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Anti-microbial Immune Profiles in Obstructive Lung Diseases

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Anti-microbial Immune Profiles in Obstructive Lung Diseases

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A thesis submitted in partial fulfilment of the requirements of the University of Northumbria at Newcastle for the degree of Doctor of Philosophy

Research undertaken in the Faculty of Health & Life Sciences and in collaboration with the Freeman Hospital and Royal Victoria Infirmary, Newcastle upon Tyne

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Abstract

Obstructive lung diseases are major causes of morbidity and mortality globally. The most common, chronic obstructive pulmonary disorder (COPD), is caused by smoke inhalation, whilst bronchiectasis (BR) is often caused by lung infection, and cystic fibrosis (CF) is due to the defective passage of fluids through lung epithelial cells. Whilst it is now recognised that the healthy lung harbours a microbial flora, obstructive lung diseases are characterised by periodic or persistent heavy bacterial colonization that can be determined using microbiological cultures of patient sputum as well as by novel genomic analysis, the most common species identified being *Pseudomonas aeruginosa* and *Haemophilus influenzae*. Adaptive immune responses generated against such organisms, in the form of antigen-specific antibodies and T cells, may be protective against infection but may also contribute to the inflammatory disease mechanism such as through IL-17-mediated neutrophil recruitment. This thesis asks whether such immune responses can serve as biomarkers of microbial colonization and of disease; whether levels of antibodies and T cells indicate lung infection and exacerbations frequency. However, high levels may simply indicate exposure to microbes. Thus, the aim of this study was to measure antibody and T cell responses against a range of lung-colonizing microbes in patients with BR (n=119), COPD (n=58), CF (n=30), asthma (n=14), and in healthy controls (n=28). Patients were clinically characterised in terms of exacerbations, lung function, sputum microbiology and underlying disease. Enzyme linked immunosorbent assays (ELISAs) were set up to measure specific antibodies in serum, whilst T cell responses in peripheral blood mononuclear cells (PBMCs) were measured by specific Enzyme-linked ImmunoSpot assay (ELIspot), flow cytometry and multiplex cytokine ELISA. Typical microbial colonization for the given disease was seen in the sputum-producing patients. The results showed that in BR, specific IgG responses against

P. aeruginosa increased according to episodes of colonization, whilst T cell responses in the form of antigen-specific IFNγ showed the opposite effect, suggesting T cell dysregulation. As well as IFNγ, T cells were shown to secrete IL-17 and IL-22 in response to microbial antigens and to possess activation and homing receptors. Antibody responses were also further characterised for cross-reactivity and Ig isotype, confirming specificity and isotype switching. In CF patients that harboured *P. aeruginosa*, high anti-pseudomonas IgG titres were detected, and T cell IFNγ responses were similar to healthy controls and were associated with greater disease stability and FEV_1. In conclusion, immune responses were successfully characterised in patients with obstructive lung diseases, and specific antibody and T cells showed some associations with colonization and clinical disease, respectively, depending upon the disease and the microbe.

Presentations and posters related to the thesis

Oral presentations

- Fathia Jaat, Clare Lanyon, Anthony De Soyza, Stephen Todryk (2015). Anti-microbial immune profiling in obstructive lung diseases. Presented at Immunology North East conference, Durham, symposium dated, 18 June 2015.

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Declaration:

I declare that the work contained in this thesis has not been submitted for any other award from the work of others, and that it is all my own work. I also confirm that this thesis fully acknowledges the work of others. The work was carried out in collaboration with the Chest Clinic and Microbiology Department at the Freeman Hospital, Newcastle upon Tyne, and Cystic Fibrosis Clinic at the Royal Victoria Infirmary, Newcastle upon Tyne.

Name: Fathia Jaat

Signature: Fathia

Date: 07/02/2018

List of abbreviations

BR	Bronchiectasis
COPD	Chronic obstructive Pulmonary Disease
CF	Cystic Fibrosis
HV	Healthy Volunteers
PBMCs	Peripheral blood mononuclear cells
PMNs	Polymorphonuclear leukocytes
IL-	Interleukin
Ig	Immunoglobulin
PRRs	Pattern recognition receptors
PAMPs	Pathogen-associated molecular patterns
TLRs	Toll-like receptors
TNFα	Tumor necrosis factor alpha
DC	Dendritic cells
MHC	Major Histocompatibility Complex
Th	T helper
APC	Antigen-presenting cell
TCR	T-cell receptor
Fc	Constant fragment
FcR	Fc receptor
ADCC	Antibody-dependent cellular cytotoxicity
LPS	Lipopolysaccharide
TGF-β	Transforming growth factor β

IFNγ	Interferon gamma
AEC	Alveolar epithelial cells
RTIs	Respiratory tract infections
BAL	Bronchoalveolar lavage
FVC	Forced vital capacity
FEV_1	Forced expiratory volume in one second
HRCT	High-resolution computed tomography
GOLD	Global initiative for chronic obstructive lung disease
Non-CFBR	Non cystic fibrosis bronchiectasis
ELISA	Enzyme Linked Immunosorbent assay
ELIspot	Enzyme-Linked ImmunoSpot assay
ANOVA	One-way analysis of variance
Mw	Molecular weight

1. Chapter One: General Introduction and Literature Review

The aim of this thesis is to examine the specific anti-microbial immune responses – both T cell and antibody – in the blood of patients with obstructive lung diseases compared to healthy volunteers (HV). The patient groups include those with bronchiectasis (BR), chronic obstructive pulmonary disease (COPD), asthma and cystic fibrosis (CF). The patients were characterized clinically to varying degrees and had microbiology carried out on any sputum they produced at the time of blood sampling. As adaptive and innate immune responses are closely linked, the literature review describes both aspects of the immune response, as well as lung structure and physiology, and then describes lung diseases in terms of the roles played by microbial infection and immune responses in their pathology.

1.1 The innate immune response

The immune system is a complex defence mechanism made up of molecules, cells and organs, which has effectively evolved to defend the body from invasive microbial diseases. Two inducible immune mechanisms are successively activated during infection and work in a coordinated manner to ensure rapid and effective microbial elimination (Albiger *et al.*, 2007). These are the innate and adaptive arms of the immune system.

Innate defence consists initially of physical defence, including the cough reflex, mucociliary clearance and the antimicrobial properties of the mucosal surface. These constitute the first barrier in the host' defence (Strieter *et al.*, 2002). In addition, phagocytic defence is provided by macrophages, neutrophils, basophils and eosinophils; which are recruited in response to infections, recognize, and respond to microbes by initiating phagocytosis and/or

degranulation. The failure of such local host defences may lead to microbial colonization and subsequent pathogenic infection (Bals and Hiemstra, 2004).

1.1.1 Mucus

Mucus is present on the moist, and internal surfaces of the human body (Knowles and Boucher, 2002). It plays an important role as an adequate mechanical protective barrier of the primary airway against many of the particles, microbes and allergens found in inhaled air (Hollingsworth and Swanson, 2004, Rajavelu *et al.*, 2015). Mucus prevents the bacteria from becoming attached to the epithelium, and those bacteria which cannot attach due to the mucus can be cleared by mucus-ciliary clearance (Quie, 1986).

Discussions of the role of the mucus layer in protection, as suggested by various studies show alteration in mucins secretion during inflammatory conditions through altered goblet cell responses in germ-free animals and by enhanced mucus secretion in response to infections (Kim and Khan, 2013).

Mucins are the main constituents of lower airway mucus, and several mucins (including MUC2, MUC5AC, MUC5B, MUC6 and MUC8) are secreted in the lower airways (Henke *et al.*, 2007). Furthermore, mucins can bind and trap inhaled particles and clear them from the lung because of the extraordinary diversity of their carbohydrate side chains. Mucin can also bind to all particles and pathogens at the cell surface of the airway and can thus neutralize pathogens and eradicate them from the lung (Kim and Khan, 2013, Knowles and Boucher, 2002).

Furthermore, polymeric mucins (glycoproteins) represent the major macromolecular components of lung mucus and they form viscoelastic gels by interacting with other mucins and proteins (Caramori *et al.*, 2009). Moreover, MUC5AC and MUC5B are the main mucins in lung secretions in patients with different obstructive lung diseases. MUC5AC mRNA

expression is associated with goblet cells in the epithelium of the conducting airway, while MUC5B mRNA is present in mucosal cells of the submucosal glands (Caramori *et al.*, 2009)

1.1.2 Macrophages

Macrophages are vital phagocytic cells that are found in all tissues and are differentiated from monocytes within circulating peripheral blood mononuclear cells (PBMCs), which migrate into the tissue in response to inflammation (Sunderkotter *et al.*, 2004). Macrophages have multiple functions in the innate immune response, including phagocytic defence (uptake and killing of microbes), recognition of exogenous and endogenous molecules, antigen presentation (AP) and activation of the adaptive immune response (Zhang and Mosser, 2008). Macrophages are involved in the clearance of cellular debris generated during tissue remodelling and effectively remove cells that have undergone apoptosis (Kono and Rock, 2008, Zhang and Mosser, 2008). Macrophages are subdivided into two subclasses: M1 macrophages and M2 macrophages. M1 macrophages, known as classically activated macrophages, exhibit enhanced microbicidal activity and secrete high levels of pro-inflammatory cytokines, such as the interleukins IL-1, IL-6, IL-23 and tumor necrosis factor alpha (TNFα). These cells are critical in protection against intracellular pathogens (Martinez *et al.*, 2008). M2 macrophages are known as alternatively activated macrophages, or wound-healing macrophages, and are important in defence against parasitic and fungal infections and for wound healing. The development of M2 macrophages is based on the production of IL-4, produced by basophils and mast cells in response to tissue injury, or microbial infections such as chitin (a structural biopolymer found in certain fungi) (Kreider *et al.*, 2007). These M2 cells also produce chitinase and chitinase-like molecules, which contribute to the clearance of parasites and fungi as well as wound healing (Mosser and Edwards, 2008).

1.1.3 Granulocytes

Polymorph nuclear leukocytes (PMNs) comprise granulocytes including neutrophils, eosinophils and basophils, which are important in the innate immune response. Neutrophils are the most abundant PMNs. They are differentiated in the bone marrow and released in to blood circulation through which they are directed by adhesion molecules and cytokines to infected tissues (Amulic *et al.*, 2012, Fuchs *et al.*, 2007). In response to microbial infections, neutrophils are rapidly recruited to sites of inflammation where they activate different antimicrobial properties, such as phagocytosis, the production of microbicidal reactive oxygen and nitrogen species (ROS and RNS) and the formation of neutrophil extracellular traps (NETs). NETs are formed of chromatin and specific granular proteins that bind and kill microorganisms (Sollberger *et al.*, 2016).

Eosinophils play a role in the allergic airway inflammatory response and are involved in host resistance to extracellular microorganisms and parasites, as well as antimicrobial activities against bacterial, viral and protozoan pathogens (Acharya and Ackerman, 2014). Eosinophils release granule proteins and toxic enzymes including major basic protein (MBP), eosinophil cationic protein (ECP), eosinophil peroxidase (EPO), eosinophil-derived neurotoxin (EDN), matrix metalloproteinases (MMPs) and reactive oxygen species, which target microbes, especially parasites, but also damage the host tissues in allergic inflammation (Forman *et al.*, 2016). Similarly, basophils are also involved in the allergic response to various IgE-dependent or -independent stimuli; they release effector molecules, including proteases, histamine, cytokines, pro-inflammatory chemokines and lipid mediators (Otsuka and Kabashima, 2015). They also contribute to immunoglobulin (Ig) synthesis and class switching and angiogenesis by producing cytokines such as IL-6 and IL-3. It has been established that they are involved in antigen presentation to $CD4^+$ or $CD8^+$ T cells via the class I or class II major histocompatibility complex (MHC) (Schneider *et al.*, 2010).

1.1.4 Toll-like receptors

The challenge for the innate immune system is discrimination between potential pathogens and the self. This is achieved by a variety of pattern recognition receptors (PRRs) expressed on the surface of innate cells which are mostly membrane-bound (Kumar *et al.*, 2009). PRRs recognize conserved microbial molecules known as pathogen-associated molecular patterns (PAMPs) generated by a wide range of pathogens. The recognition of PAMPs via PRRs leads to the activation of the pro-inflammatory pathway (Abreu and Arditi, 2004). The main category of PRRs is toll-like receptors (TLRs), which are type I transmembrane proteins characterized by extracellular leucine-rich repeat (LRR) motifs that mediate the recognition of PAMPs, transmembrane domains, and COOH-terminal signal domains, homologous to IL-1 receptors, required for signal transduction. Eleven human TLRs have been identified. These are divided into two groups, depending on their extracellular localization and respective PAMPs. For example, TLR1, TLR2, TLR4, TLR5, TLR6 and TLR11 are present on cell surfaces and have the ability to recognize microbial cell wall components such as lipids, polysaccharides and lipoproteins. The TLRs in the other group, TLR3, TLR7, TLR8 and TLR9 and IL-10 are found within intracellular vesicles, such as endosomes and lysosomes, and recognize microbial nucleic acids (Abreu and Arditi, 2004). TLRs are expressed on dendritic cells (DCs) and other antigen-presenting cells (APCs); these cells pick up the antigens, process them into small peptides, then migrate to the local lymph tissues to present the antigenic peptides on the MHC for recognition by T cells. These events involve cytokine and chemokine secretion and accessory signals. Thus, TLRs are a key component of the innate immune response and the activation of antigen-specific adaptive immune responses (Iwasaki and Medzhitov, 2004).

1.1 The adaptive immune response

The adaptive (acquired) immune response differs from the innate immune response as it is more advanced and complex, and is the antigen-specific branch of the immune system. Two main cellular subsets, T and B lymphocytes, comprise the adaptive immune response, which is characterized by antigen specificity, diversity and memory (den Haan *et al.*, 2014). It is highly regulated and slower to respond than the innate response. B cells are responsible for antibody production, while T cells control the immunological synapse and are important effectors in eliminating infection. Both B and T lymphocytes require the collaboration of the DCs for their priming (den Haan *et al.*, 2014).

B cells are named the Bursa, an equivalent organ in birds, in which they were first identified. In mammals, B cells mature in the bone marrow (bone marrow–derived). B cells are characterized by the expression of surface-bound Ig, which can bind to antigens (LeBien and Tedder, 2008). Once B cells encounter and recognize antigens and receive cytokines secreted by antigen-presenting cells and costimulatory signals from T helper (Th) cells, they rapidly differentiate into plasma cells or memory cells. Plasma cells will secrete soluble antibody that binds to the activating antigen, leading to the clearing of the infection associated with it. However, memory B cells reside in the body and provide long-term immunity to this particular antigen (LeBien and Tedder, 2008, Pieper *et al.*, 2013).

T cells (thymus-derived) represent the cell-mediated immune response and express surface T-cell receptors (TCRs). They can recognize foreign antigens previously digested and presented by APCs via MHC class I and class II molecules (Cooper and Alder, 2006). Other transmembrane proteins within the TCR complex are involved in triggering T cells. Activated T cells, if $CD4^+$, produce cytokine profiles (described later) that regulate other immune cells, while $CD8^+$ differentiate into cytotoxic T lymphocytes (Cooper and Alder, 2006, Flajnik and Du Pasquier, 2004). Broadly, Th_1 responses play a major role in killing

intracellular pathogens, whereas antibody responses destroy extracellular pathogens. Furthermore, T cells operate to alter the balance between eradicating pathogens and avoiding immune-mediated tissue injuries. The failure to clear the pathogens and dysregulated inflammation in the lung can cause bronchial wall damage (Grimwood, 2011, Strieter *et al.*, 2002). For the purposes of this thesis, antibody and T cells are discussed next.

1.2.1 Immunoglobulins (Igs) – structure and function

Immunoglobulins (Igs) were discovered in 1890 by von Behring and Kitasato when they observed substances in the blood that can neutralize the diphtheria toxin, and a year later, these substances were named antibodies (Schroeder and Cavacini, 2010). Igs are a "long range weapon" produced by activated B lymphocytes, and bind to molecular determinants (antigens), leading to neutralization and/or the removal of the antigen or associated microbe (Kepler, 2013). Each B cell produces a single specific antibody determined by its antigen-binding site. Once naïve or memory B cells are activated by antigens, and aided by Th cells, they differentiate into antibody-secreting effector cells known as plasma cells. Some plasma cells continue to secrete antibodies for years and some die after secretion (Alberts *et al.*, 2002).

Igs are gamma globulin proteins belonging to the Ig super-family (IgSF). The general structure of Igs is Y-shaped and the molecules are large, approximately 150–190KDa. The core of all Ig molecules is the same and consists of two heavy chains (HCs), each usually containing approximately 440 amino acids, and two light chains (LCs) containing approximately 220 amino acids. Genetically determined, each B cell only produces one LC sequence and one HC sequence. Therefore, the two HCs and two LCs in Ig molecules are identical. The two HCs are joined by covalent disulphide bonds and each HC is connected to an LC by an extra disulphide bond (Mak and Saunders, 2005, Schroeder and Cavacini, 2010). Each HC has only one variable (VH) domain at the N-terminus and three or four

constant domains, whereas LCs contain one variable (VL) domain and one constant (CL) domain at their N-terminus. Both VH and VL determine the specificity of the B cell receptor (BCR) in antigen recognition (Anelli and van Anken, 2013). All antibodies have two antigen-binding fragments (Fab) and thus are described as bivalent. The V regions contain three short amino acid sequences, which are highly variable between Ig molecules, called hypervariable (HV) or complementarity determining regions (CDRs) (Figure 1.1) (Mak and Saunders, 2005). If an antigen has three or four repeating antigenic determinants, an antibody molecule can cross-link into a large lattice. This lattice can be rapidly phagocytosed and degraded by macrophages (Alberts *et al.*, 2002). The function of antibodies is twofold due to the Fab and the constant fragment (Fc) of the HC, which allow the antibody to engage in different activities such as complement activation, binding to phagocytic cells and crossing the placenta from mother to foetus. Recent studies of antibody-antigen crystal structures have shown that while the VH and VL domains of an antibody are involved in forming the antigen binding site, not all antibodies use all six CDRs to interact with the antigen; for example, when the antibody binds polysaccharide or small peptide antigens (Mak and Saunders, 2005).

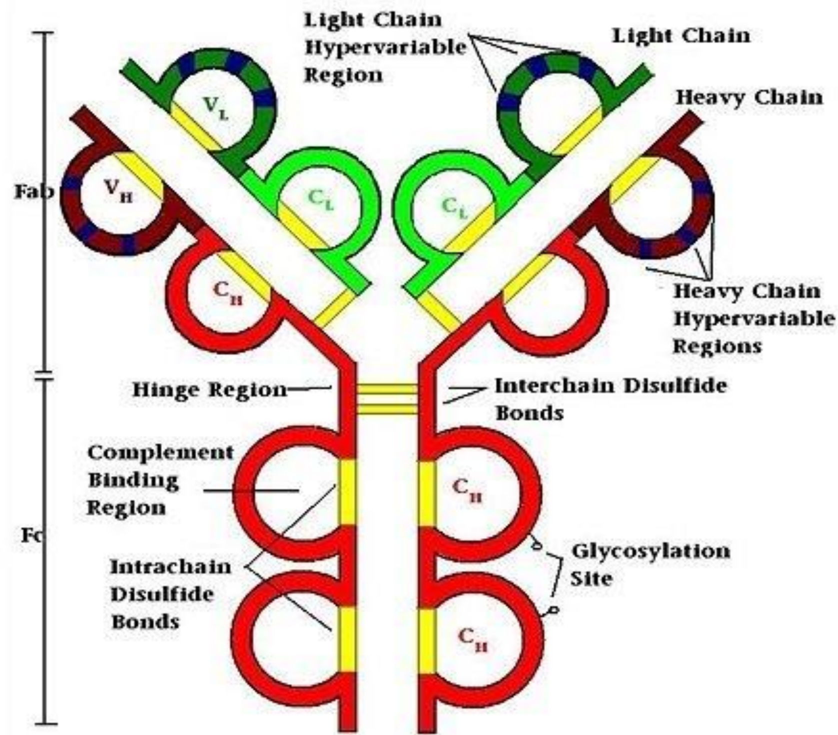


Figure 1.1: Typical structure of antibody.
An antibody is formed by two identical HCs and two identical LCs joined by covalent disulphide bonds. Each HC and LC is joined by an extra disulphide bond. Each HC has three constant (CH) domains and one variable (VL) domain. Each LC has one variable (VL) and one constant (CL) domain. The Fab is composed of one LC and part of a HC. The other part of the HC chain contains the Fc-receptor-binding sites (Martz et al., 2013)**.**

1.2.2 IgG Fc receptors

The constant region of the Fc domain of the Ig HC can bind to Fc receptors (FcRs) that are expressed on different immune cell types – particularly phagocytes – and are present as stimulatory and inhibitory pairs. The binding of Fc receptors to the antibody provides a wide range of responses, ranging from the effector function, including phagocytosis, and antibody-dependent cellular cytotoxicity (ADCC), to the secretion of cytokines or other inflammatory mediators (Molfetta *et al.*, 2014). FcγR is encoded by an IgSF of eight genes clustered on the long arm of chromosome 1. The diversity of the FcγR family allows the Ig complex to stimulate various cell functions related to autoimmunity, inflammation and host defence versus microbes and cancer (Salmon and Pricop, 2001). FcγR is capable of triggering cellular activation because it possesses intracellular activation motifs, termed

immunoreceptor tyrosine-based activation motifs (ITAMs), similar to those of BCRs and TCRs. Three different classes of human receptor for IgG (FcγR) have been identified (Figure 1.2): FcγRI (CD64), FcγRII (CD32) and FcγRIII (CD16). Stimulatory FcγR is a multichain receptor composed of a ligand-binding α subunit associated with ITAMs. This receptor has two isoforms known as FcγRI (CD64), a high-affinity FcR for IgG1 and IgG3, which binds monomeric IgG and comprises three extracellular Ig-like domains in a ligand-binding chain. FcγRIIIa is an intermediate-affinity receptor binding only multivalent IgG (Woof and Burton, 2004). In addition, there are three single stimulatory FcγRs, termed FcγRIIa, FcγRIIc and FcγRIIIb, which have only two extracellular Ig-like domains and ITAMs and bind with low affinity for IgG. However, FcγRIIB is an inhibitory isoform containing an immunoreceptor tyrosine-based inhibitory motif (ITIM) which transmits signals leading to the negative regulation of immune complex-triggered activation. Therefore, the structural diversity of FcγR leads to differences in binding capacity, different signal transduction pathways and cell type-specific expression patterns (Salmon and Pricop, 2001, Schroeder and Cavacini, 2010).

