry this association further and determine whether this polymorphism alters the protein at a functional level.

The collectin molecules, Mannose Binding Lectin (MBL), Surfactant Protein A (SP-A) and Surfactant Protein D (SP-D), have been shown to play important roles in the host response to infection by a wide variety of microorganisms and most importantly in the context of this work they are important in the host defence against *M. tuberculosis* (Crouch, 1998; Hickling et al., 2004; McCormack and Whitsett, 2002; Gaynor et al., 1995; Ferguson et al., 1999). A great deal of work has been conducted on MBL, which was described about 30 years ago (Robinson et al., 1975). Some 10 years later the protein, cDNA and genomic sequence for SP-A was elucidated and it was shown that the molecule had collectin properties (White et al., 1985; Floros et al., 1986). SP-D is the most recently described of the three (Persson et al., 1988; Persson et al., 1989).

MBL has been shown to bind to *M. tuberculosis* and acts as an opsonin, allowing the activation of the classical complement pathway resulting in the phagocytosis of bacilli by macrophages. This suggests that MBL may facilitate the entry of the pathogenic microorganisms into their preferred host environment of the alveolar macrophage. SP-A acts in a similar way to MBL in that it binds to *M. tuberculosis* and leads to the enhanced uptake of bacilli into the macrophage. Polymorphic variants within the MBL gene have been associated with low levels of MBL (Hoal-van Helden et al., 1999) and a SP-A genotype has been shown to be associated with a low to moderate level of SP-A mRNA (Karinch et al., 1997) leading one to speculate that low levels of either of these two proteins could be advantageous to the host as both lead to decreased uptake of the bacilli into alveolar macrophages.

SP-D does not function in the same way as MBL and SP-A. Upon binding *M. tuberculosis*, SP-D agglutinates the bacilli and reduces their uptake into the alveolar macrophage (Ferguson

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et al., 1999). This would appear to be more advantageous as it would decrease the chance of the bacilli reaching their preferred environment of the alveolar macrophage.

In this study the two surfactant proteins were investigated. Regions of the SP-D gene were sequenced in order to determine whether there were any novel polymorphisms within our population. We were not able to identify any new polymorphisms within the amino terminal, neck or proximal promoter regions of the gene. Concentrations of SP-D were investigated within control, previous TB and active TB groups and the concentration of SP-D was found to be significantly higher in the active TB group compared to controls, leading to the hypothesis that SP-D plays some role in a pulmonary acute phase response. Furthermore it was observed that the higher levels of SP-D were associated with a particular SP-D genotype that has been associated with susceptibility to TB as well as respiratory syncytial virus infection. These results imply that this polymorphism plays a role in susceptibility to disease, however it is not known whether this is the polymorphism that plays the main role in this observed susceptibility and influences the serum levels of SP-D or if it is fact in linkage disequilibrium (LD) with the causative polymorphism. The role of this polymorphism should be investigated further and we would propose to do this by investigating the binding and agglutinating ability of native SP-D purified from bronco alveolar lavage fluid (BALF) of individuals with the different SP-D genotypes. These experiments would indicate whether SP-D produced by individuals having the susceptibility genotype bind and agglutinates *M. tuberculosis* less efficiently than individuals having the other genotypes. A more sensitive technique seems to be required should we wish to investigate whether this polymorphism has an impact on the overall structure of the molecule.

A case control association study was carried out for polymorphisms within the collagen-like region of SP-A1 and −A2. We were able to demonstrate that there was a significant association between a single nucleotide polymorphism (SNP) in exon 4 of the SP-A2 gene with susceptibility to TB. Upon sequencing a portion of the collagen-like region of SP-A2 we identified a novel SNP in intron 4 (SP-A2 G1691A), which was not found to be associated with susceptibility to TB. LD analysis showed that there was LD between the novel SNP identified (SP-A2 G1691A) and a SNP in intron 3 (SP-A2 C1382G). The impact of these polymorphisms on the function of SP-A should be investigated further. It would be advisable to conduct a family-based study such as the transmission disequilibrium test (TDT) using SP-A2 G1649C to determine whether this is indeed a true association. It would be interesting to

determine whether the levels of SP-A are significantly increased in patients with active TB compared to previous TB cases and controls as was seen with respect to the SP-D serum concentrations (See **Chapter 6**). Furthermore the disease groups should be subdivided according to the SNPs that were investigated in this study in order to determine whether any particular genotype or haplotype in the case of SP-A2 contributes to an increase or decrease in SP-A serum concentration. Levels of SP-A have been measured in serum using a sandwich ELISA assay in numerous respiratory diseases such as PAP and IPF (Honda et al., 1995; Kuroki et al., 1993; Kuroki et al., 1998). It would be interesting to genotype individuals within this population according to the 5' splice polymorphisms that have been characterised, to determine whether the distribution of alleles is similar to those found in other populations and then to conduct LD analyses between these and the SNPs in the collagen-like region of SP-A2.

Limitations of the study:

1. The collagen-like region and carbohydrate recognition domain of the SP-D gene were not sequenced to determine whether there were any novel polymorphisms. Folding of the collagen-like region is important in the trimeric association of subunits into triple helices (King et al., 1989) and is therefore important for the overall assembly of the SP-D molecule. The carbohydrate recognition domain (CRD) binds pathogens thereby playing an important role in the elimination of microorganisms.