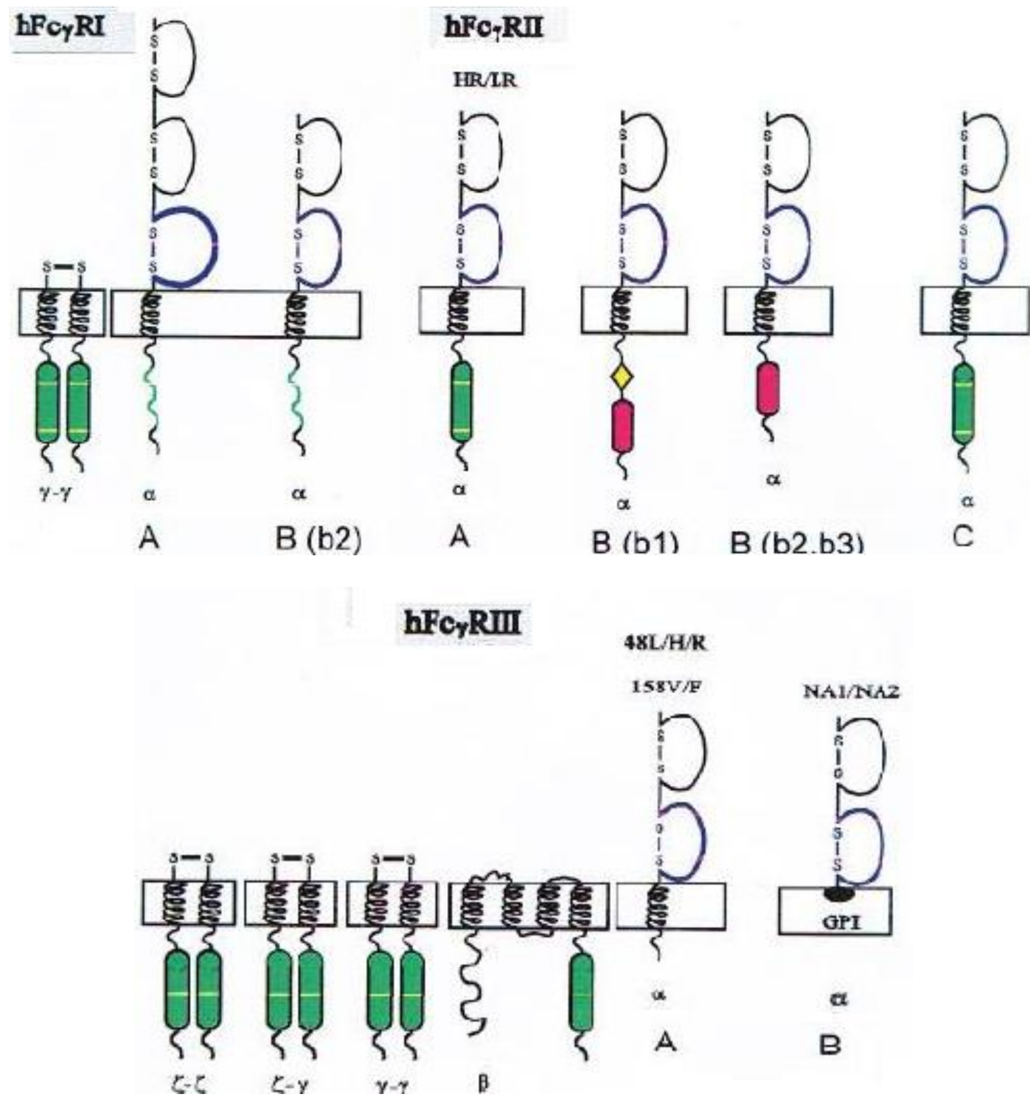


Figure 1.2: Schematic diagram of human Fcγ receptors.

FcγRI and FcγRIIIa are multichain receptors associated with ITAMs.FcγRIIa and FcγRIIc are single-chain stimulatory receptors also associated with ITAMs. FcγRIIIb is the only Fcγ lacking ITAMs. FcγRIIb is an inhibitory receptor comprising ITAMs. A, B, and C; b1, b1', b2, and b3; and α, β, γ, and ζ are referred to multiple genes in each receptor class, their alternative spliced products, and their individual subunits respectively. Adopted from (Gessner et al., 1998).

1.2.3 B cell proliferation and immunoglobulin (Ig) gene rearrangement

B cells are generated from pluripotent hematopoietic stem cells and develop in the bone marrow through successive steps in an ordered series to produce an Ig repertoire. Pro-B cells are the first irreversible committed B cell precursors, distinguished from pre-B cells by the expression of CD19 on the surface as well as increased expression of heat-stable antigen

(HSA). In these cells, the expression of Igα and Igβ heterodimer is associated with calnexin and other chaperone molecules (Wang and Clark, 2003). The developmental steps of the rearrangement of the BCR are tightly regulated by positive and negative selection steps and culminate in the formation of immature B cells that migrate to peripheral lymphoid tissues (Kato *et al.*, 2013).

Variable (V), diversity (D) and joining (J) gene segments are assembled to generate an Ig variable region gene through a site-specific somatic recombination mechanism called the V(D)J recombination (Chen *et al.*, 1993). The Ig gene rearrangement occurs primarily in the foetal liver and adult bone marrow at the pro-B cell stage, is initiated at the HC gene locus (IGH) and precedes the Ig LC gene rearrangement; D_H to J_H joining leads to V_H to DJ_H joining. This procedure is mediated by several enzymes involving different proteins operating at the DNA level and is controlled by recombination signal sequences (RSSs) that flank the V, D, and J gene segments (LeBien and Tedder, 2008). The assembly of the LC gene is generated by the joining of the V_L to the J_L segment and the expression of the Ig HC gene. The arrangement of the LC occurs at the Ig kappa locus (IGK) and, if the functional IGK rearrangement fails, the Ig lambda locus (IGL) undergoes recombination (Gonzalez *et al.*, 2007). The assembly of the IgH and IgL protein complex on the B cell surface generates BCRs and allows B cells to proceed with maturation, thus avoiding apoptosis (Gonzalez *et al.*, 2007). Immature B cells that successfully display Ig on the cell surface exit the bone marrow and migrate to the germinal centres of lymph nodes where they are then selected by antigens for activation, inactivation, or eradication (Tiegs *et al.*, 1993). In particular, the interaction between B cells, antigen-presenting cells and T cells helps B cells to carry antigen-specific receptors on their surface. Somatic hypermutation (SHM) and class-switching recombination (CSR) occur at this stage. High-affinity antibodies are produced via SHM, which allows the secreted antibodies to recognize and bind foreign antigens (Klein *et al.*, 1998). Following an encounter with an antigen, IgM is first produced and then the

mechanism of CSR switches an isotype to an IgG-, IgA- or IgE-secreting specific antibody that represents the final step in the B cell differentiation cascade (Gonzalez *et al.*, 2007).

1.2.4 B cell signalling pathways

BCRs are multimeric complexes comprising an antigen-recognition and membrane-bound (mIg) proteins, associated non-covalently with the heterodimer of Igα and Igβ. Only IgG has a long cytoplasmic tail, whereas the tails of the other five types of mIg are extremely short and lack signalling capacity (Wang and Clark, 2003). In mature B cells, following Ag interaction at the BCR, the mIg initiates events in the biochemical cascade leading to the phosphorylation of tyrosine within the ITAM (a motif found within each cytosolic tail of Igα and Igβ) by the Src family member tyrosine kinase (Fremd *et al.*, 2013). The phosphorylation of ITAM tyrosine enhances the recruitment and activation of Syk, which is the main kinase that drives many signalling pathways, including the activation of phospholipase Cγ2 and Ras. However, not all signalling requires Syk, as the activation of nuclear factor NF-κB interacts directly with one or more Src family kinases (Irish *et al.*, 2006, Niemann and Wiestner, 2013). In addition, other co-receptor molecules, such as CD19, CD21 and CD22, are associated with the BCR multimolecular complex and participate in the signalling pathway.

Other signals for B cells are created by cytokine and stimulatory molecules as B cells present their antigens on MHC class II to Th cells, to obtain additional activation signals. The CD40 ligation of CD40 on B cells with CD40L on T cells provides additional signals for proliferation and antibody secretion and it is the key molecule in human T-B interaction (Kato *et al.*, 2013). CD40–CD40L engagement induces high levels of Ig secretion by the B lymphocyte, but only in the presence of cytokines such as IL-4, IL-13 and IL-10. This process promotes the differentiation and activation of B cells to undergo class-switching (Garraud and Nutman, 1996, Kato *et al.*, 2013).

1.2.5 B cell activation through binding to proteins and polysaccharide antigens

Antigens that activate B cells and stimulate the response are classified into two groups:

- Thymus-dependent (TD or T cell-dependent) antigens
- Thymus-independent (TI or T cell-independent) antigens

1.2.5.1 Thymus-dependent (TD) antigens

T cell-dependent antigens or TD antigens are soluble proteins picked up by specialized APCs, processed by endocytic compartments and presented as peptides on the MHC class II of APCs to antigen-specific $CD4^+$ T cells. The outcome is T cell activation and the up-regulation of adhesion molecules on T cells and the expression of co-stimulatory molecules for CD40 (i.e. CD40L) (MacLennan *et al.*, 2003). T cells then interact with B cells that display the same peptide MHC II molecules and trigger them via CD40 and through the secretion of certain cytokines, such as IL-2, IL-4, IL-10 and IL-13. These cytokines have key functions in the activation of B cell growth and differentiation into plasma cells that produce high-affinity antibody of multiple isotypes. TD antigens also induce lasting immune responses, as well as forming memory B and T cells (Abbas *et al.*, 1993, Garraud and Nutman, 1996, Lesinski and Westerink, 2001).

1.2.5.2 Thymus-independent (TI) antigens

Unlike T cell-dependent antigens, T cell-independent (TI) antigens are large multivalent molecules that produce multiple domains and interact with many mIg, resulting in highly cross-linked membranes that lead to a high level of B cell activation. Thus, TI antigens have the ability to induce enhanced mIg signalling without T cell help and do not induce memory (Lesinski and Westerink, 2001). Relatively low antigen concentration is necessary to achieve the threshold level of B cell activation that is required for stimulating B cells. A lower

antigen concentration may reduce the level of modulation and disappearance of Ig from the surface of antigen-specific B cells, allowing prolonged binding between antigens and mIg, therefore regulating B cell signalling. This may explain the ability of TI antigens to induce a humoral response without T cell help (Mond *et al.*, 1995).

TI antigens are further classified into two classes: TI-1 antigens, such as bacterial lipopolysaccharide (LPS), are known to be potent B cell mitogens that lead to polyclonal B-cell activation. These antigens do not produce memory or the isotype switching of antibodies (Lesinski and Westerink, 2001). In contrast, TI-2 antigens are represented by highly repetitive structures expressed on the surface of pathogens, mainly polysaccharides. Although these reactive B cells do not require T cell help, it has been shown that without IL-2 the T cells are unable to induce Ig secretion when cultured alone with resting B cells (Haas *et al.*, 2005). After the addition of T cell-derived factors, Ig secretion is then induced. Recent studies have found that a type of natural killer (NK) cell provides help for the Ig secretory response and regulates immune responses through the release of various cytokines. These cytokines include colony-stimulating factor (CSF), transforming growth factor β (TGF-β), interferon gamma (IFNγ) , IL-1, IL-2 and IL-8, which have all been shown to be important in the immune response (Mond *et al.*, 1995). Furthermore, the polysaccharide antigen of *Streptococcus pneumoniae* can provoke B cell activation in the absence of T cell help by crosslinking BCR in a different way. A recent study found that pneumococcal polysaccharides, such as a vaccine preparation containing a TLR agonist that can be extracted by phenol, are critical for their capacity to induce antibody production. In addition, cytokines released from DCs and other leukocytes in response to such microbial products can contribute to TI-2 antibody responses, especially the response to whole pathogens (Lanzavecchia and Sallusto, 2007). The activation of B lymphocytes typically involves binding mIg to various molecules, such as a bacterial polysaccharide, LPS or proteins. The major sources of polysaccharides are the bacterial membrane and cell wall, resulting in

capsular polysaccharides and exopolysaccharides respectively (Leung *et al.*, 2006). LPS or endotoxin is the main part of the outer membrane of all Gram-negative bacteria and serves as an initial elicitor of the inflammatory response, indicating bacterial infection for the mammalian host defence system (Triantafilou *et al.*, 2001). The main differences between Gram-negative bacteria and Gram-positive bacteria are the lack of an outer membrane and the presence of a thicker peptidoglycan wall in Gram-positive bacteria.

The receptors and binding proteins of polysaccharide are associated with innate immunity, including TLRs, scavenger receptors (SRs), complement receptor type 3 (CR3) and β-glucan receptors. Indeed, TLRs are expressed on myeloid cells such as macrophages, monocytes and DCs. The main TLRs that recognize polysaccharide are TLR-2 and TLR-4. SRs are receptors on macrophages, DCs and endothelial cells. CR3 is mainly expressed on macrophages, neutrophils and some types of B and T cells. β-glucan is expressed on a wide range of cells, including eosinophils, B and T cells, monocytes and DCs (Leung *et al.*, 2006). Stimulation with LPS requires the interaction of LPS with several proteins, including LPS-binding protein (LBP), CD14, MD-2 and TLR4. LBP is a soluble shuttle protein that directly binds to LPS and helps the association between LPS and CD14. CD14 is a glycosylphosphatidylinositol cell surface 55-kD glycoprotein present on cells of the myeloid lineage, which also exists in a soluble form, mediating the transfer of LPS to the TLR4/MD-2 receptor complex and modulating LPS recognition. MD-2 is a soluble protein that non-covalently associates with TLR4, but it can directly form a complex with LPS in the absence of TLR4. Thus, stimulation by LPS involves the participation of various molecules to induce critical inflammatory cytokines, important to elicit potent immune responses (Lu *et al.*, 2008).

1.2.6 Somatic hypermutation (SHM)

SHM operates following the assembly of Ig genes on activated B cells through their corresponding antigens and costimulatory signals from Th cells. SHM occurs at a rate of 10^{-5} to 10^{-3} changes per base pair per cell generation (Li *et al.*, 2004). These mutations are targeted at the V region gene to exert various actions resulting in nucleotide changes that alter the amino acid sequence and protein structures clustered in the CDRs of Ig V-region genes and allow B cells to gain amino acid differences in the variable domain. However, silent mutations that preserve the amino acid sequence and retain the same protein structure are scattered through the V region (Murphy, 2011). Therefore, SHM increases antibody diversity and affinity maturation in response to recurrent immunization or exposure to antigen (Schroeder and Cavacini, 2010).

1.2.7 Antibody class switching

1.2.7.1 Class switching recombination (CSR)

CSR is a programmed gene rearrangement that occurs after the activation of a mature B cell. It takes place in a germinal centre through a DNA-editing enzyme called activation-induced cytidine deaminase (AID) (Figure1.3). CSR enables B cells to change the heavy-chain constant region (CH) while maintaining the same variable domains, resulting in a change of antibody class (Muramatsu *et al.*, 2000, Winter *et al.*, 2003). This change occurs by replacing the Ig CH from Cμ with one of the other CH genes (Cγ, Cα, or Cε). It occurs between the 1–10 kb tandem repetitive unit sequences, called the S regions, located upstream or in the 5′ region to each CH gene (Winter *et al.*, 2003). The Ig isotype is changed from the initial IgM that utilizes a Cμ constant region to generate IgG, IgA or IgE. Each isotype is capable of determining a way of eliminating captured antigens or the location where Ig is delivered (Kaminski and Stavnezer, 2004, Muramatsu *et al.*, 2000). The expressed V(D)J domains and

light chain do not change; therefore, the antibody specificity is not affected. Different Ig classes have been found in most vertebrates and represent the various effector functions of the humoral immune response. Mice, rats and humans have five classes, comprising IgM, IgD, IgG, IgE and IgA. IgG contains four subclasses (IgG1, IgG2, IgG3 and IgG4 in humans) and the IgA class comprises IgA1 and IgA2 (Chaudhuri and Jasin, 2007, Stavnezer, 1996).

High-affinity antibody production requires the formation of an immunological synapse between T and B cells. The synapse is generated by communication between T cell and B cell receptors through the MHC, which incorporates small peptides derived from macromolecules (particularly bacteria and viruses) processed in specialized Ag-presenting cells (APCs). This allows B cells to form a composite ligand called the peptide-MHC complex (pMHC), which can be recognized by TCRs in the immunological synapse. Since B cells use their mIg receptors to capture the antigens, then the more pMHC generated in the immunological synapse, the better the BCR affinity (Dustin, 2014). In humans, the secretion of different Ig isotypes is associated with immune responses to various pathogens. Indeed, viral infections and protein antigens lead to high production of both IgG1 and IgG3. Encapsulated bacteria expressing polysaccharide-rich Ag, such as streptococcus and haemophilus, result in the production of an IgG1, IgG2 and IgG4 response (Cavacini *et al.*, 2003). Pathogens entering the mucosal barrier elicit the response of IgA. Moreover, the IgG subclasses can be altered during an immune response. For instance, during an acute phase, virus-specific IgG1 is initially elicited, whereas IgG3 is produced later (Avery *et al.*, 2008).

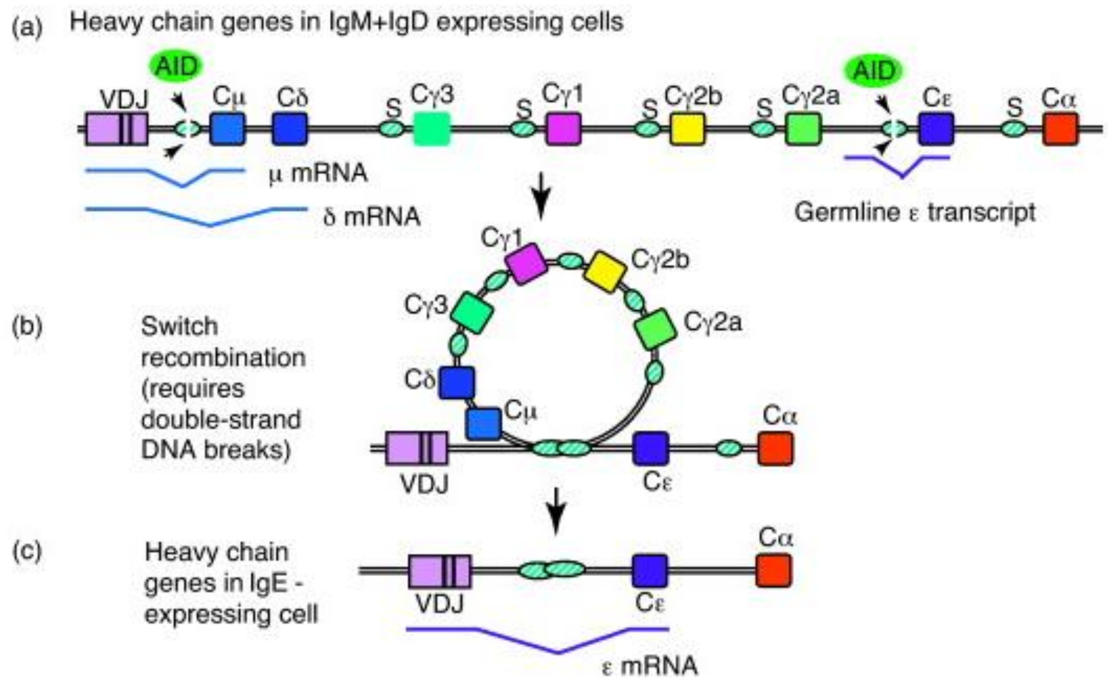


Figure 1.3: **Diagram of class switch recombination (CSR).**
(a) illustrates Ig HC genes in a B cell that expresses IgM and IgD (through alternative RNA processing), CSR occurs when AID remains within S regions that are transcriptionally active, and RNA transcripts from the un-rearranged HC genes are called germline transcripts; (b) during CSR from IgM to IgE. The intra-chromosomal deletional recombination between two switch (S) regions and the DNA in between Sμ and Sε is excised from the chromosome as a circle; (c) after CSR, the identical VH region originally expressed with the Cμ gene is now altered, with the Cε gene in cells expressing IgE (Stavnezer, 2011).

1.2.7.2 Antibody classes and isotypes

- ***IgM***

Monomeric IgM is the first isotype expressed during B cell development within the immune response and is produced before B cells undergo the process of SHM. IgM constitutes approximately 5–10% of total antibody in the serum. It is a low-affinity molecule capable of binding simultaneously to multivalent antigens such as bacterial capsular polysaccharides (Winter *et al.*, 2003). IgM is mainly found in the blood, the mucosa and to a small extent in the lymph. The low affinity of the monomeric component of IgM molecules, because of its immaturity, can be enhanced in the pentameter through multimeric antigen–antibody interactions, especially when the antigen contains multiple repeating epitopes. IgM

antibodies are also called natural antibodies, produced against a variety of cross-reactive repeating antigens (Winter *et al.*, 2003). Furthermore, IgM is effective at activating the complement system and thus combatting extra cellular infections (Janeway *et al.*, 2001).

- ***IgA***

IgA is the most abundant immunoglobulin produced in the body, providing the first line defence mechanism against many microbial pathogens that encounter mucosal surfaces (Agnello *et al.*, 2013, Twigg, 2005). Most plasma cells producing IgA reside in the lamina propria (LP) of mucosal organs, and IgA is polymeric (a dimer) with an integrated joining (J) chain associated with the polymeric Ig receptor (pIgR) on the epithelial cell layer. When transported to the apical side via a process called transcytosis, in which the pIgR is cleaved, IgA passes and is circulated into the mucosal lumen (Agnello *et al.*, 2013, Macpherson *et al.*, 2008). Secretory IgA is polymeric, whereas serum IgA is mainly monomeric. The protective functions mediated by secreted IgA take place via high-affinity interactions, inhibiting the binding of antigen to the mucosal surface and neutralizing toxins and microbes. Low-affinity interaction helps contain the dense commensal microbiota residing in the intestinal lumen (Macpherson *et al.*, 2008). Two IgA isotypes are found in humans – IgA1 and IgA2. IgA1 comprises 80–85% of total serum IgA, whereas IgA2 is mainly found in the secretions of the mucosal organs (Brandtzaeg and Johansen, 2005, Twigg, 2005). The molecular events eliciting an immune response of IgA1 and IgA2 remain unclear, but secreted IgA2 generally operates against lipopolysaccharide whereas secreted IgA1 is predominantly stimulated by protein antigens (Brandtzaeg and Johansen, 2005).

- ***IgG***

IgG is the predominant isotype in the blood and extracellular fluid and is the main characteristic of a secondary immune response produced after antibody class switching. It accounts for 80% of total Ig in the serum and is efficient in the recognition, neutralization

and removal of pathogens and other toxic antigens by virtue of its opsonizing and complement-activating properties (Kaneko *et al.*, 2006). Its small molecular size allows the IgG to cross from the blood into the tissue, as well as enabling transplacental transfer (Goldsby *et al.*, 1939). Four IgG subclasses are found in the human serum; these subclasses were discovered in the 1960s following extensive studies using specific rabbit antisera against human IgG myeloma proteins (Vidarsson *et al.*, 2014). The subclasses of IgG are distinguished by differences in HC sequences, with diverse amino acid sequences encoded by different germ-line CH genes (Figure 1.4). These subclasses – IgG1, IgG2, IgG3 and IgG4 – are ordered by their decreasing average serum concentrations in the blood of healthy individuals (Twigg, 2005). The different amino acid compositions of the subclasses affect the biological properties of the Ig (Table 1.1). Different IgG subclasses are responses to various types of antigens. The response against soluble protein antigens and membrane proteins elicits IgG1, while bacterial capsular polysaccharide antigens induce the response of IgG2 (Vidarsson *et al.*, 2014). Furthermore, IgG3 antibodies have the potential to induce effector functions that culminate in excessive inflammatory responses. IgG4 are particularly induced by exposure to allergens (Twigg, 2005, Vidarsson *et al.*, 2014).

- ***IgE***

IgE was discovered in 1967 as an Ig isotype distinct from IgG, IgA, IgM and IgD. IgE plays a major role in immediate hypersensitivity reactions and is responsible for the symptoms of hay fever, asthma and anaphylactic shock (Hamilton *et al.*, 2010). IgE is synthesized in response to an allergen, this process being initiated when the allergen is taken up by APCs, processed and presented to Th2 cell receptors, resulting in the activation of T cells and the expression of IL-4, IL-13 and CD154 (CD40 ligand). The engagement between CD154 and CD40 expressed on the APC leads to activation of the APC (mainly B cells), resulting in isotype switching and the secretion of allergen-specific IgE (Prussin and Metcalfe, 2006).

IgE responses are a key potent mechanism against nematode (worm) parasites, with the engagement of parasite antigen with IgE on mast cells promoting parasite ejection.

- ***IgD***

IgD was first discovered in 1965 in human serum as myeloma protein. More recently, it was identified on the surface of naïve mature B cells, being important in initiating BCR signal transduction (Edholm *et al.*, 2011). IgD is produced at very low levels in the serum with a short serum half-life that may be due to the sensitivity of the molecule to enzyme degradation. It is also co-expressed with IgM on the surface of mature B cells before antigenic stimulation and functions as an Ag-binding receptor (Schroeder and Cavacini, 2010). Structural differences in the H region of IgD, in length, amino acid arrangement and glycosylation provide a flexible T shape rather than the usual Y shape of other antibody isotypes, which may contribute to the different functions of IgD. It has been found that in the human upper respiratory mucosa, the constant region of IgD can bind to the protein of respiratory bacteria such as *Moraxella catarrhalis*, while their products can enhance mucosal immunity and B cell activation (Chen and Cerutti, 2011).

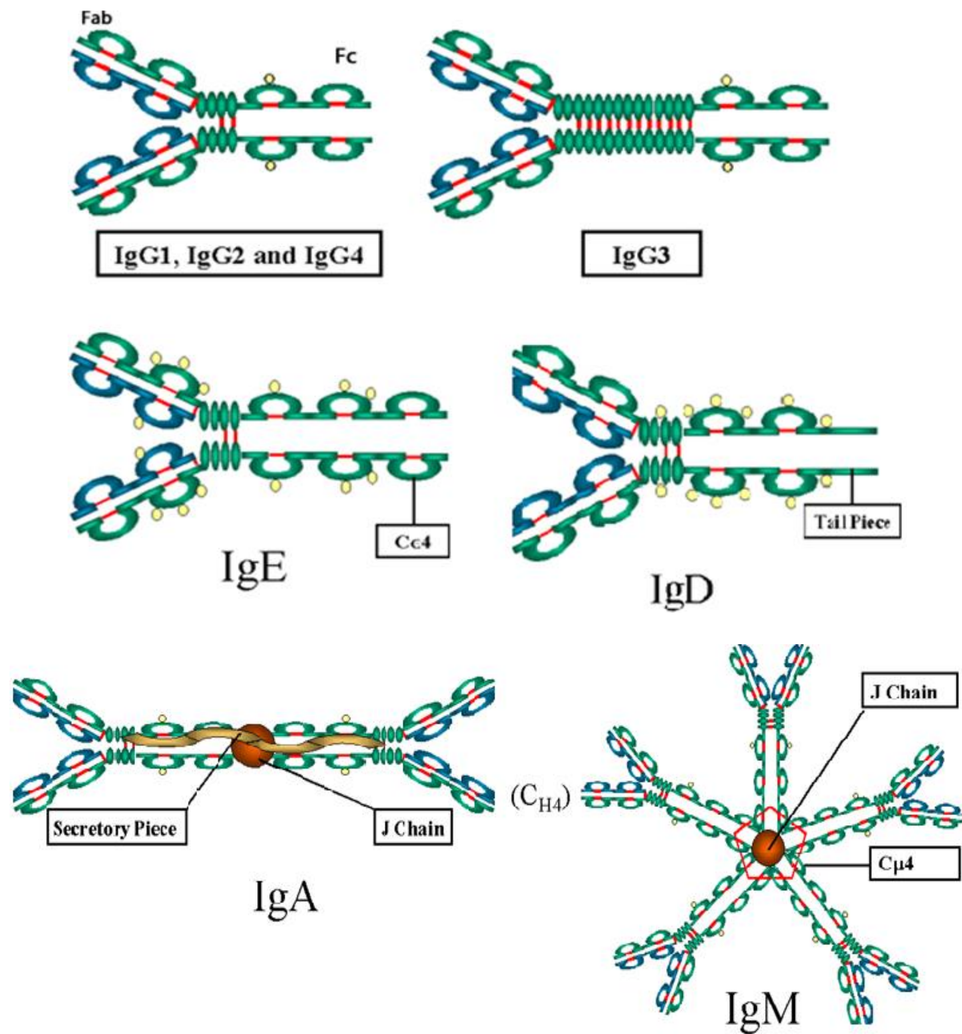


Figure 1.4: Schematic diagrams of Ig molecules.

IgG subclasses differ in terms of the number of disulfide bonds and the length of the hinge region. IgE exists as a monomer and has an extra domain in the constant region. IgD exists only as a monomer and has extra amino acids at the C-terminal end for anchoring to the membrane. IgM has an extra domain on the µ chain (CH4) and it has another protein covalently bound via an S-S bond called the J chain. IgA is a monomer and when expressed as a dimer, the J chain is associated with it (Mayer, 2015).

Table 1.1. Different IgG isotypes with specialized biological activity and unique distributions.

+ + + represents the major effector function of each isotype, + + and + represent lesser functions and minor functions respectively and - represents no function.*IgG2 is capable of acting as an opsonizer in the presence of Fc receptors (Janeway *et al.*, 2001).

Functional activity and distribution	IgM	IgD	IgG1	IgG2	IgG3	IgG4	IgA	IgE
Neutralization of toxins and microbes	+	-	+ +	+ +	+ +	+ +	+ +	-
Opsonization	-	-	+ + +	*	+ +	+	+	-
Sensitization for killing by NK cells	-	-	+ +	-	+ +	-	-	-
Sensitization of mast cells	-	-	+	-	+	-	-	+ + +
Activates complement system	+ + +	-	+ +	+	+ + +	-	+	-
Transport a cross epithelium	+	-	-	-	-	-	+ + +	-
Transport a cross placenta	-	-	+ + +	+	+ +	+/ -	-	-
Diffusion into extravascular sites	+ /-	-	+ + +	+ + +	+ + +	+ + +	+ +	+
Mean serum level (mg ml $^{-1}$)	1.5	0.04	9	3	1	0.5	2.1	$3x10^{-6}$

1.3 T cells

T cells are a form of lymphocytes that play a fundamental role in adaptive immunity. These cells can recognize wide ranges of peptide antigens presented by MHC class I or II molecules on the surface of APCs. This initiates an activating signalling cascade resulting in proliferation, differentiation and effector functions for direct or indirect pathogen clearance (Conley *et al.*, 2016, Tewari *et al.*, 2005). T cells differ from other leukocytes in the expression of either the αβ or γδ type of TCR on the cell surface (Naito *et al.*, 2011).

1.3.1 T cell development, positive and negative selection

T cells are derived from haemopoietic precursors in the bone marrow, enter the bloodstream and migrate into the thymus, where they are known as thymocytes. Thymocytes undergo the series of maturation processes based on the expression of cell surface markers. The cells here lack the expression of CD4 or CD8 receptors and are termed double negative (DN) cells ($CD4^-$, $CD8^-$) (Figure 1.5) (Cai *et al.*, 2007). Thymocyte migrants undergo the random development of multiple TCRs, which can recognize immunogenic peptides bound to MHC molecules. In the thymic cortex, the thymic cortical epithelial cells secrete factors (lymphokines) to regulate the development of the T cells. At this stage, two selection processes occur. Double positive (DP) ($CD4^+$, $CD8^+$) thymocytes undergo TCR gene rearrangement (Romagnani, 2006). Some DP cells undergo positive selection in the cortex. These cells receive a survival signal, promoting them for further development to the final stage of maturation, which is the single positive TCR^+ $CD4^+$ cell (Th-precursor) or $CD8^+$ cell (cytotoxic-precursor), based on the appropriate affinity of TCR for MHC-peptide. Most of the thymic DP cells die of neglect (no MHC-peptide recognition, no survival signals) (Petrović-Đjergović *et al.*, 2007, Romagnani, 2006). The single positive selected cells develop further in the thymic medulla; they are then released into circulation and are present in peripheral blood. Negative selection eliminates the cells that may react against self-molecules to avoid the potential cause of autoimmune reactions (Cai *et al.*, 2007).

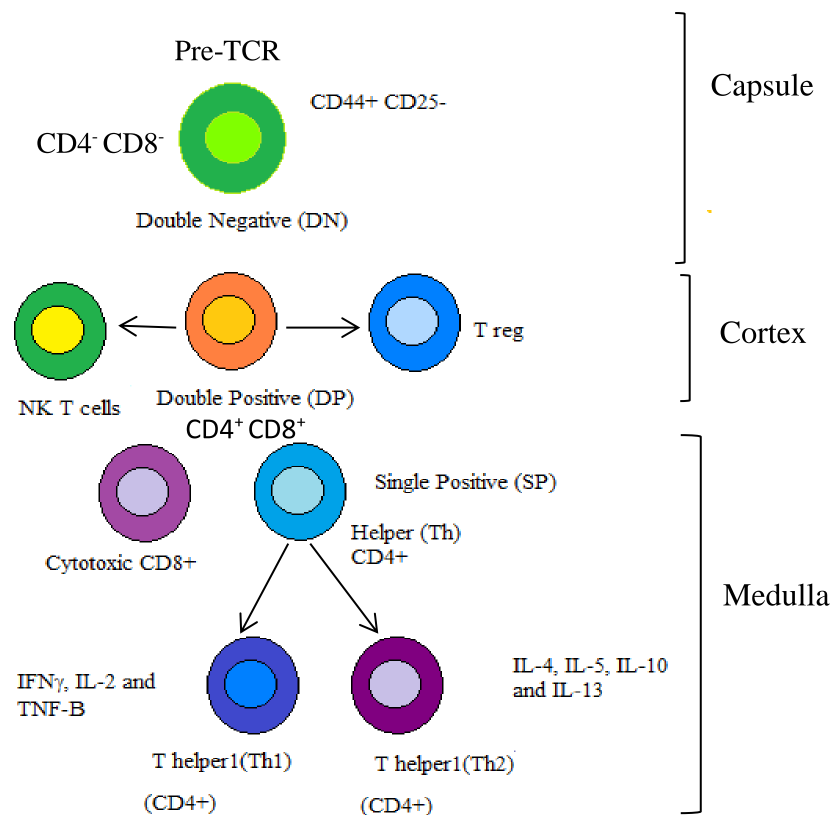


Figure 1.5: Development of T cells in the thymus.
Early T cells lack expression of CD4 or CD8 and are referred to as double negative (DN) ($CD4^-$, $CD8^-$). Double negative cells then progress into double positive (DP) ($CD4^+$, $CD8^+$). DP then further develop into either $CD4^+$ or $CD8^+$ single positive cells. In positive selection, thymocytes bind to MHC/antigens with sufficient affinity, whereas during negative selection, the binding between thymocytes and APC occurs with high affinity and so they receive an apoptosis signal. Self-drawn based on (Yeshanew *et al.*, 2015).

1.3.2 T cell receptors (TCRs) and gene rearrangement

The adaptive immune system has developed two types of T lymphocytes that detect different forms of antigen. $CD8^+$ T cells employ cell receptors (TCRs) to recognize short peptides derived from cytoplasmic antigens presented by MHC class I. $CD8^+$ undergo clonal expansion following the immune response in secondary lymphatic tissues and migrate to the site of infection to kill the infected cells, when they then die by apoptosis (Conley *et al.*, 2016). $CD4^+$ T cells use TCRs to recognize peptides from extracellular proteins presented by MHC class II during the immune response, leading to further specialization towards Th cells and the secretion of cytokines, and they provide help for $CD8^+$ T cell responses (Milam

and Allen, 2015). Each receptor is produced from a compound gene assembled by the somatic recombination of many possible gene segments on one chromosome (Tubo and Jenkins, 2014). The powerful diversity of TCRs allows the human T cells to recognize various pathogens and initiate a specific immune response. The mechanisms generating TCR gene segments are similar to Ig gene rearrangement, generated during thymocyte maturation by the recombination of specific V, D and J (Figure 1.6) segments, in the case of TCR β and δ, and V and J segments for TCR α and γ (Toor *et al.*, 2016). CDRs are the most variable part of the TCR and complement an antigen-MHC's features. The CDR consists of three regions: CDR1 and CDR2 are coded for by the V segment; CDR3 joins a part of the V segment and the D segment; J segments undertake incorporation for TCRβ, and parts of the V and J segments for TCRα (Danska *et al*., 1990). The presence of P- and N-nucleotides in all rearranged TCRα and TCRβ VDJ sequences provide further variability and antigen recognition capacity for the TCR to recognize non-self and self-peptides (Murphy, 2011).

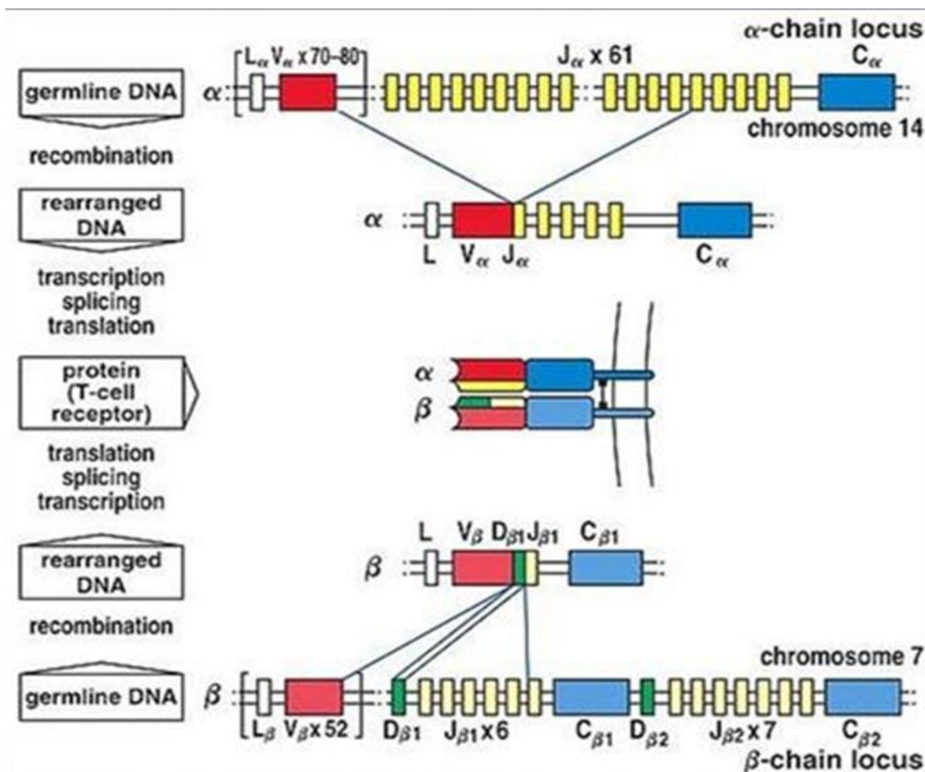


Figure 1.6: Stages of rearrangement and expression of α– and β-chain genes to generate TCRs.

The generation of the α–chain occurs through the rearrangement of Vα gene segments in a Jα gene segment to create a V-region exon. The transcription and splicing of the VJα exon to Cα generates mRNA, which is translated to create a TCR-α chain. The β chain is encoded by a recombination of Vβ, Dβ and Jβ to generate a VDJβ region, which is transcribed and spliced to join Cβ, resulting in the TCRβ chain. Both the TCRα and β chains are synthesized to generate α:β TCRs (Murphy, 2011)**.**

Table 1.2. The human TCR gene segments and sources of TCR diversity compared to Ig.

(Murphy, 2011).

Element	**Immunoglobulin**		**α:β T-cell receptors**	
	H	κ+λ	β	α
Variable segment (V)	~ 40	~70	52	~70
Diversity segment	23	0	2	0
D segments read in three frames	rarely	-	often	-
Joining segment (J)	6	5(κ)4(λ)	13	61
Joints with N- and P-nucleotides	2	50% of joints	2	1
Number of V gene pairs	1.9×10^6		5.8×10^6	
Junctional diversity	$\sim 3 \times 10^7$		$\sim 2 \times 10^{11}$	
Total diversity	$\sim 5 \times 10^{13}$		$\sim 10^{18}$	

1.3.2.1 $CD4^+$ and $CD8^+$ T-cell receptors (TCRs)

CD4 and CD8 are receptors expressed on the surface of T cells and act as co-receptors with TCRs (Figure 1.7). They interact with the antigen peptide bound to MHC molecules on antigen-presenting cells, leading to the enhancement of the T-cell signalling cascade via TCRs in association with protein tyrosine kinase p56lck (Lck) for both thymic selection and T-cell activation (Gangadharan and Cheroutre, 2004). T cells are polarized into different subsets according to priming conditions, including Th_1, Th_2, Th_{17} or Treg, whereas $CD8^+$ T cells are chiefly cytotoxic T lymphocytes (CTL) (Li *et al.*, 2013).

$CD4^+$ is a monomeric type I glycoprotein membrane molecule (58 kDa) expressed on the surface of thymocytes and Th lymphocytes, as well as macrophages and monocytes. It consists of four extracellular domains of 370 amino acid residues (D1–D4), a hydrophobic membrane-spanning region of 25 amino acids, and a short highly charged cytoplasmic tail of 38 amino acids (Vermeire *et al.*, 2006). The ligands for αβ TCRs on $CD4^+$ T cells are formed from extracellular protein peptides taken up by DCs and macrophages into endosomes by micropinocytosis for the fluid phase, and phagocytosis for particles. These endosomes contain enzymes such as cathepsins, which digest the internalized proteins into small peptides which can then be presented in association with MHCII on the surface of the APC to T cells (Tubo and Jenkins, 2014). The interaction between CD4 molecules on T cells and MHCII produces further signalling, leading to the production of cytokines by Th cells that are required for a full immune response (Yin *et al.*, 2012).