2. More polymorphisms within the SP-D gene should be typed in order to construct haplotypes and to determine whether the SP-D11 SNP is in linkage disequilibrium with another polymorphism in the gene.

3. No family based study was conducted once significance was established with SP-A2 G1649C, therefore it is not possible to state with certainty whether this is purely a chance association or whether there is population bias. A family-based study would be able to ascertain whether this was a true association or not.

4. In order to improve the SNP typing a primer extension or SNaPshot method (Pati et al., 2004; Quintans et al., 2004) using the ABI sequencer could be employed in the future, as it is not subject to bias or human error when typing SNPs. The method should improve genotyping and provide more consistent and reliable results.

TB is a complex disease with factors such as the environment and host genetic factors impacting on the outcome of disease. It is unlikely that there is one major susceptibility locus,

rather, there is probably a complicated interaction between many genetic factors including the surfactant proteins and collectin molecules as a whole. Genotypic information should be used in order to gain a better understanding of what impact these polymorphisms have on the structure and function of the proteins in question.

CHAPTER 10

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Go confidently in the direction of your dreams. Live the life you've imagined.
Henry David Thoreau

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It may be that the gulfs will wash us down,
It may be we shall touch the Happy Isles, and though
We are not now that strength which in old days
Moved earth and heaven, that which we are, we are...
Made weak by time and fate, but strong in will
To strive, to seek, to find, and not to yield.

Alfred, Lord Tennyson

APPENDICES

1. PROPOSAL FOR WHICH ETHICAL APPROVAL WAS OBTAINED

2. STUDY INFORMATION SHEET FOR BAL VOLUNTEERS

   (THIS INFORMATION SHEET WAS ALSO SUPPLIED IN AFRIKAANS)

3. PATIENT CONSENT FORM

   (THIS INFORMATION WAS ALSO SUPPLIED IN AFRIKAANS)

APPENDIX 1

PROPOSAL FOR WHICH ETHICAL APPROVAL WAS

OBTAINED

IMPACT OF THE MOLECULAR FORM OF SURFACTANT PROTEIN-D PURIFIED FROM BRONCHO ALVEOLAR LAVAGE ON BINDING TO, AND AGGLUTINATION OF, MYCOBACTERIA

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Background

Tuberculosis (TB) is an infectious disease caused by the tubercule bacillus, *Mycobacterium tuberculosis* (*M. tuberculosis*). Worldwide, TB causes approximately 3 million deaths annually, which is estimated to be the most deaths caused by a single infectious agent (Harth and Horowitz, 1999). Furthermore, it is believed that one third of the human population is latently infected with TB, of whom 10% are expected to develop the disease in their lifetime (Pym and Cole, 1999). The World Health Organisation predicts that without changes in the present TB control strategies, 200 million people living today will eventually develop the disease (Grange and Zumla, 1999).

In developing countries, such as South Africa, TB is a major problem. It is known that infection with *M. tuberculosis* does not always lead to clinical disease and the question that arises is: are some people more susceptible to the development of clinical disease than others? Although socio-economic status is a major factor, there is evidence that the progression to clinical TB, as well as susceptibility to TB, is affected by interactions between environmental and genetic factors (Schluger and Rom, 1998; Fine, 1981). It is therefore important to investigate the genetic aspect by either performing linkage analyses, requiring large families or many families with affected sibling pairs (Blackwell *et al.*, 1997; Bellamy *et al.*, 2000), or association studies with candidate genes that are potentially involved in the host response to *M. tuberculosis* infection. Genes that have been shown to be associated with susceptibility to TB include; HLA-DR2 (Singh *et al.*, 1983), natural resistance associated macrophage protein 1 (NRAMP1) (Bellamy *et al.*, 1998a), mannose binding lectin (MBL) (Hoal-van Helden *et al.*, 1999), vitamin D receptor (Bellamy *et al.*, 1999) and surfactant protein A1 and A2 (Floros *et al.*, 2000).

Pulmonary Surfactant

Pulmonary surfactant is a complex mixture of lipids and proteins important for normal respiratory function (Rooney et al., 1994), playing a distinct role in the regulation of alveolar macrophage function and inflammation and contributing to innate immune defence of the lung. Its major role is to stabilise the alveolar walls and prevent collapse of the alveoli during exhalation (van Golde et al., 1988). It is composed of four proteins; SP-A, SP-B, SP-C and SP-D whose classification as surfactant proteins is based on the fact that they are found in lavage fluid, synthesised by alveolar type II cells, and are present in large amounts specifically in the lung.

SP-A and SP-D belong to the group of mammalian lectins, designated collectins (Bezouska et al., 1991). Collectins are a group of multimeric proteins organised into either 'bundles of tulips' or 'X-like' structures. These molecules are assembled at their amino termini by disulphide bonds; possess a collagen-like region and clusters of three lectin domains at the end of the collagen-like triple helices (Lu, 1997). Other members of this family include MBL (Turner, 1996), which activates the serum complement system, bovine serum conglutinin and bovine serum lectin 43 (CL-43) (Holmskov et al., 1995).

Agglutination of microorganisms is thought to be an important part of innate immune defence. Agglutinated bacteria may be cleared more readily from the respiratory tract by the mucocillary system or may have altered interactions with host cells (Pikaar *et al.*, 1995; Kuan *et al.*, 1992). SP-A and SP-D are able to bind a wide variety of non-self structures ranging from bacteria, viruses and fungi to allergens and environmental inorganic substrates (Lawson and Reid, 2000). SP-D has been shown to be able to effect the aggregation of various microorganisms and fungi, including certain strains of *Escherichia coli, Streptococcus pneumoniae, Staphylococcus aureus* (Hartshorn *et al.*, 1998), influenza virus (Hartshorn *et al.*, 1997; Hartshorn *et al.*, 1994; Eda *et al.*, 1997), *Cryptococcus neoformans* (Schelenz *et al.*, 1995), *Aspergillus fumigatus* (Mandan *et al.*, 1997a) and *Candida albicans* (van Rozendaal *et al.*, 2000). These findings suggest that collectins recognise carbohydrate structures in the cell wall of the initial infectious forms of fungi and bacteria (Schelenz *et al.*, 1995).

By binding to microorganisms SP-D is able cause agglutination, allowing more efficient removal of pathogens from the lung (Lawson and Reid, 2000). SP-D is able to act as an

opsonin although this does not always promote phagocytosis. For example, SP-D is able to enhance the binding of *Pneumocystis carinii* to alveolar macrophages but it does not promote phagocytosis (O'Riordan *et al.,* 1995). Surfactant proteins act as chemotactic agents by recruiting or curtailing phagocytic cells at the sites of inflammation and immune reaction. SP-D acts as a potent chemoattractant for phagocytes, neutrophils and monocytes (Madan *et al.,* 1997b; Crouch *et al.,* 1995; Cai *et al.* 1999). The mechanism and receptors behind this process are not fully characterized. The surfactant collectins have been shown to enhance phagocytosis in some cases. SP-D is able to bind and enhance macrophage phagocytosis of mucoid and non-mucoid strains of *Pseudomonas aeruginosa* but it is unable to aggregate either strain, which suggests that SP-D enhances phagocytosis through receptor-mediated opsonisation and killing (Restrepo *et al.,* 1999). The mucoid strains of *P. aeruginosa* are important in the pathogenesis of chronic lung disease associated with cystic fibrosis and this could be related to the decreased levels of both SP-A and SP-D that have been reported in lung lavages of at least some patients with cystic fibrosis (Postle *et al.,* 1999).

Transgenic mice studies (SP-D knockouts) have been conducted using SP-D. These studies have provided some information about the function of SP-D in agglutination, phagocytosis and killing of numerous bacterial and fungal species. LeVine *et al.,* (2000a) showed that SP-D$^{-/-}$ mice, infected intratracheally with group B streptococcus (GBS), were able to kill the bacteria as efficiently as the wild-type mice and the number of bacteria associated with macrophages was lower than observed in the wild-type mice. In the presence of SP-D there was increased association of GBS with alveolar macrophages but phagocytosis was not altered. The same group also infected SP-D$^{-/-}$ mice with *Haemophilus influenzae* and observed a similar situation in that these mice were able to kill this bacterium as efficiently as the wild-type, that the number of bacteria associated with and internalised by alveolar macrophages was significantly less in SP-D$^{-/-}$ mice compared to the wild-type mice. This suggests that there is a defect in opsonization and/or phagocytosis. In both cases SP-D$^{-/-}$ mice showed increased inflammation and inflammatory cell recruitment into the lung after infection. Le Vine *et al.,* (2000b) showed that SP-D$^{-/-}$ mice were susceptible to respiratory syncytial viral pneumonia. The uptake of viral particles by alveolar macrophages and the clearance of the virus from the lung were deficient in SP-D$^{-/-}$ mice. Studies of this nature have not yet been conducted in connection with *M. tuberculosis* but would be useful in elucidating the function of SP-D in the progression of TB.