$CD8^+$ is a glycoprotein surface molecule expressed on the surface of T cells, which exists in two isoforms: CD8αβ and CD8αα. CD8αβ is found on αβ TCR thymocytes and CTLs and is a heterodimer type I transmembrane glycoprotein member of the IgSF. The α and β chains are composed of an Ig variable-like domain connected to the transmembrane domain by a stalk and a cytoplasmic tail (Li *et al.*, 2013). It has been revealed that CD8αβ plays a

fundamental role in the positive selection of $CD8^+$ T cells in the thymus, and in triggering $CD8^+$T cells in the periphery. Meanwhile, the CD8αα homodimer is present on γδ T cells, intestinal intraepithelial T lymphocytes, NK cells and DCs comprising the α chain only (Li *et al.*, 2013). It has been shown that CD8αβ functions as an efficient TCR co-receptor due to its interaction with the ectodomain of CD8β, enhancing the interaction between membrane-bound CD8 and MHCI. The cytoplasmic tail of CD8β increases the association between CD8α and intracellular signalling p56lck LAT. These two physical mechanisms of the CD8β domain enhance the dependence of CD8 co-receptors on each other (Cheroutre and Lambolez, 2008). Unlike CD8αβ, CD8αα is a less effective TCR co-receptor and does not enhance the positive selection of conventional MHCI-restricted T cells; it has been associated with the negative regulation of intestinal intraepithelial cells (Li *et al.*, 2013). The hallmark of CD8αα is as an adhesion molecule able to increase the strength and the affinity for MHCI ligands, because it depends only on the interaction of its extracellular region with MHCI (Cheroutre and Lambolez, 2008). Although both isoforms have similar affinities for MHCI, and the Lck interacts with the cytoplasmic region of CDα, the distinct activity may be attributable to the location of CD8αβ in membrane rafts (Rybakin *et al.*, 2011).

The ligands for $CD8^+$ T cells are generated in the cytosol by the proteasome, through which proteolysis degrades intracellular proteins into small peptides. Peptide degradation products then translocate into the endoplasmic reticulum through the transporter associated with antigen processing (TAP) where they bind with MHCI (Conley *et al.*, 2016). The peptide-MHCI complex is transported to the cell surface so that MHCI can display intracellular proteins to cytotoxic T cells (CTLs). However, MHCI can also display extracellular proteins in a process known as cross-presentation, mediated mainly by DCs(Tubo and Jenkins, 2014).

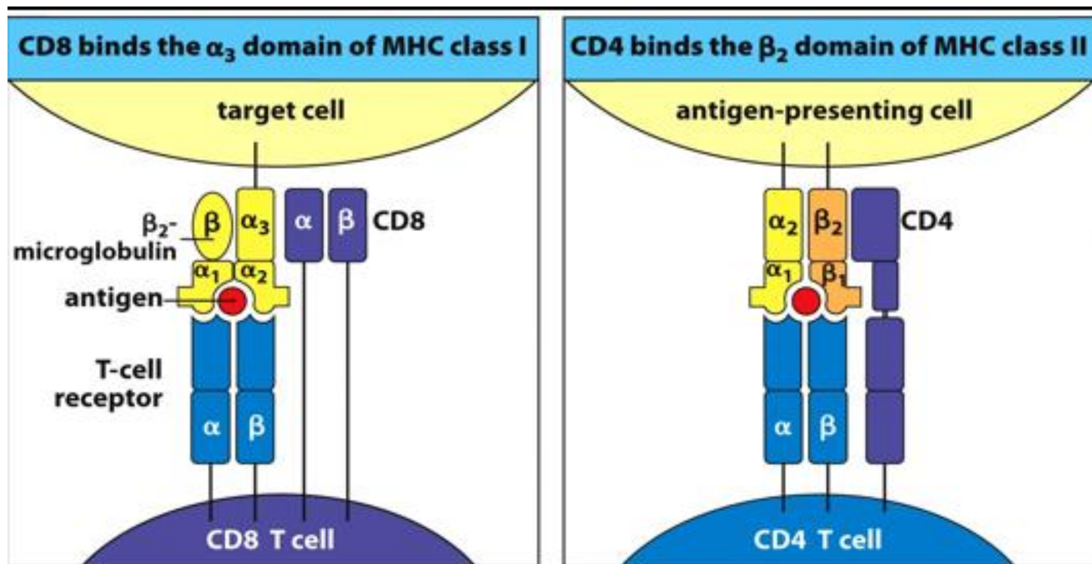


Figure 1.7: Schematic diagrams of MHCI and MHCII interactions with T cells.
The left panel shows antigens processed by target cells and presented to the $CD8^+$ T cell via MHC class I in association with $CD8^+$ co-receptors to activate $CD8^+$. The right panel shows APC-digested antigens presented via MHCII in association with $CD4^+$ co-receptors to activate $CD4^+$ cells (Parham, 2009).

1.3.3 Generation of T-cell responses and T-cell subsets

Pathogen recognition begins with the cells of the innate immune system, such as DCs and macrophages. These cells recognize potential pathogens through TLRs that are expressed on their surface. Stimulation of TLRs via pathogen components induces the secretion of anti-microbial peptides and proinflammatory cytokines through nuclear factor kappa-light-chain (NF-KB) and mitogen-activated protein kinases (MAPKs). TLR signals activate mature DCs to become more efficient in antigen uptake and processing, as well as up-regulating MHCI and II on the cell surface (Pei *et al.*, 2012). Following pathogen recognition and uptake of pathogen antigen, DCs migrate to the secondary lymph organs where they encounter T cells. Furthermore, CD28 on T cells binds to CD80 and CD86 expressed by the activated APC, leading to the activation of the co-stimulatory signalling that is necessary for T cell proliferation and survival (Leahr and Bevan, 2004). Naïve $CD4^+$ T cells are further polarized to differing Th effector subsets, based on the ability of the APC to produce specific cytokines in response to TLR signalling. Th_1 responses are initiated when DCs are stimulated through

their TLRs, leading to the cytokine production of IL-12. The resulting Th_1 secretes mainly IFNγ, as well as TNFα. In contrast, the Th_2 response is initiated after DCs interact with multicellular parasites and allergens. Th_2 cells produce various cytokines, including IL-4, IL-5 and IL-13 (Figure 1.8) (Leahr and Bevan, 2004, Romagnani, 2006). Th_1 cells control cell-mediated responses, such as the activation of macrophages, whereas Th_2 cells are responsible for stimulating B cells to switch to IgE. Th_1 differentiation is regulated by cytokines through the activation of signal transducer and activator of transcription 1 (STAT-1) and T-bet. Thus the production of IL-12 by DCs leads to IFNγ induction and the inhibition of IL-4 and IL-5. In contrast, the differentiation of Th_2 originates by activating the STAT-6 signalling pathway through IL-4 production. It has been suggested that the early IL-4 may either be produced by naïve T cells after interaction between their Notch receptors and the Jagged ligand of DCs, or by unknown cell types. The GATA-binding protein 3 (GATA-3) and c-Maf (transcription factor Maf) are also associated with Th_2 development and promote IL-4, IL-5 and IL-13, as well as inhibiting IFNγ production (Nicol *et al.*, 2016, Romagnani, 2006). Thus, cytokines cross-regulate each other's differentiation (mainly IL-4 and IFNγ) by promoting one type and suppressing the opposite subsets (Anderson *et al.*, 2003, Romagnani, 2006).

Activated naïve $CD4^+$ T cells can also differentiate into Th_{17} cells, which are regulated by different cytokines. It has been found that the combination of IL-6 and transforming growth factors-β (TGF-β) induces related orphan nuclear receptors (RORγt and RORα) transcription factors (Nicol *et al.*, 2016). IL-1β and IL-23 have also been shown to have roles in human Th_{17} cell differentiation (Ganjalikhani Hakemi *et al.*, 2011). These transcription factors are essential for developing the Th_{17} lineage. In addition, STAT-3 regulates the IL-6 induced expression of both RORγt and RORα and the secretion of IL-17, whereas the activation of STAT-1 inhibits Th_{17} differentiation (Kimura and Kishimoto, 2011). Recently, it has been

found that these cells express distinct markers, such as the chemokine receptor CCR6 and CD161 and CD49d (the α-chain of the integrin VLA-4) (Kleinewietfeld and Hafler, 2013). Furthermore, Th_{17} cells produce various cytokines, including IL-17, IL-17F, IL-21 and IL-22, which are essential in the defence against various bacteria and fungi, especially at the mucosal surface, through the recruiting and activation of neutrophils (Ivanov *et al.*, 2007). Cytokines produced by Th_{17} cells also stimulate fibroblasts, endothelial cells, epithelial cells and macrophages to produce chemokines, granulocyte colony-stimulating factors (CSFs) and granulocyte-macrophage (Romagnani, 2006). Although under homeostatic conditions Th_{17} cells are detected in the lamina propria of the small intestine, during extracellular bacterial or fungal infection these cells also migrate to other tissues (Kleinewietfeld and Hafler, 2013).

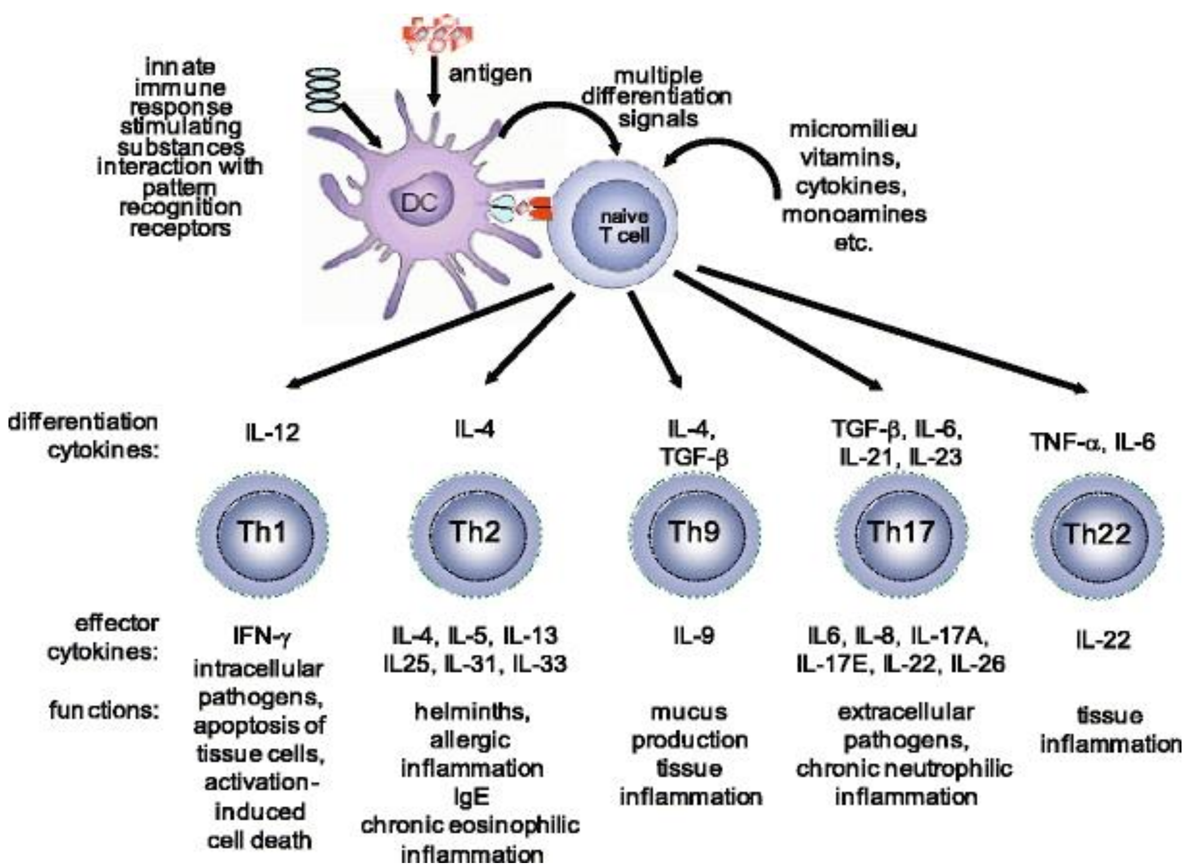


Figure 1.8: Generation of naïve T cells.

Following antigen recognition and help from APC, cytokines and co-stimulatory signalling with other cells, naive T cells can differentiate into effector subsets, including Th_1, Th_2, Th_9, Th_{17} and Th_{22}. These cells produce different inflammatory responses and cytokine profiles. Adopted from (Jutel and Akdis, 2011).

1.3.4 T cell activation phenotypes, and potential roles in lung disease

Activated $CD4^+$ cells in particular play a pivotal role in the pathophysiology of bronchiectasis (BR) (Gaga *et al.*, 1998), a disease of dilated bronchi (see section 1.6.1). However, the activation of $CD4^+$ T cells along with B cells is an important mechanism for inducing high affinity neutralizing antibodies. In general, the activation of T cells involves a variety of co-stimulatory molecules which provide the second signal that is critical for the enhancement of T cell differentiation, the facilitation of TCR signaling and modulation of T cell trafficking (Zhong *et al.*, 2010). These co-stimulatory molecules are classified into three groups. The first are the immunoglobulin superfamily members including CD28 and CD152 (CTLA-4) and their ligands CD80 and CD86. The second and third groups are the tumour necrotic factor receptor (TNFR) superfamily including CD134 (OX40), CD137 (4-IBB), CD137L (4-IBBL), CD27 and CD30 (Ki-1); and finally the cytokine receptors such as IL-2R, -7R, -15R, -1R and -6R (Giscombe *et al.*, 2006). Many of these stimulatory molecules are, therefore, likely to contribute to the T cell pathology seen in BR.

For instance, $CD69^+$, an early activation molecule, belongs to the natural killer cell gene complex on mouse chromosome 6 and human chromosome 12. It is rapidly expressed on thymocytes during positive selection or on cells that have completed this process. Furthermore, CD69 is also expressed on neutrophils, eosinophils, epidermal Langerhans cells, and bone marrow myeloid precursors (De Maria *et al.*, 1994).CD69 is selectively expressed in chronic inflammation at the sites of active immune responses *in vivo* (Maino *et al.*, 1995, Sancho *et al.*, 2005). It has been shown that the antibody cross-linking of CD69 releases several cellular responses in humans, including nitric oxide (NO), monocytes, neutrophil degranulation, T cell proliferation, the production of TNFα and NK cell cytotoxicity. Functional CD69 receptors are expressed on the surface of circulating monocytes, and signals generated following CD69 cross-linking monocyte activation. This leads to the production of inflammatory mediators and the initiation of cytotoxic activity (De Maria *et al.*, 1994). CD69 is therefore likely to have a potential pro-inflammatory role in the pathogenesis of numerous inflammatory conditions, including pulmonary fibrosis (Yamauchi *et al.*, 2011).

PD-1 (CD279) is an inhibitory receptor on hematopoietic cells which belongs to the CD28/CTLA-4 receptor superfamily and is expressed on T cells, B cells, NK cells and myeloid cells. During the normal effective immune response, PD-1 is up-regulated on T cells to suppress the excessive activation of immune cells and thus prevent injury to tissue (MacFarlane *et al.*, 2014). Besides, it mediates the signaling which inhibits cytolytic effector cells in the peripheral organs and tissues, thereby protecting the tissue from damage (Dolan and Gupta, 2014). Thus, the measurement of PD-1 may indicate the progression of lung disease and provide useful information on disease stage.

CD49d (alpha4-integrin) is an important molecule which acts as an adhesive and signaling receptor, and it plays an important role in the immune system through leukocyte migration, activation and survival. CD49d is also involved in B cell development and mediates the signalling that up-regulates the anti-apoptotic B-cell lymphoma gene (BCL2 family), consequently influencing B-cell survival (Majid *et al.*, 2011). Furthermore, the up-regulation of CD49d can aid the recruitment of mononuclear leukocytes to sites of inflammation, and it is implicated in the pathogenesis of chronic inflammatory diseases including asthma, multiple sclerosis, hypersensitivity and rheumatoid arthritis (Majid *et al.*, 2011, Rose *et al.*, 2002).

OX40 (CD134, TNFRSF4) is a 50-kDa type 1 transmembrane protein. It is a co-stimulatory molecule belonging to the tumor necrosis factor receptor (TNFR) superfamily and is expressed on T cells (mainly Th_2), as well as on natural killer T cells and neutrophils. OX40 signaling promotes T cell proliferation and survival and the expression of IL-4 cytokine (Hirano *et al.*, 2016, Kaur and Brightling, 2012, Lane, 2000). OX40 induces co-stimulatory signals that augment the clonal expansion of T cells rather than initiating T cell activation (Gramaglia *et al.*, 2000).The OX40 ligand OX40L (CD252) is expressed by different cell types, including dendritic cells, B cells, macrophages, and Langerhans cells (Siddiqui *et al.*, 2010, Wang and Liu, 2007). The interaction between OX40 and OX40L is important in T cell activation, survival and inducing memory T cells from the effector cells (Ruby *et al.*, 2008, Wang and Liu, 2007). Additionally, OX40L promotes naïve T cells to undergo Th_2 polarization and the release of cytokines such as IL-4, IL-5, and IL-13. These cytokines are associated with the pathogenesis of allergic eosinophilic asthma (Kaur and Brightling, 2012). OX40/OX40L interaction is involved in different conditions where both CD4 and CD8 are mainly

associated with orchestrating the immune response, including many inflammatory and autoimmune diseases (Croft *et al.*, 2009).

In addition to the role of co-stimulatory molecules in T cell activation and migration, chemokine receptors also play a fundamental role in leukocyte activation by controlling the effector functions of immune cells and maintaining the adaptive immune response in both physiological and inflammatory conditions (Ito *et al.*, 2011). Chemokine receptors are a group of transmembrane (TM) proteins of the superfamily of G-protein-coupled receptors that are involved indirectly in leukocyte biology throughout the body under both physiological and inflammatory conditions (Choi and An, 2011). They are categorised as CCR, XCR, CXCR, or CX3CR and numbered according to the order of identification (Choi and An, 2011, Szczepanska *et al.*, 2015). For instance, CX3CR is a receptor for three chemokines expressing the CXC chemokine family including CXCL9 (a monokine induced by interferon-γ (IFN-γ), MIG), CXCL10 (IFN-γ-inducible protein 10, IP-10) and CXCL11 (IFN-γ-inducible T cell α chemoattractant, I-TAC) (Szczepanska *et al.*, 2015). CCR6 is an important receptor that is involved in controlling mucosal immunity by mediating and recruiting immature and mature dendritic cells and antigen presenting cells (APCs) to the sites of epithelial inflammation (Ito *et al.*, 2011). CCR6 is expressed on immature DCs, most B cells, subsets of $CD4^+$ and $CD8^+$ T cells and NKT cells. This receptor can bind only one chemokine ligand, CCL20, which is expressed by different epithelial cells such as keratinocytes, pulmonary epithelial cells, and intestinal epithelial cells (Krzysiek *et al.*, 2000, Kucharzik *et al.*, 2002, Reibman *et al.*, 2003). Lung epithelial cells express high levels of CCL20, which in turn result in immature $CCR6^+$ DCs being recruited into the lung (Lundy *et al.*, 2005). It has been shown that CCL20 regulates the recruitment of DCs in the airways of patients with COPD, and the increased expression of CCL20 in patients with COPD compared with healthy controls has been demonstrated at the RNA and protein levels in different areas of the lungs (Demedts *et al.*, 2007).

Therefore, it was hypothesized that the analysis of key chemokines produced by different inflammatory cell and stimulatory molecules may provide useful evidence in determining the immunopathogenesis of patients with BR and other obstructive lung conditions.

1.4 The human lungs

The human lungs are complex organs in which the vascular system is combined with epithelial-lined tubes and sacs to enable effective gas exchange. The lungs are known to have the largest epithelial surface of the body, at the interface between the internal and the external environment of the human host (Loscertales *et al.*, 2016, Zhang *et al.*, 2000). The lungs consist of different parts known as lobes (Figure 1.9). The right lung comprises three lobes: upper, middle and lower. In contrast, the left lung has two lobes: the upper and lower. Human lungs are conical in shape and are situated on either side of the mediastinum, surrounded by pleural cavities (Seeley *et al.*, 2008). The right lung is larger than the left and weighs an average of 620 g, whereas the left lung is generally 560 g in weight. Both lobes of the lung have a central region, referred to as the hilum, on the medial surface of the lung, where the main bronchus, blood vessels, nerves and lymphatic vessels enter and leave the lung. These structures, passing through the hilum, are termed the root of the lung. The bronchi branch from the trachea into primary bronchi, which enter the right and the left lungs and then divide into small secondary bronchi. These bronchi continuously subdivide into segments and small tubes called bronchioles and then terminal bronchioles. The bronchi are supported by cartilage in their wall, whereas the bronchioles have smooth muscle tissue; both are lined with ciliated columnar epithelial cells. Furthermore, the bronchioles continue to divide, terminating in the air spaces of alveoli, where the actual gas exchange occurs between the air and the blood (Seeley *et al.*, 2008).