Structure of SP-D

The surfactant genes: two SP-A genes, one SP-A pseudogene and the SP-D gene, are found on chromosome 10 (10q22.2-q23.1) in close proximity to MBL (Crouch *et al.*, 1993; Hoover and Floros, 1998). These collectin molecules are composed of four domains. Firstly, a short amino terminal region containing two conserved cysteine residues (Cys15 and Cys20), which are important in the formation of interchain disulphide bonds that stabilise the dodecameric structure of SP-D (Crouch *et al.*, 1994a). The long collagen like domain accounts for the pronounced linear characteristic of the molecules (Fig 1). There is a coiled-coil neck region and a calcium dependent carbohydrate recognition domain (CRD) that is necessary for carbohydrate binding (Sastry and Ezekowitz, 1993; Botas *et al.*, 1998). Each member of the collectin family has a different degree of higher order oligomerisation. (Lawson and Reid, 2000). Hartshorn *et al.*, (1998), were able to show that the degree of multimerisation of SP-D was a critical determinant of both aggregating activity and potency in enhancing bacterial uptake, and Zhang *et al.*, (2001), demonstrated that the activity of SP-D *in vivo* is dependent on the oligomeric structure of the SP-D molecule. Conglutinin and SP-D have a characteristic cruciform structure that is assembled as tetramers of three identical polypeptides. It is thought that the agglutination activity is dependent on the oligomerisation and their ability to bridge
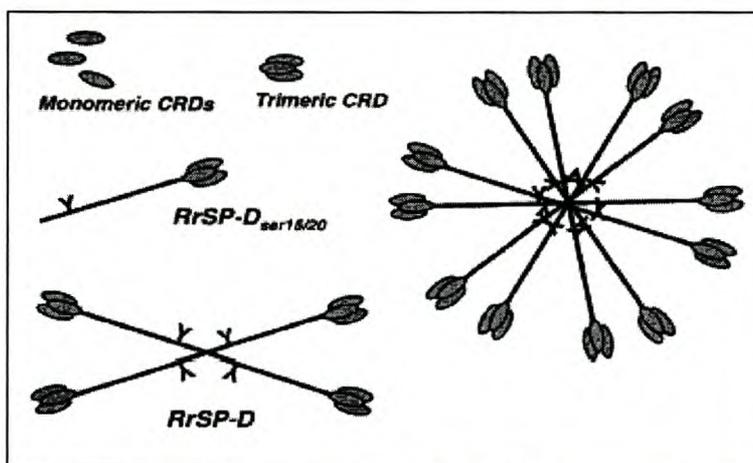
the large distances between organisms (Eda *et al.*, 1997).

Figure 1: The alternative molecular forms of SP-D. Monomeric carbohydrate recognition domains (CRDs), trimeric CRDs, a recombinant rat single arm mutant (RrSP-Dser15/20), the natural dodecameric structure (RrSP-D) and an SP-D multimer or 'astral body'. Monomeric

CRDs have a weak binding affinity to various ligands. Trimeric CRDs and single arm mutant forms of SP-D cannot mediate effective agglutination of microorganisms, the minimal structure required for this is the dodecameric form of SP-D (Crouch, 1998).

SP-D and *Mycobacterium tuberculosis*

The initial immunologic responses to *M. tuberculosis* in the lung are important, as entry of this inhaled pathogen into the alveolar macrophage is a defining event in disease pathogenesis (Ferguson *et al.*, 1999). *M. tuberculosis* is likely to come into contact with surfactant when inhaled into the alveolus and it is proposed that the interaction between *M. tuberculosis* and surfactant components alters the ultimate fate of the bacterium (Ferguson *et al.*, 1999). Ferguson *et al.*, (1999), investigated the binding of SP-D to *M. tuberculosis* and the effect of this binding on the adherence of *M. tuberculosis* to human macrophages, and demonstrated that specific binding of SP-D to *M. tuberculosis* is saturable, calcium dependent and carbohydrate inhibitable. On incubation with SP-D, the virulent Erdman strain of *M. tuberculosis* agglutinated but there was minimal binding to the avirulent strain of *Mycobacterium smegmatis*. SP-D was found to bind predominantly to the lipoarabinomannan (LAM) of *M. tuberculosis* Erdman, but was unable to bind to the AraLAM of *M. smegmatis*. It was observed that SP-D was able to agglutinate *M. tuberculosis*, however, SP-D was shown to decrease the adherence of the bacteria to human macrophages. This result contrasts with the findings regarding SP-A (Gaynor *et al.*, 1995), which showed that SP-A acts to enhance *M. tuberculosis* adherence to macrophages as well as phagocytosis of the bacteria. Therefore, mechanisms that enhance entry of *M. tuberculosis* into the alveolar macrophage may be detrimental to the host, whereas mechanisms that reduce the entry of *M. tuberculosis* may be protective. Recently Jounblat *et al.*, (2004) showed that SP-D was able to bind to and agglutinate various strains of *Streptococcus pneumoniae* however; the extent of binding as well as agglutination differed between strains. This led us to the hypothesis that SP-D binding to and agglutination of *M. tuberculosis* could differ among *M. tuberculosis* strains.

Sumiya *et al.*, (1991), showed that children carrying mutations in MBL were more susceptible to recurrent infections, which supports the concept that collectins are part of the innate immunity against microbial pathogens. In support of this, Matsushita (1995), found that polymorphisms in the nucleotide sequences of the collagen domain of the MBL gene altered oligomerisation and that it was associated with defects in host defence function. It is therefore

thought that increased levels of mutant SP-D that are unable to form higher orders of multimerisation, might influence the function of SP-D which, in turn, could contribute to the pathogenesis of various lung diseases including TB (Zhang *et al.*, 2001). Kondo et al (1998) found that patients with active pulmonary TB had approximately three-fold the concentration of serum SP-D as healthy controls.