1.4.1 Alveoli

Alveoli are the main part of the respiratory system and form most of the lung parenchyma. Over 350 million alveoli are found in each adult human lung and each alveolus has a membrane surrounded by many capillaries facilitating gas transfer to the blood through the diffusion process. The surface area of adult human lungs is approximately 60–70 m^2,

providing an ample surface for gas exchange to be effective. Alveoli are similar to bubbles lined with a basement membrane (Figure1.9). Several groups of alveoli are confluent with an alveolar duct and terminate at the alveolar sac (Carola *et al.*, 1990). A histological image of the respiratory tract shows that most of the upper airway epithelium, from the trachea to bronchioles of human lungs, is lined with pseudostratified epithelium, consisting of equal proportions of basal cells, secretory cells and ciliated cells (Carola *et al.*, 1990). The smallest bronchioles comprise simple columnar or cuboidal epithelia, containing secretary cells referred to as goblet cells, and ciliated cells. These cells form the physical barrier against foreign substances through the process of mucociliary clearance (Donne *et al.*, 2015). The alveolar epithelium is composed of a single layer of epithelial cells – type I and type II alveolar epithelial cells, called AEC1 and AEC2 respectively. Airway epithelial cells play a critical role in host defence mechanisms, in which they regulate innate immunity and produce molecules that interact with immune cells to activate immunity. Furthermore, airway epithelial cells (AECs) secrete various microorganism-killing effectors, such as mucins, anti-microbial peptides (AMPs) and reactive oxygen species (ROS). These cells also produce proinflammatory cytokines, growth factors and chemokines, which recruit and activate phagocytes, eliminating pathogens through phagocytosis (Li *et al.*, 2012).

Type I alveolar epithelial cells (AEC1) are squamous, large, thin cells, which represent around 40% of the alveolar lining and combine with other epithelial cells to form an effective barrier between the air space and the septal wall (Donne *et al.*, 2015). Type II cells (AEC2) are cuboidal cells, smaller than type I cells, which form around 60% of the alveolar lining. These secretory cells produce detergent-like phospholipids called surfactant (dipalmitoyl lecithin), which is essential for reducing the alveolar surface tension and keeping the alveoli inflated. In addition, the surfactant modulates the lung immune response against inhaled pathogens (Ross and Pawlina, 2011). Type II cells also have the ability to generate both type I and type II cells after lung injuries; thus, they are also an important marker of alveolar

injury and the repair of alveoli. Alveolar macrophages, neutrophils and monocytes are also found in the alveoli. Alveolar macrophages represent the initial line of phagocytic defence against invaders that gain access to alveoli and escape physical defences. These cells have the ability to engulf and digest bacteria and initiate the inflammatory immune response (Zhang *et al.*, 2000), facilitating the gas exchange between the atmosphere and the blood (Carola *et al.*, 1990, Donne *et al.*, 2015, Ross and Pawlina, 2011). In the normal state, an array of innate immune receptors, such as TLRs, NLRs and C-type lectins, are expressed by the epithelium, as well as microbicidal small cationic peptides, lysozyme, lactoferrin and mucins. Epithelial cells also produce cytokines, chemokines and growth factors as a result of infection, inflammation, stress and injury (Ramakrishna *et al.*, 2012).

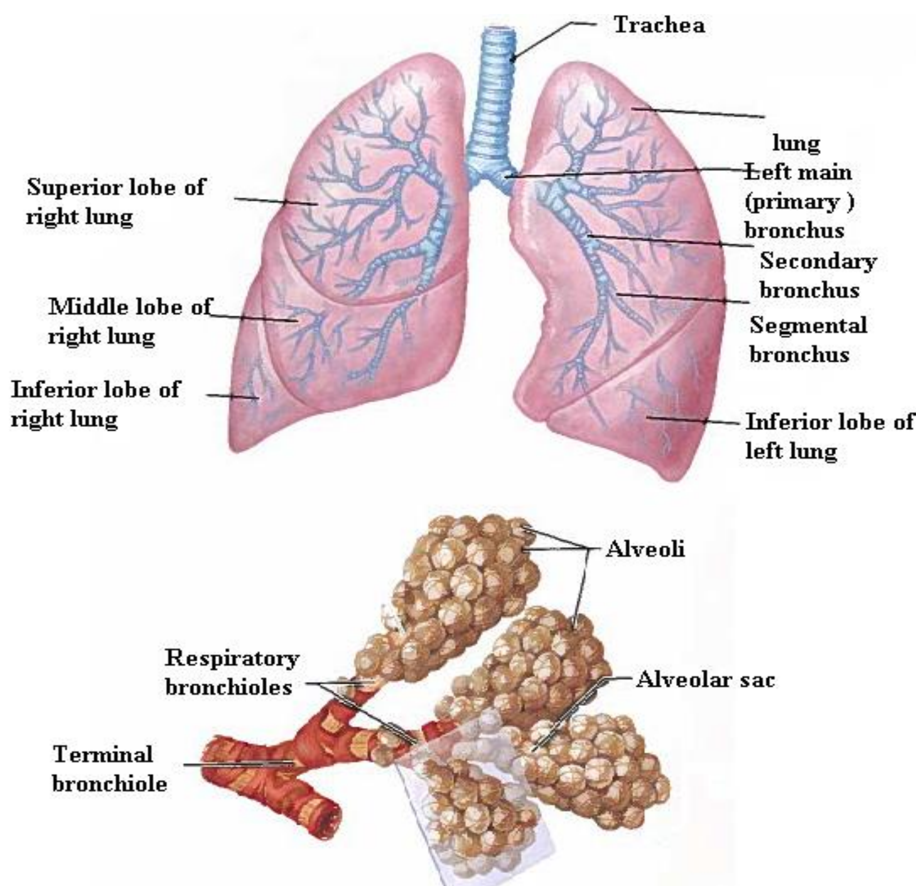


Figure 1.9: Diagrammatic view of the human lung and alveoli.
Diagrammatic view of the human lung and alveoli. The air pathway consists of the trachea and the main, secondary and segmental bronchi, which divide into smaller bronchi and bronchioles, terminating in the terminal bronchioles and alveoli. Adopted from (Marieb and Hoehn, 2007)**.**

1.4.2 Lung infections

The lungs of the respiratory system are exposed to a large number of microbes and organic and inorganic particulate material as a result of the daily inhalation of 10,000 litres of air (Bals and Hiemstra, 2004). Healthy individuals are less susceptible to lung infections, despite bacteria frequently being inhaled, due to the adequate host defences, which include mucociliary clearance, antimicrobial peptides produced by epithelial cells, and the sophisticated innate and adaptive immune systems. However, patients with lung disorders, such as bronchiectasis (BR), chronic obstructive pulmonary disease (COPD) and cystic fibrosis (CF), are characterized by airway inflammation associated with increased mucus production and the reduction or inhibition of mucociliary clearance. This is usually associated with lung infection -episodes or chronic disease. The recurrent cycles of inflammation and tissue damage result in lung damage and reduced lung function and/or further respiratory tract infections (RTIs) (Cullen and McClean, 2015).

RTIs have become the main cause of human morbidity and mortality globally, at great economic cost to health care resources (Braido *et al.*, 2007); they are increasing in incidence and cases are estimated by the World Health Organisation (WHO) to have exceeded HIV, cancer and heart disease (Bomberger *et al.*, 2011). The main cultured pathogens associated with RTIs are bacterial infections, such as *Pseudomonas aeruginosa (P. aeruginosa)*, *Haemophilus influenzae (H. influenzae)*, *Streptococcus pneumoniae (S. pneumoniae)*, *Moraxella catarrhalis (M. catarrhalis)*, *Staphylococcus aureus (S. aureus)*, *Chlamydophila pneumoniae (C. pneumoniae)* and *Mycoplasma pneumoniae (M. pneumoniae)*. Viral infections include influenza, parainfluenza viruses and respiratory syncytial virus (RSV). Fungal infections are mainly caused by aspergillus and candida (Braido *et al.*, 2007).

The interaction between pathogens and the host defence depends on factors such as the virulence of the pathogen and the susceptibility of the host. The host immune response may

eliminate the pathogen and improve immunity against further infection through immune memory. However, in the case of bacterial infection with *P. aeruginosa*, the interaction between the host and the bacteria may produce a cycle of infection, allowing the bacteria to adhere to the epithelium and colonize the lung in the long term, resulting in tissue damage and lung destruction (Bourke, 2002). Obstructive lung diseases associated with frequent infections include conditions such as BR, COPD, CF and asthma.

1.5 Laboratory features

1.5.1 Lung function test

The lung function test, or pulmonary function test (PFT), is an important diagnostic test in the evaluation of patients with different lung diseases, including chronic obstructive lung diseases and asthma (Rasam *et al.*, 2015). Spirometry is the most common lung function test used to measure limitations in airflow, specifically measuring the volume of air forcibly exhaled (forced vital capacity [FVC] and the volume of air exhaled during the first second (forced expiratory volume in one second [FEV_1] to provide the FEV_1/FVC ratio. This is a measure simple and has been employed in various clinical studies. PFTs are also defined by the Global Initiative for Chronic Obstructive Lung Diseases (GOLD), which is classified into four stages: stage 1 (mild, $FEV_1 \geq 80\%$ predicted function); stage 2 (moderate, $FEV_1 =$ 50–80% predicted function); stage 3 (severe, $FEV_1 =$ 30–50% predicted function); and stage 4 (very severe, $FEV_1 < 30\%$ predicted function) (Decramer *et al.*, 2012). The ratio between FEV_1 and slow vital capacity (VC), i.e. FEV_1/VC, is also calculated. These measurements are then evaluated through comparison with reference data based on age, sex and height (Decramer and Vestbo, 2014). Typically, the presence of a post-bronchodilator FEV_1/FVC less than 0.70 confirms airflow limitation and persistent chronic obstruction (Vestbo *et al.*, 2013).

1.5.2 Bronchoalveolar lavage (BAL)

Bronchoalveolar lavage (BAL) is a research tool in the assessment of the lung diseases that are associated with viral and bacterial infections, including COPD, BR and asthma (Garbino *et al.*, 2009). BAL is very useful in evaluating airway inflammation, being a safe way of investigating the sampling of cells and mediators from the lower airways (Gorska *et al.*, 2009). This provides reliable data in patients for whom it is difficult to detect microorganisms in the sputum (Ratjen, 2006). The analysis of BAL fluid in patients with CF has revealed a significant increase in concentrations of local pro-inflammatory cytokines, such as IL-1, IL-6, IL-8, and TNFα. Elevated inflammatory markers are also observed in the BAL fluid of infants with CF in the absence of infection with CF-related pathogens (Courtney *et al.*, 2004). Activated neutrophils and macrophages are increased in the sputum and BAL fluid of patients with COPD, suggesting their role in airway destruction through the secretion of inflammatory mediators, enzymes and reactive oxygen species (Barnes *et al.*, 2003). The analysis of BAL fluid has shown higher eosinophil counts in asthmatics compared with COPD cases, which may contribute to airway constriction (Gorska *et al.*, 2009).

1.6 Respiratory tract diseases

1.6.1 Bronchiectasis (BR)

Bronchiectasis (BR) was initially described by Laennec in 1819 as a disease of the bronchi associated with chronic mucus production and repeated coughing (Neves *et al.*, 2011). BR is also defined as a heterogeneous condition characterized by airway inflammation that leads to irreversible dilation of the bronchi and persistent sputum overproduction (Boyton, 2008). Recently, BR has been diagnosed using high-resolution computed tomography (HRCT), which allows diagnosis at earlier stages, thus demonstrating an increase in the prevalence of BR. Furthermore, there is an overlap between BR and COPD, where both conditions share

common characteristics in terms of their pathophysiology and clinical behaviour. In 2014, GOLD described BR as one of the comorbidities of COPD (Du *et al.*, 2016). It has been reported that the occurrence rates of BR in COPD are between 29% and 50% (Neves *et al.*, 2011). Patients with BR may be asymptomatic during the mild phase, but in early-defined exacerbations they suffer from a chronic cough, daily mucopurulent sputum production, chest pain, weight loss, bronchospasm, dyspnoea, breathlessness, wheezing, tiredness and impaired physical performance. Exacerbations are due to viral, or bacterial infections, often associated with fever, malaise, increased coughing and sputum production (Boyton, 2012, Rademacher and Welte, 2011). The pathophysiology of this disease is defined by infiltrating inflammatory cells surrounding the parenchyma, leading to damaged bronchial epithelium. The destruction of the elastic and muscular layers of the bronchi leads to the in dilation and the impairment of mucociliary clearance, allowing mucus to accumulate and favouring bacterial colonization, leading to chronic inflammation. Furthermore, microorganisms release inflammatory mediators which cause airway obstruction and progress lung damage (Angrill *et al.*, 2002, Rademacher and Welte, 2011).

1.6.1.1 Aetiology of bronchiectasis (BR)

BR is classified into CF BR, which is a genetic disease caused by a mutation in the CF transmembrane conductance regulator (King *et al.*, 2006) and non-CF BR (nCFBR), caused by multiple conditions. For example, BR may occur following serious infections by fungi, viruses and bacteria. BR also progresses due to physical obstruction, producing localized areas for disease development (Redding, 2011, Shoemark *et al.*, 2007). Furthermore, the incidence of BR is associated with abnormalities in pulmonary host defences, such as immune disorders, ciliary dyskinesia and autoimmune disease. In 50% of patients (idiopathic), a certain cause is never found (Dogru *et al.*, 2005, Shoemark *et al.*, 2007). Whatever the cause, BR leads to a risk of bacterial colonization, a decline in lung function

and recurrent exacerbations, resulting in significant morbidity. Some complications associated with BR are stated in Table 1.3 (King, 2011, King *et al.*, 2006). The identification of causative factors may aid management and the early treatment of the condition (Shoemark *et al.*, 2007).

Table 1.3. Various conditions associated with bronchiectasis (BR).

(Adopted from (Bandyopadhyay *et al.*, 2004, Whitters and Stockley, 2012)).

Post infective	Viral (adenovirus, measles, HIV)
	Bacterial (pneumococcus)
	Mycobacterium tuberculosis
	Whooping cough
Host immune defects	Allergic bronchopulmonary aspergillosis
	Host immune defects: humoral immune deficiency (IgA, IgG, IgM)
	Selective IgG subclass deficiency
	Specific antibody deficiency
Mucociliary clearance defects	Primary ciliary dyskinesia
	Kartagener's syndrome (a genetic disease affecting the airways, involving primary ciliary dyskinesia and situs inversus)
	Young's syndrome (repeated airway infections and congenital epididymis obstruction)
Inflammatory disease	Rheumatoid arthritis
	Inflammatory bowel disease
	Chronic obstructive pulmonary disease
Others	Foreign body inhalation
	Middle lobe syndrome
	α1-Antitrypsin deficiency
	Toxic inhalation

1.6.1.2 Diagnosis of bronchiectasis (BR)

BR can be diagnosed through the clinical history of patients, in particular childhood and respiratory illnesses, and also sputum analysis, pulmonary function tests and biochemical analysis of blood samples in association with radiographic changes (Neves *et al.*, 2011). High-resolution computed tomography (HRCT) is the best tool for diagnosing BR, allowing

detection of the disease at an early stage. This includes ‘tree-in-bud’ abnormalities, indicating the distribution of infection in the bronchi (Figures 1.10 and 1.11) (Scullion and Holmes, 2013). Bronchoscopy and HRCT demonstrate the structural changes in the airways, including in shape, size and separation, and evaluate the quality of lung parenchyma in both lungs. Sputum analysis for microbial colonization is also recommended as a diagnostic procedure since the disease is characterized by airway inflammation induced by bacterial infections (Ibralic and Beslic, 2009).

Another process for diagnosing BR is spirometry, in which forced expiratory volume in one second (FEV_1) <80% at ages <14 years at first diagnosis indicates the disease (Rademacher and Welte, 2011) . Most, if not all, patients with BR have mild to moderate airflow obstruction that worsens over time (King, 2011). The high production of mucus and an increase in the thickness of bronchi in the airways also provide markers for BR (Scullion and Holmes, 2013). These investigations reveal underlying aetiology and frequently lead to a change in disease management (Neves *et al.*, 2011).

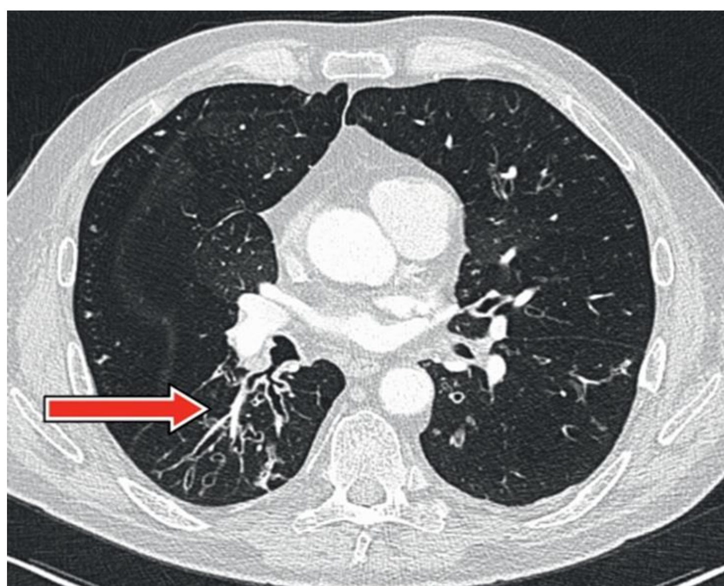


Figure 1.10: Transverse image of the lung of a BR patient using HRCT.
The arrow indicates diseased areas (Rademacher and Welte, 2011)**.**

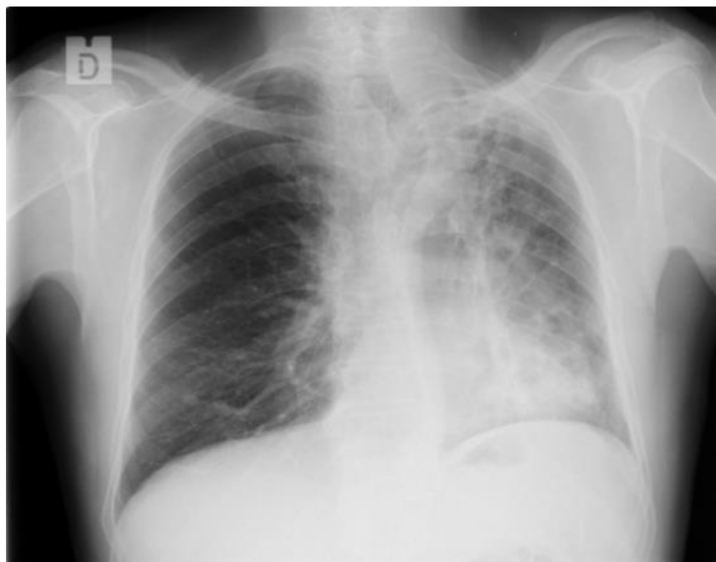


Figure 1.11: Radiograph of a patient with severe non-CF BR.
Severe cystically enlarged bronchi are marked in the left lung. This radiograph also demonstrates crowding of the bronchi, loss of definition of the bronchovascular markings and oligemia (Neves *et al.*, 2011).

1.6.1.3 Microbial infection in bronchiectasis (BR)

Patients with BR are often colonized with potentially pathogenic microorganisms. These are risk factors for lung disease and they induce inflammatory mediators, leading to bronchial wall damage and airway obstruction (Angrill *et al.*, 2002). The hypothesis of a "vicious cycle" has been proposed, in which the associated failure of the host defence and induction of a host chronic inflammatory response leads to the amplification of the problem (Whitters and Stockley, 2012). Pathogens release mediators that can inhibit ciliary function and damage ciliated epithelium, leading to the stimulation of chemokines such as IL-8, which recruits neutrophils to the site of inflammation in the lung (King, 2011).

The high production of mucus in the airways of BR patients attracts the growth of bacteria. Gram-negative bacteria are the predominant bacteria that have been detected in the sputum of patients with BR. These include non-typeable *H. influenzae* (NTHi), which are non-encapsulated and found in around 47% of patients. *P. aeruginosa* and *M. catarrhalis* are found in 12% and 8% of patients respectively(McShane *et al.*, 2013). *H. influenza* and *P.*

aeruginosa produce mediators that impair ciliary clearance and damage the epithelium. They also stimulate chemokines, such as IL-8, to attract neutrophils to the site of inflammation via the release of glycoproteins (King, 2011). Once these bacteria are established, it is difficult to eradicate them because they have the ability to form robust, antibiotic-resistant biofilms. These biofilms make the bacteria more challenging for anti-microbial defences and therapy because their hydrated matrix of extracellular polysaccharide and proteins covers the organized communities of bacteria and protects them from the host environment. Biofilms allow the bacteria to survive in the host through several means. First, within the biofilm, there are zones of anoxia, acidity, or nutrient depletion that promote the bacteria, enabling them to be increasingly antibiotic resistant. Second, biofilms delay the rate of antibiotic diffusion, allowing bacteria more time to sense and respond to their environment. Finally, biofilms may protect the bacteria from phagocytosis and various host defences (McShane *et al.*, 2013). *P. aeruginosa* colonization has been shown to correlate with a higher decline in lung function and the exacerbation and progression of disease (McShane *et al.*, 2013). Non-typeable *H. influenzae* strains, which lack the polysaccharide capsule of a typeable strain, have been found to be one of the main bacterial pathogens detected in respiratory secretions. This microorganism can cause recurrent or chronic lower airway infection in BR and persists in the lung through different strategies. The microorganism expresses multiple adhesions with individual specificities for different cell types in the respiratory tract. The ability to survive within some innate cells, such as macrophage and epithelial cells, allows the bacteria to undergo antigenic drift, thus avoiding adaptive immune responses. Furthermore, the bacteria also form biofilms on the mucosal surfaces and effectively resist the host defence mechanism. The appearance of *M. catarrhalis*, also detected in BR, in the respiratory tract is due to the capacity of this bacterium to invade respiratory epithelial cells, induce biofilms and avoid complement activation (Grimwood, 2011). A recent study found that, in a proportion of BR patients colonized by *P. aeruginosa*, their antibodies surprisingly protect

the bacterium from complement-mediated killing (Wells *et al.*, 2014). These bacteria produce lipopolysaccharide containing O-antigen and the impaired antibody-mediated killing is due to the high production of O-antigen-specific IgG_2 antibodies. As the O-antigen can be in other Gram-negative bacteria, the failure of IgG to kill the bacteria may be found in different Gram-negative bacteria. The depletion of IgG_2 to O-antigens increases the patient serum's ability to kill such strains. These findings are important in understanding the protection produced by natural infection and the design of vaccines (Wells *et al.*, 2014).