Previous work in this laboratory: We have shown in a case-control association study, (317 cases and 228 controls), that a polymorphism in the amino terminal region of SP-D was significantly associated with susceptibility to tuberculosis (unpublished). From this we hypothesized that this polymorphism could lead to a structural change in the molecule causing decreased agglutination of and binding to *M. tuberculosis* due to inefficient oligomerisation of the SP-D molecule. Subsequently, serum samples were taken to the University of Oxford (MRC Immunochemistry Unit of Prof Ken Reid) and purified by our student, T. Roos (Med. Biochem) on a Superose 6 HR 10/30 column to partially purify SP-D from serum obtained from healthy volunteers. The aims of the investigation were to identify, by ELISA, which fractions, collected from the column, contained SP-D using purified rabbit antibodies to recombinant SP-D, and to separate proteins in a SDS-PAGE gel under reducing conditions and then Western blot to detect SP-D. The results thus far look promising.

We would like to further refine this study by using BAL fluid, from which we can directly purify SP-D in the form required for bacterial agglutination as opposed to using a complex mixture of proteins such as plasma.

Aim of the study and hypothesis

Hypothesis: SP-D purified from individuals with different SP-D genotypes shows variant forms which result in differential agglutination of *M. tuberculosis*. In addition, a given SP-D form binds to a greater or lesser extent to different strains of *M. tuberculosis*.

Aim: To test this hypothesis we propose to purify SP-D from the BAL of individuals of the three possible genotypes and measure the binding to, and agglutination of, *M.tuberculosis*. We will therefore take a small blood sample from volunteers to determine their genotypes. These same volunteers will undergo BAL and from this BAL fluid we will purify SP-D. We intend to recruit approximately 20 to 30 volunteers and are aiming to obtain at least 6 volunteers in each genotypic category (CC, CT, TT). This should allow us to detect whether

there are measurable differences in the molecular form of SP-D, which impact on the binding of SP-D to *M. tuberculosis* strains and their ability to agglutinate *M. tuberculosis*. From the allelic frequency we estimate that this sample size will be sufficiently large to yield the numbers required in the genotype categories. When 6 to 10 individuals in each category have been identified and sufficient BAL fluid obtained, recruitment will be stopped.

Study design

Structure

This is a cross-sectional observational study.

Study population

Twenty to thirty, adult, non-smoking volunteers, aged 18-45 years and not taking any regular medication will be recruited. Only patients scheduled for bronchoscopy where this is indicated for diagnostic reasons (e.g. cancer, fibrosis) will be approached and asked to participate in the study. Patients undergoing broncho alveolar lavage for any disease other than infectious disease will also be recruited, and the lavage fluid not required for assessment of the patient will be brought to the laboratory and used for this project. Other suitable candidates will be patients with mass lesions of the lung requiring further investigation, without collapse of any lobe in order to avoid BAL contamination with proteins resulting from the infective process.

Exclusion criteria

Smoking, known asthma, current tuberculosis or other infectious disease, or other current or previous serious lung, cardiac or other systemic disease will be exclusion criteria.

Pre Study Procedures

Preparation of volunteers

After written informed consent has been obtained from the volunteer, eligible volunteers will be allocated a study number in numerical ascending order. Volunteers will be briefed on the necessary preparation for BAL and anaesthesia and given a date and time for the next visit. Volunteers will be questioned to ensure that they remain fit for anaesthesia and continue to fulfil the inclusion and exclusion criteria. An intravenous catheter will be inserted and

preserved with heparin. BAL will then be carried out, followed by blood sampling as specified below.

BAL and Blood Sampling

The broncho alveolar lavage of the right middle lobe will be performed in the standard manner using a fibre-optic flexible bronchoscope under mild sedation with intravenous midazolam and fentanyl after premedication with intravenous atropine. This procedure will be carried out in the endoscopy suite of Tygerberg Hospital by Prof Walzl or similarly qualified persons delegated by him.

A venous blood sample of 10ml will be collected in a VACUTAINER® tube with EDTA.

Laboratory tests

DNA Isolation

The VACUTAINER® tube with EDTA will be stored at room temperature for less than 24 hrs until centrifugation. If DNA extraction is not performed that day, the white cell pellet will be stored at -20° C until the time of DNA extraction, which will be within one week.

Genomic DNA will be isolated from the venous blood sample using the Nucleon BACC3 Kit for blood and cell cultures (Amersham Biosciences) and stored at -80°C until required.

Purification of SP-D from BAL Fluid

This will be carried out according to Madan *et al.* (1997b) using affinity chromatography of the BAL fluid on maltose agarose columns.

Binding and Agglutination Assays

These will be carried out according to the protocol of Scott Ferguson *et al.* (1999). Briefly a bacterial ELISA assay will be used to determine the extent of SP-D binding to various mycobacterial strains. Bacteria will be fixed into the wells of a microtiter plate and killed by 5mM nitrite at pH 4.5 or formaldehyde. In the case of *M. tuberculosis* strains being investigated; this step will be carried out in the P3 facility in the Dept. Medical Biochemistry. After blocking, the wells will be incubated with recombinant or native SP-D at the appropriate concentration, thereafter the wells will be incubated with biotinylated rabbit anti-human SP-D IgG. After incubation substrate will be added and once the reaction is complete the absorbance will be read using a microplate spectrophotometer, and the degree of binding of SP-D to the bacteria determined.

Similarly the agglutination assay will be carried out according to Scott Ferguson *et al.* (1999). *M. tuberculosis* will be incubated with an appropriate concentration of native human SP-D. Thereafter aliquots will be dried on glass coverslips, fixed with formaldehyde, stained with a fluorescent stain and viewed by fluorescence microscopy.

Personnel

Professor Walzl will make all clinical assessments and perform or supervise the taking of blood samples and the broncho alveolar lavage. Laboratory tests (SP-D purification and DNA extraction) will be performed by Dr Hoal's team in the Dept. Medical Biochemistry.

Data analysis

All data will be analysed using a software package (SPSS or Graphpad Prism). The degree of agglutination effected by SP-D from individuals of different genotype will be compared. This type of comparison has not been done before, and we therefore cannot predict the magnitude of the differences that will be seen, if any. If the differences are modest, these sample numbers will not provide sufficient power, but even a descriptive study will be novel. An initial ANOVA test will be performed and any significant differences between the groups will be further investigated by appropriate grouping into two categories, either T- or C-containing genotypes, followed by a t-test.

Ethical considerations

Approval is requested from the institutional ethical committee at Tygerberg Hospital. All subjects will receive a detailed information leaflet regarding the study and all possible dangers regarding blood sampling and bronchoscopy will be explained. Fibre-optic bronchoscopy under mild sedation and employing local anaesthesia can be used with low risk even in severely ill patients and should pose a very small risk for serious adverse events in healthy young adults when performed by an experienced operator. Written consent will be obtained. Subjects will be informed of their right to cease participation in the study at any point in time and confidentiality will be maintained at all times.

Importance of the study

This study will yield novel results. Some agglutination studies have been done, but the functional comparison of SP-D from individuals of different genotype has not been performed. Other workers in this field have suggested that the polymorphism resulting in the different genotypes may affect the form and/or function of the molecule, and we have preliminary results from column chromatography suggesting that this may be true. If this study confirms our hypothesis, it will indicate the importance of the mode of entry of *M. tuberculosis* into the macrophage in subsequent disease development.

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APPENDIX 2

STUDY INFORMATION SHEET FOR BAL VOLUNTEERS

(THIS INFORMATION SHEET WAS ALSO SUPPLIED IN AFRIKAANS)

INFORMATION SHEET FOR VOLUNTEERS

Study: IMPACT OF THE MOLECULAR FORM OF SURFACTANT PROTEIN-D PURIFIED FROM BRONCHO ALVEOLAR LAVAGE ON BINDING TO, AND AGGLUTINATION OF, MYCOBACTERIA

Principal Investigators: Dr Eileen Hoal, Prof Gerhard Walzl

SU/MRC Centre for Molecular and Cellular Biology and Department of Medical Biochemistry, Faculty of Health Sciences, Stellenbosch University.

Background

Tuberculosis (TB) is a major health problem in the world and especially in the Western Cape. It is a disease that can be cured with medication, but we need more scientific information on the nature of the disease to be able to come up with better ways of diagnosis, prevention and treatment. We also need more information on the manner in which healthy people fight off the disease. One of the ways the body combats the TB bacterium is to use a protein in the lungs called Surfactant Protein-D to bind to the bacteria and make it easier for the macrophages (one of the types of white cells in the blood) to take them up and destroy them. We have been working on the gene that produces different types of this protein and have found that people with a certain genotype may be more likely to get TB. We would now like to investigate whether the different forms of the protein bind the bacteria more or less efficiently, which may explain why some people seem to be more able to resist TB.

Design of Experiments

We aim to purify Surfactant protein-D from the lungs of people without TB. Thirty volunteers will be recruited. In order to obtain samples of fluid from the lung we will use a process called bronchoscopy and bronchoalveolar lavage. You have already been scheduled to undergo bronchosopy and/or bronchoalveolar lavage, and we are therefore asking for only a small additional procedure i.e. the bronchoalveolar lavage if you were not scheduled to undergo this. If you consent to take part in this research project, you will be asked to undergo this process once only. A small blood sample will be taken at the same time to extract DNA and type the genes influencing TB susceptibility, especially the form of the surfactant protein-D gene.

Procedures

Blood samples will be obtained from a vein in the arm using a sterile needle and syringe. The amount of blood drawn will be 15ml (3 teaspoons) divided into two or three tubes. This is not an amount that poses any dangers to a child or an adult. All blood samples will be labelled with numbers to ensure that none of the researchers are able to recognise who the samples came from, to maintain confidentiality. Only the Principal Investigators at Stellenbosch University and the nursing sister who takes consent from you will have access to the information that links the blood samples to the nominal data (names and addresses). Confidentiality will be protected by the Health Professional Council of South Africa's guidelines.

The **bronchoscopy** and washing of one lung segment (lavage) will be performed by an experienced physician. This is a straightforward and safe procedure and provides a simple means for sampling the cells and fluid in the airways of the lungs. It involves the passage of a fine flexible tube through the nose or mouth and into the lung. A volume of sterile solution is then instilled into a lobe of the lung, followed by the sucking up of the accumulated contents. This procedure usually lasts about five minutes. A mild sedative will be administered before this procedure to help make the test a more comfortable experience.