1.6.1.4 Innate and adaptive immunity in bronchiectasis (BR)

1.6.1.4.1 Neutrophils

Neutrophils are the most abundant cells found in the pulmonary tissues of BR patients. They rapidly migrate into the site of inflammation and provide effector mechanisms, such as phagocytosis, reactive oxygen mediators and other anti-microbial properties. Activated neutrophils also release proteases, neutrophil elastase and pro-inflammatory mediators, including IL-1β, IL-8 and TNFα. Neutrophils and epithelial cells express TLRs. Once TLRs encounter specific pathogen-associated molecules, they provoke a signalling cascade, leading to the activation of nuclear factor (NF)-kB and other transcription factors (Fuschillo *et al.*, 2008). Furthermore, neutrophils release matrix MMPs, which may augment the inflammation and cause airway remodelling. High levels of MMPs which have been found in the BAL fluid of patients with CF and non-CF BR may contribute to the progression of the disease (Bergin *et al.*, 2013).

1.6.1.4.2 Macrophages

Although the role of macrophages in patients with BR is still unclear, there is a higher proportion of macrophages within the entire lamina propria of patients compared to healthy controls. Also, higher numbers of macrophages are seen in patients with regular sputum production, suggesting macrophages may be implicated in disease activity (Fuschillo *et al.*,

2008). Moreover, increased TNFα levels have been observed in the airway lamina propria of these patients. This is attributed to the activation and stimulation of macrophages by LPS from bacteria (Zheng *et al.*, 2001).

1.6.1.4.3 Lymphocytes

Infiltrating T cells have been found in the lamina propria and the epithelium of both humans with BR and in experimental models of idiopathic BR (Fuschillo *et al.*, 2008). Increased $CD4^+$ and $CD8^+$ T cells found in the lungs and BAL fluid of patients with BR may contribute to the inflammatory response (Boyton, 2008, Fuschillo *et al.*, 2008). The principal role of cytotoxic T cells in idiopathic BR is supported by observation of bare lymphocyte syndrome type 1, which is caused by a mutation in the gene of MHCI molecules. Consequently, these patients lack expression of MHCI and $CD8^+$ T cells, which leads to recurrent respiratory infections and airway damage, resulting in BR (Fuschillo *et al.*, 2008).

Furthermore, research has revealed an association between idiopathic BR and the human leukocyte Class II antigen HLA-DR1, DQ5. This suggests that the recognition by $CD4^+$T cells of these MHC molecules presenting antigen from respiratory bacterial pathogens is affected, thus increasing susceptibility to lung infections (Grimwood, 2011).

Th17 cells are also important, as the airway submucosa shows greater levels of IL-17^+ cells which may correlate with neutrophil numbers, leading to the vicious cycle of infection and inflammation (Boyton *et al.*, 2012). It has been found that patients with BR have higher pro-inflammatory cytokines, such as tumour necrosis factor α (TNFα), IL- 6 and IL-8 in the sputum, BAL fluid and plasma (Martínez-García *et al.*, 2008). Primary antibody deficiency syndromes (PADS) have been implicated in BR, in which there is inability to mount a proper and competent antibody response to pathogen antigens. This can involve either the partial or complete absence of one or more Ig classes or subclasses (Whitters and Stockley, 2012),

including IgM, IgG, IgA and the IgG subclasses: IgG1, IgG2, IgG3 and IgG4. Furthermore, a reduction in the antibody response to encapsulated pyogenic bacteria, such as *H. influenzae* and *S. pneumoniae* (pneumococcus), has also been found in patients with BR (Stead *et al.*, 2002). In response to *H. influenzae*, Th_2 was the predominant T cell response in patients with BR, rather than the Th_1 found in healthy controls (Grimwood, 2011).

1.6.2 Chronic obstructive pulmonary disease (COPD)

Chronic obstructive pulmonary disease (COPD) is a major cause of illness and death throughout the world. It affects around 10% of the general population, with increasing incidences of morbidity and mortality (Cosio *et al.*, 2009). COPD has become the fourth leading cause of death in industrialized countries and is expected to be the third leading cause of death worldwide by 2020 (Kheradmand *et al.*, 2012). COPD is characterized by a limitation of air flow, which is not fully reversible, in association with an abnormal inflammatory response of the lungs to noxious particles and gases such as those found in cigarette smoke (Dutta, 2013, Gadgil and Duncan, 2008). The structural abnormalities that are found in bronchioles with a diameter of less than 2 mm are characterized by increased airway wall thickness with peribronchial fibrosis, and by luminal reduction (Hogg, 2006). Exposure to smoke promotes inflammatory infiltrates in the mucosa and alveolar walls, increased mucus secretion, epithelial cell hyperplasia and the destruction of alveoli (Figure 1.12). These factors appear to contribute to recurrent bacterial infections and inflammatory responses (Decramer *et al.*, 2012, Hogg, 2006, Kheradmand *et al.*, 2012).

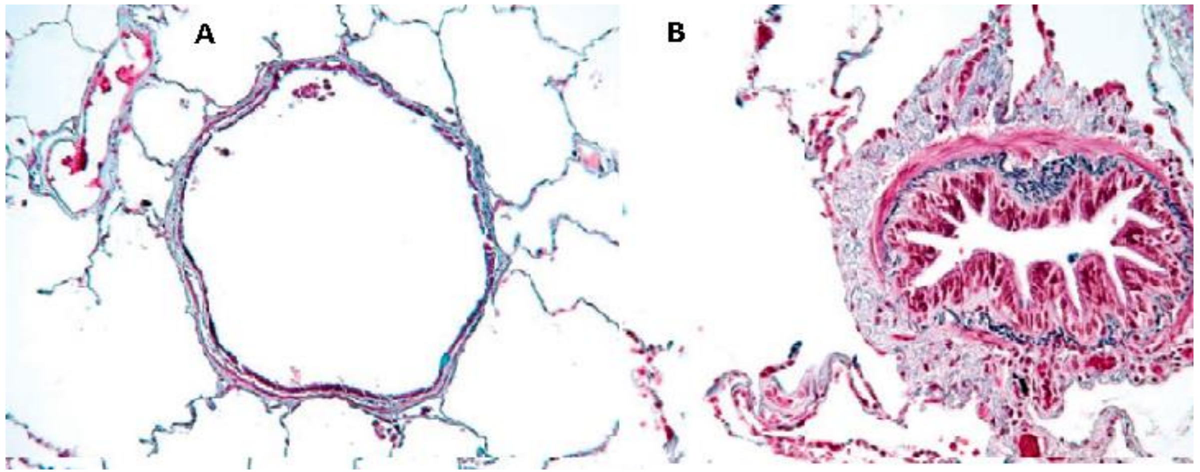


Figure 1.12: **Histological image of airways of a healthy subject and a subject with COPD.** (A) clear normal airways; (B) airways affected by COPD have inflammatory cell infiltration, mucosal hyperplasia and increased thickness of connective tissue in the peribronchiolar space. Modified from (Decramer et al., 2012)**.**

1.6.2.1 Aetiology of COPD

1.6.2.1.1 Smoking risk factors

Cigarette smoke is the main risk factor for COPD, which probably accounts for ~80–90% of COPD cases in the US (Yoshida and Tuder, 2007). Cigarettes contain a mixture of carcinogens, irritants and free radicals and when these components are introduced into the lungs, excessive inflammatory responses disturb the normal lung defence mechanisms (Nikota and Stämpfli, 2012). The chemical components of cigarettes generate reactive and unstable free radicals, such as superoxide anion, nitric oxide, peroxynitrite (ONOO-) and hydroxyl radicals, leading to a series of chain reactions resulting in tissue damage due to oxidation (Figure 1.13). Several studies have shown the presence of oxidative stress and free radical biomarkers in patients with COPD. High levels of 8-hydroxy-deoxyguanosine have been found in the urine of COPD patients and increased levels of 3-nitrotyrosine and F2α isoprostanes have been detected in the lungs. These biomarkers are associated with disease severity (Yao and Rahman, 2011). The gas and tar phase of cigarette smoke contains reactive oxygen and nitrogen species (ROS, RNS), as well as phenols and quinone (Yoshida and Tuder, 2007). ROS/RNS are also induced by the inflammatory immune response of cells

such as epithelial cells, resident macrophages, neutrophils, monocytes, B and T lymphocytes amplified by cytokines, and chemokines. The main ROS-generating enzyme in inflammatory cells is nicotinamide adenine dinucleotide phosphate-oxidase (NADPH) and other enzymes are involved, such as xanthine oxidase. Likewise, RNS in the form of nitric oxide (NO) is generated by nitric oxide synthase. A study of a mouse model showed that NADPH oxidase causes enlargement of the airspace, suggesting that ROS derived from NADPH oxidase actively contributes to the signalling pathway of tissue homeostasis. Therefore, the use of an NADPH oxidase inhibitor to alter the oxidant/antioxidant imbalance in COPD may be damaging (Yao and Rahman, 2011). Cigarette smoking has various effects on the mechanical barrier of the lung, reducing the frequency of ciliary beat and promoting squamous metaplasia. This may lead to a reduced number of ciliated cells and the stimulation of goblet cell and submucosal gland secretions. The high production of mucus in the airways and impaired mucociliary clearance promotes bacterial growth and colonization in the airways (Beasley *et al.*, 2012). Once bacteria have become established, they increase mucus secretion, disrupting the ciliary activity and amplifying the chronic cycle of infections. Chronic bacterial infection, which produces inflammation, contributes to progressive airflow obstruction and lung destruction. Cigarette smoking also impairs the ability of innate immune cells, such as macrophages and neutrophils in the airway, to eradicate pathogens by phagocytosis (Beasley *et al.*, 2012, Sethi, 2010).

Pulmonary emphysema is the key feature of COPD and it is much more likely to be found in cigarette smokers than in non-smokers. Emphysema is defined as a permanent enlargement of air space distal to the terminal bronchioles. It is associated with the irreversible destruction of alveolar walls and contributes directly to airflow reduction (Horio *et al.*, 2017). The process includes the disruption of the epithelial barrier and thus interfering with mucociliary clearance, resulting in an increase of inflammatory mucous in the small conducting airways and accumulations of connective tissue in the airway wall (Hogg, 2004).

Emphysema is characterized by different inflammatory cells including neutrophils, alveolar macrophages, $CD4^+$ and $CD8^+$ lymphocytes and imbalances of proteinase and anti-proteinase within alveolar space, leading to the permanent loss of airspace and destruction of alveoli (Cheng *et al.*, 2009, Goldklang *et al.*, 2012).

It has been shown that activated macrophages and neutrophils produce proteases, including matrix metalloproteinases (MMPs), which degrade elastin, collagen, proteoglycans and other structural proteins leading to alveolar wall damage and contributing to the aetiology of emphysema (Cheng *et al.*, 2009, Ostridge *et al.*, 2016). The expression of MMP is also found in the airways of COPD subjects (Ostridge *et al.*, 2016).

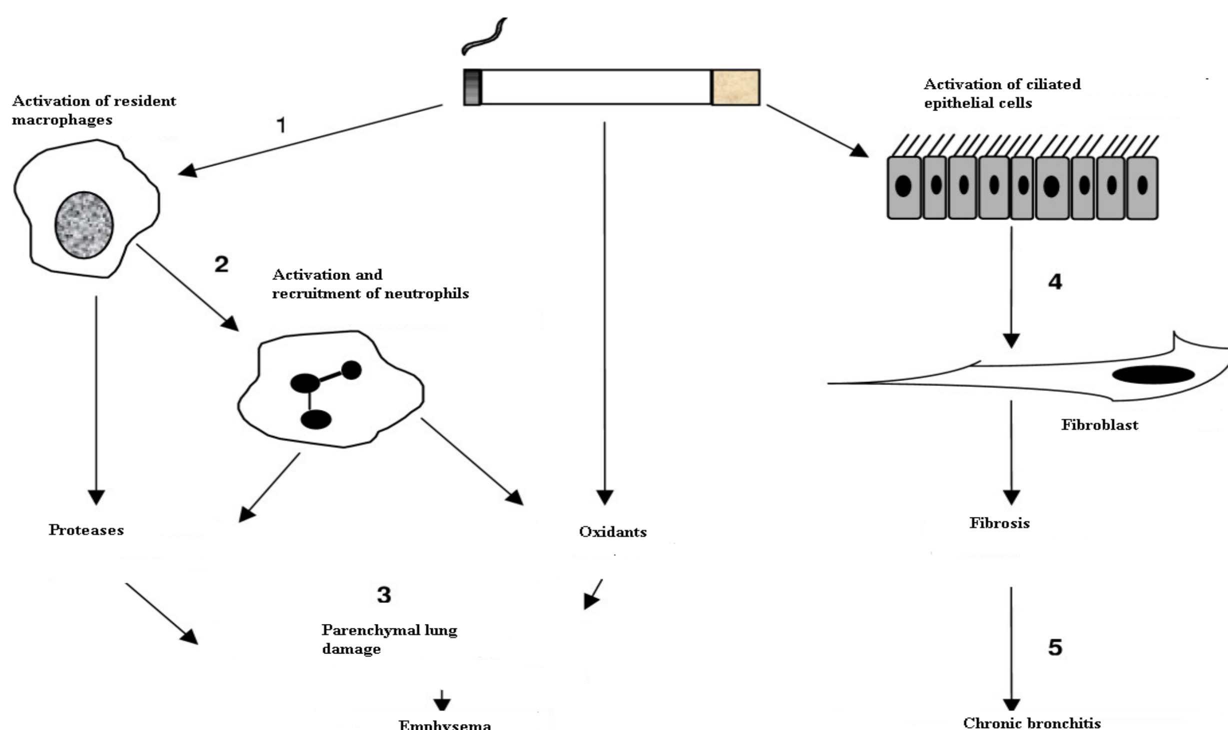


Figure 1.13: The pathogenesis of COPD.
Cigarette smoke induces inflammatory responses, which in turn activate macrophages (1) and neutrophils (2) to release proteases. The chemical components of cigarette smoke generate oxidants, resulting in parenchymal lung damage (3) and over time induce emphysema. In addition, activated epithelial cells stimulate fibroblast activity, resulting in chronic bronchitis (5). Adapted from (Wood and Stockley, 2006).

1.6.2.1.2 Non-smoking risk factors

Although the major environmental risk factor for COPD is tobacco smoke, the cellular and molecular mechanisms that are involved in the pathogenesis of the development of COPD have not fully been explained. It has been suggested that genetic factors are major contributors, such as the rare hereditary deficiency of α 1-antitrypsin (AAT), a disintegrin, metalloproteinase 33 (ADAM33), antioxidant superoxide dismutase (SOD) and pro-inflammatory mediators, such as TNFα (D'hulst *et al.*, 2005, Yao and Rahman, 2011). AAT is a serine protease inhibitor, which is synthesized in the liver and belongs to the group of acute phase proteins associated with inflammatory infection. It is elevated in 1–3% of patients with COPD (Mannino and Buist, 2007, Wozniak *et al.*, 2015). Other factors, such as indoor air pollution from burning biomass fuels, are also associated with an increased risk of COPD in developing countries (Cosio *et al.*, 2009). Indoor air pollution is probably responsible for an estimated 2 million deaths and 4% of the global health burden in developing countries. Exposure to various levels of indoor pollutants released from cooking, baking and heating with a biomass fuel, such as coal, straw, animal dung and wood, suggests that this chronic exposure is associated with chronic airflow obstruction in adults and acute respiratory infection in children (Liu *et al.*, 2007). Furthermore, exposure to dust, chemicals and fumes in the workplace is another risk factor that is associated with COPD (Mannino and Buist, 2007).

1.6.2.2 Diagnosis of COPD

In terms of diagnosis, the examination of medical history and spirometry are critical steps. Patients with COPD complain of a cough, sputum production, dyspnoea and wheezing. Spirometry is used to measure airflow limitation, which is helpful in assessing the severity of COPD. To increase the accuracy of spirometry, the test should be performed when the patient is stable or 8 weeks following exacerbation (Dutta, 2013). Chest X-rays are useful to

show pockets of trapped air that are associated with emphysema. Sputum production analysis is not very useful in diagnosing patients with COPD because the majority of patients produce a small amount of sputum with coughing (Dutta, 2013).

1.6.2.3 Innate and adaptive immune cells in COPD

1.6.2.3.1 Neutrophils

Neutrophils are short-lived cells and the first line of defence recruited from the circulation to the airways of the lungs. The role of neutrophils in the pathogenesis of COPD has been established. Activated neutrophils are increased in the sputum and the BAL fluid of patients with COPD. These cells produce serine proteases, including neutrophil elastase (NE), cathepsin G, proteinase-3, matrix metalloproteinase (MMP)-8 and MMP-9, which in turn leads to inflammation and alveolar destruction (Barnes *et al.*, 2003). Neutrophils also contribute to the production of mucosal metaplasia in chronic bronchitis and the destruction of lung tissues in emphysema. Studies focused on the analysis of airway smooth muscle have found an association between neutrophil infiltration and both computed tomographic measurements of air trapping and the severity of airflow. Such studies explain that exposure to inflammatory mediators leads to smooth muscle contraction, mucus induction and peripheral airway dysfunction in COPD (O’Donnell *et al.*, 2006).

1.6.2.3.2 Macrophages

Macrophages are part of the innate immune system, playing a major role in the airway defence mechanism against infections and potentially contributing to the pathophysiology of COPD. Macrophages are markedly increased in the airways, lung parenchyma, BAL fluid and sputum of COPD patients (Barnes *et al.*, 2003). Macrophages release inflammatory compounds, such as reactive oxygen species, chemotactic factors, inflammatory cytokines, smooth muscle constrictors, mucus gland activators, extracellular matrix proteins and a

range of matrix metalloprotease enzymes (MMPs). These mediators are stimulated by the components of cigarette smoke. Specifically, alveolar macrophages induce elastolytic enzymes, including MMP-2, MMP-9 and MMP-12, resulting in emphysema. It has been shown that alveolar macrophages from COPD patients have a high level of elastolytic activity at baseline compared with normal smokers (O'Donnell *et al.*, 2006).

1.6.2.3.3 Epithelial cells

Airway epithelial cells are the first line of defence and provide a physical barrier preventing invasive infection, as well as producing a range of antimicrobial compounds, such as defensins and other cationic peptides. These cells have numerous PRRs that are necessary for pathogen recognition. In addition, these cells secrete antioxidants and antiproteases, such as secretory leukoprotease inhibitor (SLPI) (Barnes *et al.*, 2003, Parker and Prince, 2011). Airway epithelia can be affected by environmental stimuli, such as airborne pollutants, allergens, cigarette smoke and microbes, leading to a change in epithelium defence mechanisms, such as the secretion of mucus and reduced efficiency of ciliary beating. Once the epithelial cells are activated, they produce IFNα and TNFα. These mediators prompt epithelial cells to release secondary mediators, including lipid mediators, reactive oxygen species (ROS), IL-1β, GM-CSF and IL-8, all of which increase inflammation and contribute to the pathophysiology of COPD (Barnes *et al.*, 2003, Pettersen and Adler, 2002).

1.6.2.3.4 T lymphocytes

T lymphocytes have a variety of activities involved in protective immunity against lung infection. They provide help for proliferation and functioning of other cell types. There are different subsets of T cells involved in COPD including $CD8^+$, $CD4^+$, T regulatory cells and $Th17^+$ T cells, all potentially contributing to the pathogenesis of COPD (Grumelli *et al.*,

2004). Tobacco smoking with COPD also stimulates the humoral and cellular components of the adaptive immune response (D'hulst *et al.*, 2005).

In COPD, $CD8^+$ lymphocytes secrete a predominantly Th_1 cytokine pattern that includes increased production of IFNγ, interferon-inducible protein-10 (IP-10) and monokine produced by interferon-gamma (MIG). These mediators can lead to tissue injury through the up-regulation of matrix metalloproteinase (MMP) (Brusselle *et al.*, 2011, Gadgil and Duncan, 2008). Furthermore, $CD8^+$ T cells can cause cell death by secreting cytotoxic mediators such as granzyme and perforins, suggesting a potential role in COPD pathophysiology (Brusselle *et al.*, 2011). Various studies have reported high levels of $CD8^+$ in the sputum of smokers with and without COPD compared with non-smokers, and that these cells are highly active in producing lytic substances such as perforin. Increased levels of $CD8^+$ T cells have also been reported in the lung parenchyma and within the smooth muscle of airways of COPD patients (Sohal *et al.*, 2013).

$CD4^+$ T cells are also important in the release of activating cytokines and in focusing and increasing inflammatory responses by other immune effectors such as macrophages, neutrophils and NK cells. $CD4^+$ T cells provide help for the activation of B cells and may facilitate the production of IgG autoantibodies in COPD. They also provide help for isotype switching from IgM to strong and avidly binding IgG antibodies, in particular against protein antigens (Gadgil and Duncan, 2008). Grumelli *et al.* (2004) found that reduced lung function in COPD patients is strongly associated with $CD4^+$ and $CD8^+$ T lymphocytes expressing a specific repertoire of chemokine receptors, such as CCR5 and CXCR3 (markers for Th_1 but not Th_2). Furthermore, peripheral lung lymphocytes from ex-smokers with COPD show that both $CD4^+$ and $CD8^+$ T cells are polarized to the Th_1 phenotype compared to cells from the lung tissue of normal individuals. The destruction of alveolar tissue observed in the lungs of

patients suggests an autoimmune component involving $CD4^{+}$T cells in the pathogenesis of COPD (Vargas-Rojas *et al.*, 2011).

It has been shown that, in a COPD cohort, TNFα, IL-13 and IL-17 are produced by peripheral T cells in response to non-typeable *H. influenzae* (NTHi). These pro-inflammatory mediators have been proposed to contribute to inflammation (King *et al.*, 2013). Th_{17} cells are a critical component of the adaptive immune response, which is implicated in chronic inflammatory and autoimmune diseases. These cells are important in the clearance of pathogens that are not cleared by Th_1 and Th_2 cells, such as extracellular bacteria and fungi. It has been shown that the differentiation of naive T cells into Th_{17} depends on the presence of TGFβ1 and IL-6. Elevated levels of TGFβ1 in the serum of COPD patients and increased IL-6 in both serum and sputum during COPD exacerbation may lead to higher Th_{17} cells observed in patients with COPD compared to smokers without COPD and healthy individuals. Also, a large number of Th_{17} cells may predict the severity of airflow, as the decline in lung function is associated with increased Th_{17} cells (Cazzola and Matera, 2012).

1.6.2.4 Exacerbation of BR and COPD

An exacerbation of BR and COPD are defined as an acute worsening of respiratory symptoms associated with events of physiological instability, including dyspnoea, cough, sputum production, large volumes of purulent sputum and wheezing, and may include systemic complaints such as fever and fatigue for >24 h. These symptoms contribute to the progressive decline in lung function and accelerate morbidity, mortality and poor health status (Dickson *et al.*, 2014, Metaxas *et al.*, 2015). Acute exacerbation (AE) is more common in older individuals with COPD, who experience worsening of symptoms for >48 hours leading to the acceleration of lung function decline, respiratory failure and often death (Liao *et al.*, 2015). The causes of exacerbation are not well-defined and are described as heterogeneous events because various factors are implicated, such as respiratory virus and

bacterial infections of the airway, often associated with the progression of airflow obstruction (Gompertz *et al.*, 2001, Wedzicha and Seemungal, 2007). In COPD, the most frequent viruses associated with an exacerbation are rhinoviruses, coronavirus, respiratory syncytial virus, influenza and parainfluenza viruses. It has been found that exacerbations caused by respiratory viral infection are more severe and require a longer recovery time (Wedzicha and Seemungal, 2007) than those caused by other factors. Traditional culture-based methodologies have demonstrated that approximately 50% of COPD exacerbations are associated with bacterial infections, such as *S. pneumoniae, H. influenzae* and *M. catarrhalis*, leading to increased symptoms of coughing, sputum production and inflammatory changes, particularly in acute exacerbations. However, these pathogens can also colonize the airways between exacerbations (Han *et al.*, 2012 , Mohammad and Dhanashree, 2012, Pragman *et al.*, 2012). Tissue deterioration and inflammation in the lung impairs local immune responses and provides a suitable microenvironment for additional complicated pathogens, such as *P. aeruginosa* and *Stenotrophomonas maltophilia (S. maltophilia)* (Lode *et al.*, 2007). These bacteria stimulate inflammatory mediators, such as neutrophils, IL-8 and TNFα, in the sputum (Beasley *et al.*, 2012). However, recent studies have suggested that bacterial and viral infections may not be the main causative factor in the development of BR exacerbations (Guan *et al.*, 2015, Metaxas *et al.*, 2015). *P. aeruginosa* colonization has predominantly been associated with a higher number of exacerbations, poor quality of life and increased hospital admissions compared with patients colonized with other bacterial infections, accelerating lung function decline (Chawla *et al.*, 2015).

1.6.3 Asthma

Asthma is a long-term chronic inflammatory condition, comprising airway limitation, in which many cells and cellular elements play a critical role (Gillissen and Paparoupa, 2014). Globally, asthma affects more than 300 million people, with approximately 250,000 annual attributable deaths (Kudo *et al.*, 2013). The Health and Social Care Information Centre 2013 showed that asthma affects an

estimated 3.8 million individuals in the UK and incidence has increased worldwide in the past 30 years (Hazeldine, 2013). Asthma often starts in childhood, both due to inherited genetic traits and in association with exposure to common inhaled allergens. Around one million children in the UK have been diagnosed with asthma (Reed and Neil, 2013). It is characterized by recurrent episodes of shortness of breath, coughing, chest tightness, sputum production and wheezing. These symptoms increase at night or in the early morning, as well as after exposure to environmental antigens or stimulus (Racusin *et al.*, 2013). The predominant cause of asthma is genetic disposition, together with exposure to environmental antigens and factors such as house dust mites, plant pollens, animal dander, airborne pollutants, viral infections, physical factors (i.e. exercise and cold air) and some drugs, such as aspirin (Clancy and Blake, 2013). Despite some similarities between asthma and COPD, there are obvious differences in the airway physiology, pathogenesis and different patterns of inflammation. COPD shows persistent airflow limitation that is not fully reversible (Kitaguchi *et al.*, 2016, Tzortzaki *et al.*, 2011). However, asthma is characterized by chronic inflammation and airway remodeling leading to airflow obstruction that is often reversible spontaneously or by treatment (Tzortzaki *et al.*, 2011).

The chronic inflammation within the respiratory tract are associated with the involvement of different inflammatory cells and cytokine expression (Aoshiba and Nagai, 2004).

1.6.3.1 Pathology of asthma

The clinical hallmarks of the pathogenesis of asthma are airway inflammation with reversible airflow limitation, collagen deposition under the epithelium, increased airway smooth muscle and thickening of the sub-mucosal basement membrane (Barnes, 2008). Collagen types I and III are produced by activated fibroblasts, such as myofibroblasts, resulting in significant structural changes in the airways (Chanez *et al.*, 2004). The loss of ciliated epithelium cells is also associated with asthma, as well as increased mucous gland hypertrophy in the submucosa. Airway obstruction in asthmatics can affect both the large and the small airways and lead to the early closing of the smaller airways and a decrease in expiratory flow rates (Fireman, 2003). The main feature of asthma is declining lung function,

with a reduction in peak expiratory flow (PEF) or forced expiratory volume in 1 second (FEV_1) (Edwards *et al.*, 2012). Abnormalities in the general immune response and local immune dysregulation in the mucosal surface are also implicated in asthma. Airway remodelling occurs in asthmatic patients because of repeated episodes of inflammation and through the production of matrix proteins and growth factors induced by inflammatory cells. Recurrent epithelial damage followed by tissue repair is another cause of airway remodelling (Barrios *et al.*, 2006).

1.6.3.2 Immunopathogenesis

Asthma is an inflammatory disorder of the pulmonary airways associated with the innate and adaptive immune response that involves both cellular and humoral immunity result in the clinical expression of reversible lower airway obstruction (Fireman, 2003, Holgate, 2012). It is classified into several phenotypes, including an allergic asthma phenotype characterized by allergen sensitization and involving an adaptive immune response resulting in the induction of allergen-specific Th_2 and IgE responses (Holgate, 2012). In contrast, non-allergic asthma phenotypes are associated with exposure to environmental factors such as air pollution and cigarette smoke, involving innate immunity. These include macrophages, neutrophils, natural killer T cells and innate lymphoid cells (Yu *et al.*, 2014). The airway epithelium contains a large population of airway epithelial cells that interact with inhaled allergens and pathogens and can activate the immune response by influencing antigen-presenting cells such as DCs. These cells express various extracellular and intracellular PRRs, such as TLRs. Mature DCs that have taken up allergens migrate to draining lymph nodes, where they process the allergens into small peptides, and then present them via MHC class I and MHC class II to T cells (Papazian *et al.*, 2015). Co-stimulatory molecules and epithelial-derived cytokines and chemokines such as IL-33, IL-25, CCL17 and CCL22

induce the activation of DCs and Th_2 maturation, which then migrate into the mucosa (Holgate, 2012).

Group 2 innate lymphoid cells (ILC2s), another population of innate cells associated with Th_2 responses, are similar to lymphocytes but they lack expression of T-cell or B-cell antigen receptors. These cells produce signature cytokines associated with Th_1, Th_2, or Th_{17} cells, including IL-5 and IL-13. It has been shown that ILC2 are implicated in lung inflammation and mediate allergic inflammation in response to the papain allergen (Papazian *et al.*, 2015). Furthermore, humoral immune responses play a critical role in the immediate allergic response upon exposure to allergens. Isotype switching from IgM to IgE involves both cytokines (IL-4 or IL-13) and ligation of CD40 and CD40L modulated by Th_2 cells (Holgate, 2012).

The eosinophils, mast cells, T cells (in particular Th_2 $CD4^+$) and macrophages are the major cells described in asthma aetiology (Aoshiba and Nagai, 2004). Mast cells are also important in the pathology of asthma, in which Fc receptors bind with high affinity to IgE. Upon exposure to antigen, binding mast cells are stimulated to release histamine and tryptase and to synthesize leukotrienes, prostaglandins and platelet-activating factor. These mediators promote smooth muscle contraction and increase secretion from mucus glands (Fireman, 2003) (Figure 1.14). Studies in animal models of asthma have shown that Th_2 cells are associated with the pathogenesis of asthma through the recruitment of eosinophils and induce goblet cell hyperplasia. Th2 cytokines such as IL-5, IL-9 and IL-13 activate eosinophils to produce inflammatory mediators, such as leukotrienes, platelet-activating factor and major basic protein. These derived mediators damage the mucosa and accelerate pulmonary obstruction (Lacy and Moqbel, 2000). Furthermore, IL-5 is essential in eosinophil proliferation, differentiation and maturation, migration to tissue sites and survival. Thus, in the case of asthma associated with eosinophils, targeting IL-5 inhibition

(through neutralizing mAbs) may be an effective method for treatment of especially severe asthma (Garcia *et al.*, 2013).

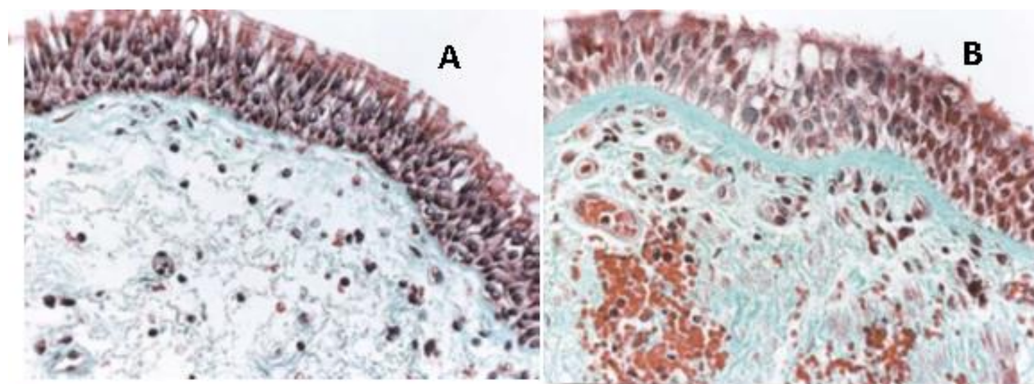


Figure 1.14: Histological image of bronchial mucosa in a normal individual (A) and a patient with asthma (B).
Obvious differences in the asthmatic compared to the healthy individual are the goblet cell hyperplasia in the epithelial cell lining, the thickening of the sub-basement membrane and the collagen deposits and cellular infiltration (Busse and Lemanske, 2001).

1.6.3.3 Microbial infections in asthma

Bacterial and viral infections are associated with the exacerbation of asthma by impairing mucociliary clearance and inducing increased mucus production. *S. aureus, H. influenzae* and *Chlamydia pneumoniae* (*C. pneumoniae*) are pathogens often isolated from the sputum of asthmatics. The persistence of these bacteria is perhaps due to the reduced phagocytic capacity of macrophages in eradicating bacteria, leading to accelerated chronic inflammation and exacerbations (Liang *et al.*, 2014). *S. aureus* is frequently isolated from the respiratory tract and is found in 87% of patients with intrinsic asthma (Stentzel *et al.*, 2016). Infection with these bacteria is characterized by the high production of inflammatory mediators such as TNFα, IL-8 and IFNγ; these mediators activate local immune cells such as Th_2 and macrophages, leading to structural changes in the airways through the regulation of MMPs (Liang *et al.*, 2014). *Mycoplasma pneumoniae* has been identified in respiratory samples of adults and children affected with asthma and is associated with the severe persistent acute exacerbation of asthma. *M. pneumoniae* infection promotes the host inflammatory response

via the activation of neutrophils, lymphocytes and plasma cells, resulting in pulmonary injury. The protein membrane components of *M. pneumoniae* activate PRRs, including TLR-1, TLR-2 and TLR-6, and increase the production of proinflamatory cytokines such as IL-8 and TNFα (Chaudhry *et al.*, 2016). *C. pneumoniae* also inhibits the apoptosis of the infected cells, which ensures the longevity of the host cells and increases chronic inflammation due to persistent infection (Gillissen and Paparoupa, 2014). Furthermore, viral infections such as respiratory syncytial virus (RSV) are very common in individuals with asthma. RSV causes an imbalance between Th_1 and Th_2, with high levels of IFNγ and low levels of IL-4. Increased levels of IL-4 and IFNγ in cord blood are associated with a diagnosis of asthma at 6 years of age, so the measurement of cytokines may be helpful in diagnosing individuals at risk of asthma (Castro *et al.*, 2008).

1.6.4 Cystic fibrosis (CF)

CF is one of the most common life-threatening autosomal recessive conditions in Caucasian populations (Milagres *et al.*, 2009). Genetically, CF is caused by a mutation in the gene that codes for the CF transmembrane conductance regulator (*CFTR*) protein. The defective gene was cloned in 1989 and is located on the long arm of chromosome 7 (Jackson and Pencharz, 2003). CF is also defined as a disease of exocrine gland function, initiating several pathological and clinical complications (Jacquota *et al.*, 2008). The main function of the *CFTR* protein is the transport of chloride ions and the regulation of other ion channels through the epithelial cell membrane, such as the inhibition of the transport of sodium ions through the sodium channel, the regulation of adenosine triphosphate (ATP) channels, the exchange of bicarbonate-chloride and others (O'Sullivan and Freedman, 2009). The failure of chloride ion transport into the bronchial mucosa leads to abnormally high viscosity secretions and mucus plugging, resulting in an impaired volume of ciliary movement and the inhibition of mucociliary clearance, which promotes bacterial colonization and

significantly reduces life expectancy in CF (Bourke, 2002, Henrique, 2007). Structural abnormalities found in CF lungs include BR, the accumulation of airway mucus, peribronchial inflammation and fibrosis associated with high levels of MMPs and glycosaminoglycan (Cigana *et al.*, 2016).

1.6.4.1 Microbial infections in CF

The hallmarks of CF lung disease are acute and chronic bacterial infection and subsequent excessive inflammatory immune responses. High sodium chloride levels on the airway surface disrupt mucociliary transport and may inhibit anti-microbial molecules, such as lysosome and β-defensins, thus enhancing bacterial growth (Ratjen, 2006). The most commonly isolated pathogens in CF are *S. aureus, P. aeruginosa*, *Burkholderia cepacia complex (B. cepacia)*, *S. maltophilia* and non-tuberculous mycobacteria (de Vrankrijker *et al.*, 2010). It has been suggested that *S. aureus* is the main pathogen colonizer during early CF infection, whereas *P. aeruginosa* is eventually established and persists for decades because of its ability to survive in various environments and develop resistance to different antibiotics (Nguyen *et al.*, 2016). Furthermore, early *P. aeruginosa* infection together with *S. aureus* colonization significantly influences the progress of CF pathology due to its virulence factors and the ability to evade immune responses and antibody treatment (Yonker *et al.*, 2015). Group members of *B. cepacia* have also been isolated from older CF patients and can cause nosocomial infections in people with CF. This group is highly resistant to antimicrobial compounds and antibiotic treatment. Therefore, it is necessary to identify the strains early on and to ensure that patients colonized with *B. cepacia* are isolated from other patients to reduce the risk of transmission (Baldwin *et al.*, 2005). Several studies have shown that most of the bacteria isolated in biofilms contain exopolysaccharides and host mucin. It is suggested that airway inflammation caused by these pathogens is induced by the release

of bacterial components, which access the airway epithelium and stimulate immune cells in the lung, rather than by direct contact between organism and host cells (Sadikot *et al.*, 2005).

The acquisition of fungal species also occurs in the respiratory tract of patients with CF. *Candida* species and *Aspergillus fumigatus (A. fumigatus)* are the most common fungi isolated from the airways of CF patients, with a prevalence of *A. fumigatus* ranging from 16% to 45.7% and *Candida dubliniensis* being present in 11.1% in CF patients (Bouchara *et al.*, 2009).

1.6.4.2 Inflammatory cells and cystic fibrosis

The inflammatory response in CF involves both innate and adaptive responses. Neutrophils migrate to the site of infection and release inflammatory mediators and pro-inflammatory cytokines, such as TNFα, IL-8, and IL-1β, causing lung injuries (Figure 1.15). Neutrophils also release oxidants and protease mediators, such as neutrophil elastase. Neutrophil elastase can digest the elastin fibres and other matrix proteins in the airway, increasing mucus secretion, stimulating the generation of chemoattractants and promoting degradation and tissue damage (Courtney *et al.*, 2004). *CFTR* dysfunction results in impaired human macrophages and increases pro-inflammatory cytokine released by macrophages, such as IL-1α, IL-6, G-CSF and IL-8 (Yonker *et al.*, 2015). IL-8, TNFα and IL-1β produced by macrophages in response to bacterial LPS may activate neutrophils and aid development of Th_1 or Th_2 response in CF (Conese *et al.*, 2003).

The role of T lymphocytes against infections is pathogen-specific (Yonker *et al.*, 2015). Most patients with CF and chronic *P.aeruginosa* colonization have increased antibody responses and are dominated by Th_2 immune responses, characterized by IL-4, IL-5, IL-6 and IL-10 production, as well as IgG1 and IgE antibody (Song *et al.*, 2003). Furthermore, a high anti-Pseudomonas-specific IgG response associated with defective STAT1 signalling is found in CF respiratory epithelium. Increased levels of IgG response appear insufficient

to clear the bacteria from the lung or prevent the further acquisition of new *P. aeruginosa*. However, specific IgG is likely to be important in preventing the systemic spread of *P. aeruginosa*, as infected patients rarely develop pseudomonal bacteremia or sepsis (Amin *et al.*, 2010). γδT-cells from the peripheral blood cells of CF patients colonized with *P. aeruginosa* produce high TNFα and interferon-γ levels. Thus, T cell responses to *P. aeruginosa* potentially contribute to the progression of respiratory disease and failure (Dubin *et al.*, 2007).

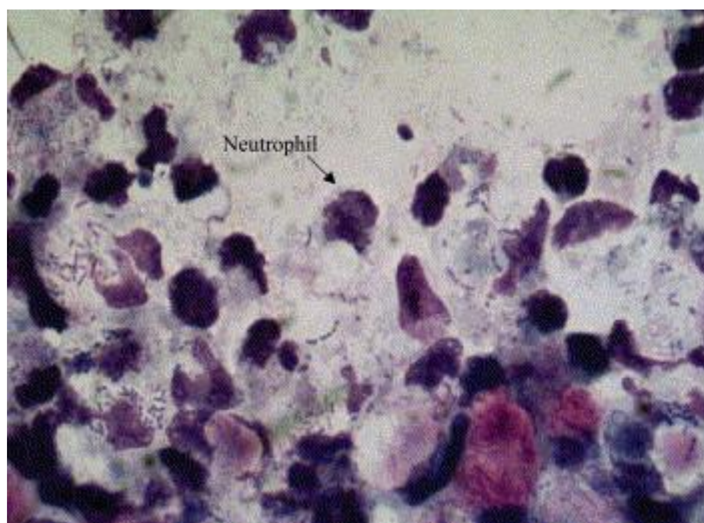


Figure 1.15: Neutrophil infiltrate in the sputum of a patient with CF.
Activated neutrophils recruited to the site of infections release pro-inflammatory cytokines (Courtney et al., 2004).

Recent studies have shown that Th_{17} are present in the airway submucosa of young CF patients. These cells produce the signature cytokine IL-17, which is critical in the activation and migration of neutrophils in the lung. However, other IL-17-related cells, such as neutrophils, γδ T cells and natural killer T cells, have also been found. The IL-17 protein is important for the eradication of some pathogens that fail to be cleared by Th_1 and Th_2 (Tan *et al.*, 2011). IL-22, a member of the IL-10 family of cytokines produced by both Th cells and innate lymphoid cells, is involved in mucosal host defence, including in the lung, by inducing various antimicrobial peptides and promoting epithelium surface protection and repair (Bayes *et al.*, 2016). It has been found that healthy individuals and CF patients have

similar levels of Th_{22} specific to *P. aeruginosa*. These cells have been found to be positive for mucosal chemokine receptors, $CCR6^+$, but lack the skin-homing receptors CCR4 and CCR10 that have previously been detected on human Th_{22} (Bayes *et al.*, 2014).

The infected lungs of CF patients also show elevated proteolytic enzyme activity and high levels of chemokines such as IL-8 (Jacquota *et al.*, 2008). Although the main source of IL-8 in the airway is still unclear, several studies have shown that in response to some stimulus (i.e. bacteria-derived or autocrine stimulus TNFα), respiratory epithelial cells may be the predominant source, as bronchial epithelial cells from CF patients produce detectable levels of IL-8 and IL-6 compared with healthy controls (Gwyer Findlay and Hussell, 2012).