Possible side-effects of these procedures

The only side-effects that you might experience are an irritating cough during the procedure, occasional sore throat and mild fever afterwards. All these symptoms are short lived. In the 1970's a study was reported that investigated the side effects of the procedure in patients, many of them seriously ill and therefore more likely to develop problems. The number of patients that developed mild symptoms were 3 in 10 000, a further 3 in 10 000 developed more serious side effects like bleeding from the lung and there was a mortality of 1 in 10 000. We would like to point out that these were seriously ill patients who also had additional procedures like biopsies (the removal of small pieces of lung tissue), which do not form part of this experiment. As you are not seriously ill, we do not expect any serious problems. Because we administer a sedative, you will not be able to drive or operate machinery for the rest of that day.

Additional information

While the research project may not be directly beneficial to you, the results of the research will help us to understand how the body protects itself against TB in the lungs and this information may help us to develop a better treatment to prevent the development of TB.

You are completely free to choose whether or not you decide to take part in this study. If you decide to take part you may withdraw at any time without having to give a reason. Refusal to participate will not involve any penalty or change in the standard of medical attention for you or your family in the hospital or clinic. We will use the samples exclusively for research on TB and lung disease and will not do other experiments with it. You will not receive any financial reward for participating in this study. All personal information obtained from this study will be strictly confidential. The results of the study will be published in medical journals, but without mentioning the name of any person on whom blood was taken. Should you have any questions you are free to contact Prof Gerhard Walzl at the Dept Medical Biochemistry Tel No. 938 9158 (office hours).

All proposals for research using human subjects are reviewed by an ethics committee before they can proceed. This proposal was reviewed by the Stellenbosch University Ethics Committee and will be in accordance with recognised standards of good clinical practice; in particular the Declaration of Helsinki and the ICH/GCP guidelines.

We are covered by insurance against any expenses due to injury caused by participation in the study.

If you experience any problems after the procedure, please contact Prof Gerhard Walzl at Tygerberg Hospital Tel No0825923212 or the Department of Medical Biochemistry Tel No 938 9158 during office hours or the registrar on call at the Lung Unit at Tygerberg Hospital at telephone no 938 4911 after hours.

APPENDIX 3

PATIENT CONSENT FORM

(THIS INFORMATION WAS ALSO SUPPLIED IN AFRIKAANS)

**INFORMATION AND INFORMED CONSENT DOCUMENT AND
INFORMATION AND INFORMED CONSENT DOCUMENT FOR DNA
ANALYSIS AND STORAGE**

TITLE OF THE RESEARCH PROJECT: **IMPACT OF THE MOLECULAR
FORM OF SURFACTANT PROTEIN-D PURIFIED FROM BRONCHO
ALVEOLAR LAVAGE ON BINDING TO, AND AGGLUTINATION OF,
MYCOBACTERIA**
REFERENCE NUMBER:

……………………………………….………

………………..

PRINCIPAL INVESTIGATORS: Dr Eileen Hoal, Prof. Gerhard

Walzl………….

Address: Dept Medical
Biochemistry………….……………

                                                          Faculty of Health

Sciences……………………..

                                                          Stellenbosch

University…………………………..

**DECLARATION BY OR ON BEHALF OF PATIENT/*PARTICIPANT:
I, THE UNDERSIGNED,**

……………………………………………………………….. *(name)*

[ID No: …………………..…………] the patient/*participant or* in my capacity as

……………………………………………. of the patient/*participant [ID No:

…………………...] of …………………………………..……………………….

……………………………..………………………………………………………...

…………………………………….. *(address).*

A.   HEREBY CONFIRM AS FOLLOWS:

1.   I/*The patient/*participant was invited to participate in the abovementioned research project which is being undertaken by the Department of Medical Biochemistry, Faculty of Health Sciences, Stellenbosch University.

2.   The following aspects have been explained to me/*the patient/*participant:

2.1 **Aim:** To investigate the binding of different forms of Surfactant Protein- D (purified from human broncho alveolar lavage fluid) to

mycobacteria. Additionally, this project aims to collect genetic material (blood) to analyse for differences in the DNA (polymorphisms) and to store excess material for future research.

2.2 **Procedures:** I will be requested to provide information about my medical history. Blood (15*ml*, about 3 teaspoonfuls) will be collected from me. The investigators will perform broncho alveolar lavage once.

2.3 **Genetic considerations:**

➢ The blood or tissue may be used to create a cell line, which can grow indefinitely and can be used to synthesize more of my/*the participant's DNA at any time in the future;

➢ The DNA may be stored for several years until the technology for meaningful analysis becomes available;

➢ The DNA will be maintained indefinitely, unless I/*the participant request(s) to have it and/*or the stored clinical data destroyed by contacting the investigator conducting the present study, Dr Eileen Hoal at Tel No. 938 9412 or the Chairperson of the Research Subcommittee C/Ethics Committee at Tel No 938 9207 if the former cannot be located;

➢ The analyses in the current study are specific to the investigation or disease mentioned above and cannot determine the entire genetic make-up of an individual;

➢ Even under the best conditions, current technology of this type is not perfect and could lead to unreliable results.

2.4 **Risks:** Possible side-effects I might experience from the broncho alveolar lavage are an irritating cough during the procedure, occasional sore throat and mild fever afterwards. In taking blood for DNA, there are no more than minimal medical or psychological risks associated with this study:

*Delete where not applicable

> ➢ I/*The participant may feel some pain associated with having blood withdrawn from a vein and may experience discomfort, bruising and/or slight bleeding at the site;

> ➢ As some insurance companies may mistakenly assume that my/*the participant's participation is an indication of a higher risk of a genetic disease which could hurt my/*the participant's access to health or other insurance, no information about me/*the participant or my/*the participant's family will be shared with such companies as this investigation cannot be regarded as formal genetic testing for the presence or absence of certain genes.

2.5 Benefits:

> ➢ Although there may not be any direct benefits to me/*the participant by participating at this stage, family members and future generations may benefit if the researchers succeed in scientifically delineating certain disorders further. Thereby the rational approach to the clinical diagnosis and therapy of its manifestations may be facilitated. The identification and location of the genes involved in such disorders, could in the end lead to the development of methods for prevention and to forms of new treatment aimed at curing or alleviating these conditions;

> ➢ In the unlikely event that the research may lead to the development of commercial applications, I/*the participant or my/*the participant's heirs will not receive any compensation, but profits will be reinvested into supporting the cause of further research which may bring benefits to my/*the participant's family and to the community, such as health screening, medical treatment, educational promotions, etc;

*Delete where not applicable

2.6 **Confidentiality:** My/*The participant's identity will be kept confidential throughout. Information will not be associated with my/*the participant's name. The research staff will use only a coded number, access will be limited to authorized scientists and any scientific publications, lectures or reports resulting from the study will not identify me/*the participant by name.

2.7 **Access to findings:** I understand that I may apply to the Principal Investigators to share their findings with me.

2.8 **Voluntary participation/refusal/discontinuation:** I understand that participation is voluntary and that the I may consequently refuse to participate and that the I may discontinue participation at any time and that such refusal or discontinuation would not prejudice my future treatment at this institution and that the investigator may withdraw me from the study should he/she feel that it would be in my best interest.

2.9 **Permission for further studies:** Before my/*the participant's material is used in further projects in the future, the written approval of the Research Subcommittee C/Ethics Committee, Faculty of Health Sciences, will be obtained.

3. The information above was explained to me/*the patient/*participant by …………………………………………………………... *(name of relevant person)* in Afrikaans/*English/*Xhosa/*Other ………….……………………… and I am/*the participant/*patient is in command of this language/*it was satisfactorily translated to me/*him/*her by …………………………………. *(name of translator).* I/*The participant/*patient was given the opportunity to ask questions and all these questions were answered satisfactorily.

4. No pressure was exerted on me/*the patient/*participant to consent to participation and I/*the participant/*patient understand(s) that I/*the participant/*patient may withdraw at any stage without any penalization.

5. Participation in this study will not result in any additional costs to myself/*the participant/*patient.

*Delete where not applicable

B. **HEREBY CONSENT VOLUNTARILY TO PARTICIPATE IN THE ABOVEMENTIONED PROJECT/*THAT THE PATIENT/*POTENTIAL PARTICIPANT MAY PARTICIPATE IN THE ABOVEMENTIONED STUDY.**

Signed/confirmed at ……………………………….. *(place)*

on………………………… 20 …..... *(date)*

………………………… ………………………

Signature or right thumb print of   *Signature of witness*

patient/*representative of the patient/*participant

STATEMENT BY OR ON BEHALF OF INVESTIGATOR(S):

I, ……………………………………………………….……………………………,

declare that

- I explained the information given in this document to

  ……………………………………. *(name of the patient/*participant)* and/*or

  his/*her representative …………………….……… *(name of the representative);*

- he/*she was encouraged and given ample time to ask me any questions;

- this conversation was conducted in Afrikaans/*English/*Xhosa/*Other ……...……… and no translator was used/*this conversation was translated into ……………………... *(language)* by ………………………………… *(name).*

  Signed at ………………………………..….. *(place)*

  on……………………..………...20 …. *(date)*

………………………………

Signature of investigator/*investigator's representative

………………………………

Signature of witness

DECLARATION BY TRANSLATOR:

I, …………………………………..…..………………………………… *(name)*, confirm that I

- translated the contents of this document from English into …………………….. *(indicate the relevant language)* to the patient/*the patient's representative/*participant;

- explained the contents of this document to the patient/*participant/*patient's representative;

- also translated the questions posed by …………………….………………… *(name),* as well as the answers given by the investigator/*the investigator's representative; and

- conveyed a factually correct version of what was related to me.

Signed at …………….……………………… *(place)* on …………………………. 20 …… *(date)*

……………………….……   ……………………...

Signature of translator   *Signature of witness*

IMPORTANT MESSAGE TO PATIENT/*REPRESENTATIVE OF PATIENT/*PARTICIPANT:

Dear patient/*representative of the patient/*participant,

Thank you for your/*the patient's participation in this study. Should, at any time during the study,
- an emergency arise as a result of the research, or

- you require any further information with regard to the study, kindly contact Prof Gerhard Walzl at telephone number 9389158 (office hours) or the Lung Unit at telephone number 938 4911 (after hours).

19/02/02

bd/Guidelines

*Delete where not applicable