Table 1.4. **Summary of the characterization of chronic obstructive lung diseases**

Diseases	BR	COPD	Asthma	CF
Features	Chronic lung disease	Chronic conditions mostly caused by smoking	Inflammatory condition	Genetic disease
Microbial infection	*P. aeruginosa* *H. influenzae*	*H. influenzae* *P. aeruginosa*	*S. aureus* *H. influenzae*	*P. aeruginosa* *S. aureus*
Sputum production	High	Low	Low	High
BAL content	Neutrophils and pro-inflammatory cytokines	Neutrophils and macrophages	Eosinophils	Neutrophils and pro-inflammatory cytokines

1.7 Hypothesis and Aims

1.7.1 Research hypothesis

It has been shown that microbial infection is involved in the pathogenesis and exacerbation of obstructive lung diseases. This study hypothesizes that microbe-specific immune responses (T cell and antibody) are biomarkers, having a positive association with colonization, disease severity (lung function) and the exacerbation of obstructive lung diseases. It is expected that patients with chronic obstructive lung disease (COPD) will have higher anti-Haemophilus responses, cystic fibrosis (CF) patients have higher anti-Pseudomonas responses and bronchiectasis (BR) patients have a mixture of responses; moreover, exacerbation and severity are associated with higher immune responses for all groups.

1.7.2 Aims and objectives

The aim of this PhD research project is to investigate specific immune responses, both T cell and antibody, against colonizing microorganisms in patients with obstructive lung diseases – BR, COPD, non-infectious lung disease (asthma) and CF – compared to healthy controls. The data generated may provide a useful indication of colonization and severity, as well as of infection-induced disease exacerbation if the quality and quantity of specific responses correlate with microbiological analysis of sputum samples for the patients and their clinical parameters. Through the detailed phenotyping of reactive T cells, their contribution to the immunopathology of obstructive lung diseases will be explored.

2 Chapter Two: General Materials and Methods

2.1 Ethical approval

Ethical approval for the project was granted to Dr Anthony De Soyza, Department of Respiratory Medicine, Freeman Hospital by the local NHS Research Ethics Committee, the NRES Committee North East – County Durham & Tees Valley (ref 12/NE/0248) (see Appendix I). Internal ethical approval was obtained from Northumbria University, the School of Applied Sciences Ethics Committee (RE-HLS-HLS-12-100513). The ethical approval for the Manchester specimens was given to the Manchester Allergy, Respiratory and Thoracic Surgery Biobank Ethics Committee (REC reference: 10/H1010/7).

2.2 Human samples

Patients with BR and COPD, and healthy controls, were recruited by Dr Anthony De Soyza at the Chest Clinic, Freeman Hospital, Newcastle upon Tyne. Heparinized blood samples were taken from patients attending the Chest Clinic and transferred directly to the tissue culture lab at Northumbria University for processing. The sputum samples were analysed using the culture-based technique in the Microbiology Department at the Freeman Hospital. Sputum samples from BR patients were also subjected to genomic analysis for microbial colonization by Dr Clare Lanyon, Northumbria University.

Blood samples from CF patients were obtained by Dr Stephen Bourke from patients attending an adult CF clinic at the Royal Victoria Infirmary, Newcastle upon Tyne. In addition, sputum samples were collected and submitted to the Microbiology Laboratory for microbial analysis.

2.2.1 Clinical data

Patients were clinically stable at the time of assessment. They underwent spirometry testing to obtain forced expiratory volume in 1 second (FEV_1), FEV_1 % Pred, VC, FEV1/VC % and VC % Pred. The bronchiectasis severity index (BSI) score was assessed for 86 BR patients; these patients were classified as either hospitalized (>5 exacerbations) or as outpatients (2–3 exacerbations). (Appendix 11). The exacerbations were determined for the preceding 12 months. All clinical data were obtained by the research team at the Sir William Leech Clinical Trials Centre and calculated during routine medical follow up of the patients.

2.2.2 Separation of peripheral blood mononuclear cells (PBMCs) and plasma

15 ml of Lymphoprep (Axis-Shield, Norway) was added to sterile Leucosep tubes (Greiner Bio-One, Stonehouse.UK) and centrifuged for 20 second at 1500 rpm to obtain the Lymphoprep below the glass filter. Heparinized peripheral blood obtained from patients and healthy volunteers was added to the tube, on top of the glass filter, and the tube was centrifuged for 12 min at 2100 rpm. The tube then has three layers (Figure 2.1): the plasma as a yellow liquid at the top, the peripheral blood mononuclear cells (PBMCs) forming a cloudy layer located between the plasma, and Lymphoprep underneath and the other denser white blood cells and erythrocytes collected at the bottom of the tube. The plasma is removed, aliquoted and stored at −20 ºC. The PBMCs layer is carefully removed using a plastic pipette and washed in supplemented RPMI-1640 (R0, Appendix 6) by re-suspending and centrifuging at 1700 rpm for 10 min. If not used immediately, the cell pellet is re-suspended in freezing medium.

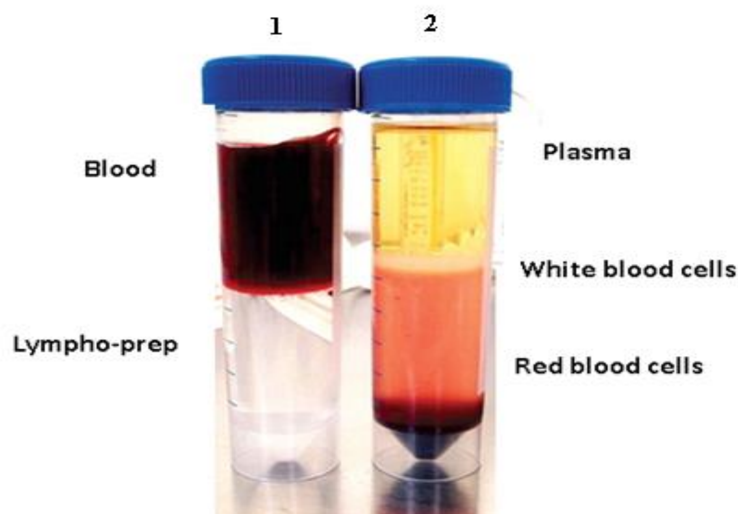


Figure 2.1: Separation of blood into different layers.
(1) Before centrifugation and (2) after. The top yellow layer is plasma, while the opaque layer is PBMC and the red layer at the bottom is composed of red blood cells.

2.2.3 Freezing the cells

The freezing medium was prepared by adding 90% of Fetal Bovine Serum (FBS) (Biosera, E. Sussex.UK) (Appendix 6) and 10% Dimethyl Sulfoxide (DMSO) (Sigma-Aldrich, Poole.UK) in a 7ml bijou tube. For 2 vials, 1.8 ml of FBS plus 0.2 ml of DMSO was prepared. The freezing medium was then added to the re-suspended pellet of the PBMCs and mixed gently using a sterile pipette. The cells and the freezing medium were aliquoted in labelled micro tubes and placed in a Mr Frosty controlled freezing box at -80 ^{o}C for 24 h. The frozen cells were then transferred to longer storage in a freezer at -150 ^{o}C (Thermo Scientific, Loughborough, UK).

2.2.4 Thawing the cells

When needed, the micro tubes containing the frozen cells were taken from -150 ^{o}C and quickly thawed by gently swirling in a water bath at 37 °C. The cells were then transferred to a sterile universal tube containing 10 ml of R0 and centrifuged at 1500 rpm for 5 min. The supernatant was decanted and the cell pellet re-suspended in 1 ml of R10 (Appendix 6) and then counted using a CASY® cell counter (Roche Diagnostics, Burgess Hill, UK).

2.2.5 Setting the CASY counter for cell counting

The CASY® cell counter (Roche Diagnostics, Burgess Hill, UK) (Figure 2.2) was used to count the PBMCs obtained from patients and healthy volunteers. Once the device is switched on, all required initialisation procedure is automatically performed. The system then completes a self-test with all memory modules. After the initialisation procedure is completed the CASY then displays a blank size distribution on the screen. Measurement of the PBMCs is initiated by filling a CASY cup with clean CASYton solution and by selecting the appropriate setting according to the parameters for the selected measuring capillary that contains the external electrode. The CASYton solution under the measuring capillary is used to monitor the background and clean the cell counter system before and after each sample run through the CLEAN key holding down 3 times. Once the background is clear, a new CASY cup containing 10 ml CASYton and 10µl of cell suspensions was placed on the measuring capillary and measurement is started by pressing the key ENTER. The cell size ranges for counting are: Lymphocytes 5.4-9.2 µm and Monocytes 9.3-20 µm as validated by the flow cytometer's scatter of lymphocytes and monocytes. All cell counts were adjusted to $8x10^{6}$cells/ml to obtain optimal T cell responses and maintain consistency among samples.

Figure 2.2: The CASY® cell counter for PBMCs counting.
Adopted from (Sabanci University Nanotechnology Research and Application, 2013)

2.2.6 Cell counting

The CASY® cell counter was first cleaned with CASY®clean (Roche Diagnostics GmbH, Mannheim, Germany) to remove any residual cells. The cells were re-suspended in 1 ml of R10 thoroughly to disperse any clumps. Subsequently, 10 µl of the cell suspension was transferred into a CASY cup containing 10 ml of CASY®ton (Roche), which was mixed thoroughly and run on the CASY counter. Lymphocytes, monocytes and total PBMCs were measured based on the cell diameter with a readout of cells per ml.

2.3 Preparation of bacterial lysate

Bacterial strains were provided by Dr John Perry, Microbiology Department, Freeman Hospital, cultured with the help of Dr Andrew Nelson, Northumbria University. The strains were cultured from a single colony in 10 ml of broth, the type depending on the species (Table 2.1) and incubated with shaking at 200 rpm overnight at 37 ^{o}C. Then, 1 ml of the culture (Appendix 2) was transferred into broth in a 200 ml flask and incubated with further

shaking for 2 h at 37 ℃ until the optical density (OD) of the culture reached 0.8. This culture was then decanted into 50 ml universal centrifuge tubes and the cells harvested by centrifugation at 4000 rpm for 15 min at 4 ℃. The supernatant was removed to leave the bacterial cell pellets and 50 ml sterilized PBS (Appendix 5) was added to the pellets to wash them. This wash was repeated 3 times and the final pellet was suspended in 5 ml of PBS. Using the Sonicator (Soniprep 150, Henderson Biomedical, UK), equipped with a microtip, the suspension was sonicated on ice in 10 seconds bursts at high intensity with 10 seconds cooling periods on ice between each burst. The cells lysate was decanted into microfuge tubes and centrifuged at 13000 rpm at 4 ℃ for 20 min to remove debris. The soluble lysate was aliquoted and stored at -20 ℃.

2.4 Determining protein concentration using the Bradford assay

The concentration of protein in the lysate was measured using the Bradford assay. A 50 µl aliquot of the original lysate was diluted in PBS. 10-fold dilution steps of bovine serum albumin (BSA – Sigma-Aldrich, Poole. UK) in 500 µl of PBS, ranging from 0.1 mg/ml to 1.4 mg/ml produced the series of standards. Then 50 µl of this protein standard and 50 µl of lysate samples were added to a 96-well plate. Subsequently, 200 µl of Bradford Dye Reagent (B6916 – Sigma-Aldrich, Poole. UK) (Appendix 2)was added to each well. The plate was incubated for 30–45 min at room temperature (RT) and then read on an ELISA Plate Reader (EL808, Bio-Tek Vermont, USA) at 595 nm. The concentration of unknowns was read from the standard curve.

2.5 Separation of protein using SDS-PAGE electrophoresis

The components of the SDS-PAGE 12% resolving gel (Appendix 3) were combined, mixed and poured between two clean glass plates of the dimensions 10.1x8.2 cm, separated by a 1 mm spacer ridge on the larger of the two plates. The plates were held together with clamps and checked to ensure the bottom edges of the plates were flush. The resolving gel was then

slowly pipetted into the space between the plates. A layer of deionized water was added to cover the top of the resolving gel to prevent oxygen from inhibiting polymerization. The resolving gel was then allowed to polymerize for 45 min. Once the gel was set, the water was removed from the top of the gel by means of absorption with filter paper. The components of the stacking gel (Appendix 3) were then combined, mixed and loaded on the top of the resolving gel. A 10-toothed comb (1.1x0.75 cm) was then inserted carefully into the gap between the plates at an angle to prevent air bubbles being trapped between the comb and the stacking gel and was allowed to set for 45 min. The comb was then removed to reveal the wells, which were immediately rinsed with deionized water to remove any gel debris. The gel was placed vertically in the electrophoresis rig (Bio-Rad). SDS-PAGE (1X) (Appendix 3) running buffer was added to the tank with caution to ensure that the level of the buffer in the space between the two gels was higher than the level on the outside of the gels. Loading samples were prepared for SDS-PAGE by adding 5 µl of loading buffer to 20 µl of each sample. The samples were boiled for 5 min.

Then, 20 µl of samples and 10 µl of the molecular weight markers were loaded into the wells using a P20 pipette. The apparatus was connected to the power supply at 120 mA and run at 150 V for 45 min. Once all the protein migrations had completed, the power supply was turned off and the gel was removed from the plate and placed in Coomassie blue for 5–10 min on a rocking table and stained with Coomassie blue (Appendix 3). The gels were then immersed in de-staining buffer (Appendix 3) and left overnight on a rocking table at RT until bands appeared. The gels were photographed using a gel documentation system (Bio-Rad Gel Doc2000) using Quantity One™ software. Hard copies of the gel photographs were printed using a Mitsubishi video copy processor (Model P91, attached to the gel doc system).

Table 2.1. Microbial species used for antigens in the antibody and T cell assays

Number	**Abbreviation code**	**Species**	**Gram**	**Strain ID and/or source**
1	BPT	*Bordetella pertussis*	Neg	NCTC15434
2	BCEN	*Burkholderia cenocepacia*	Neg	LMG18829
3	BMULTI	*Burkholderia multivorans*	Neg	LMG18822
4	MCAT	*Moraxella catarrhalis*		Wild9 (in house)
5	PINT	*Provetella intermedia*	Neg	Wild (in house)
6	CF2	*Pseudomonas aeruginosa*	Neg	Wild CF2 (in house)
7	CF3	*Pseudomonas aeruginosa*	Neg	Wild CF3 (in house)
8	PSA	*Pseudomonas aeruginosa*	Neg	NCTC10662
9	SPN	*Streptococcus pneumoniae*	Pos	DMSZ11865
10	NT Hi	Non-typeable *Haemophilus influenzae*	Neg	MQCL 491
11	HBO	*Haemophilus influenza* serotype type B	Neg	(HbO-HA antigen) NIBSC, Potters Bar,UK
12	ASP	*Aspergillus fumigatus*	fungus	ALK-Abello Ltd, Reading, UK
13	ALT	*Alternaria .alternata*	fungus	ALK-Abello Ltd, Reading, UK

Note: Strains either were taken from the National Collection of Typed Cultures (NCTC), Laboratory of Microbiology, University of Ghent (LMG) Belgium, or were fully identified wild in-house isolated and characterized strains.

2.6 Measurement of lipopolysaccharides (LPS – Endotoxin) in antigens

A procedure was carried out to detect LPS in Gram-negative bacteria using *Escherichia coli* (*E. coli*) endotoxins as controls.

2.6.1 Preparing the standard stock solution

The solution of (endotoxin unit) 1.0 EU/ml of endotoxin standard (Thermo Scientific, Leicester, UK) in vial (A) was reconstituted by adding 0.05 ml of *E. coli* endotoxin standard stock to endotoxin-free water (Thermo Scientific) according to the endotoxin concentration of the vial (e.g. if the concentration is X/ml, the volume of endotoxin-free water is (X-1)/20).

A 0.25 ml of the 1.0 EU/ml standard from vial (A) was transferred to 0.25 ml of endotoxin-free water in a small eppendorph tube (B), which was then vortexed for 1 min (Appendix 4). Then, 0.25 ml from the standard vial (A) was transferred into 0.75 ml of endotoxin-free water to prepare 0.25 EU/ml in vial (C). 0.1 ml of vial (A) was then transferred to 0.90 ml of endotoxin-free water to prepare vial (D).

2.6.2 Microplate assay

A sterile 96-well microplate (Greiner Bio-one) was labelled for the standard and unknown samples. A 40 µl of endotoxin-free water (Thermo Scientific) was added to appropriate well of the blank wells. 40 µl the standard from the (A), (B), (C) and (D) vials was added to the appropriate well. Then 50 µl of the unknown samples (1in5 and 1in500) was added to each well of the plate and incubated for 5 min at 37 ^{o}C. Limulus Amebocyte Lysate (LAL) (Thermo Scientific) was reconstituted in 3.4 ml of endotoxin-free water and 50 µl volumes were added to wells of the plate. The plate was then incubated for 10 min at RT. Subsequently, 100 µl of chromogenic substrate solution (Thermo Scientific) was added to the plate prior to incubation for 10 min at RT (Appendix 4). Then, 50 µl of stop reagent

(25% acetic acid) was added and the absorbance was read at 405 nm using the plate reader (Synergy HT lab tech).

2.7 The basic protocol of Enzyme-Linked Immunosorbent Assay (ELISA) for serum antibody detection

An indirect enzyme-linked immunosorbent assay (ELISA) method was used to determine the antibody specificity and the optimal dilutions for the coating of microbe-derived antigens, to initiate screening and undertake titration for total IgG and for the measurement of other subclasses.

ELISA with 96-well plates (Nunc Maxisorp, Sigma-Aldrich, UK) were coated with selected antigen diluted in PBS, and incubated overnight at 4°C (Figure 2.2). The plates were washed 3 times with PBS containing 0.05% Tween 20 (PBST) (Appendix5). Washing was always followed by blotting on tissue paper. The plates were then blocked with 100 µl of 5% milk powder in PBST (Appendix5) for 30 min at RT. Next, the plates were washed 3 times and incubated with100 µl of diluted sera in PBST containing 0.5% milk powder (diluent) for 2 h at RT. After 6 washes, the plates were incubated for 1 h at RT with 50 µl of anti-human IgG/HRP (Dako polyclonal Rabbit P0214 Dako, Ely, Cambridgeshire) at a dilution of 1in5000 diluent. Following 6 washes, 100 µl of OPD substrate solution (Appendix 5) was added to each well and the reaction was stopped after 10 min by adding 25 µl of 2M sulphuric acid (Sigma-Aldrich, UK). Absorbance was read at 490 nm in the ELISA reader (EL808, Bio-Tek Instrument, US) using KC Junior software.

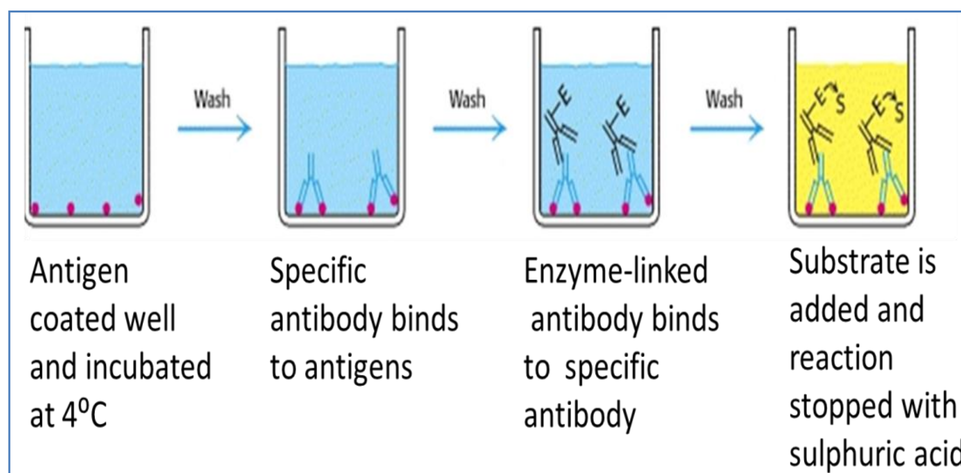


Figure 2.3: Schematic diagram of the indirect ELISA procedure for antibody screening and titration.

The plate is coated with appropriate antigen and incubated at 4 ˚C overnight. The plate is washed and blocked. Diluted sera are added and any antibody present binds to the antigens. The plate is washed again and the secondary (detection conjugate) antibody is added. After incubation and another 6 washes, the substrate is added and the reaction is stopped with 25 µl of 2M sulphuric acid. E = enzyme, S = substrate. Adapted from (Chhabra, 2014)**.**

2.7.1 Optimal coating concentration for each antigen used in the project

Each antigen was diluted in PBS in microfuge tubes through double dilution starting from 1 in 20. 100 µl of each of diluted antigen was added to 96-well ELISA plates in triplicate. A serum containing specific antibody activity was added in a single dilution. The ELISA procedure was then carried out as described in section 2.7 and the results were expressed graphically as absorbance vs coating concentration.

2.7.2 Screening of sera against antigens to establish starting titration

Serum dilution ELISA showed that absorbance at a single dilution was not sufficient to give an accurate measurement of the antibody level. It was decided to carry out a 4-step process for each serum sample and obtain an end-point titre. Screening of sera at a 1in 25 dilution was initially carried out to establish the starting dilution of the sera. Table 2.2 shows the initial dilution for sera titration based on the mean of absorbance obtained in the screening.

Table 2.2. Establishing the initial dilution for titration

The starting sera dilution for titration was estimated by calculating the mean absorbance for sera at 1 in 25 dilutions with the mean of the blank subtracted.

Absorbance	Initial dilution
<0.2	1/25
0.2–0.4	1/50
0.4–0.6	1/100
0.6–0.8	1/200
0.8–1.0	1/400
>1.0	1/800

2.7.3 Antibody titration for all groups in the study

Extended serum dilution was undertaken to establish the linear part of the curve from which the endpoint titre can be extrapolated. Serum titration was performed to determine the endpoint titre of antigen-specific antibodies. The 96-well plates were coated, blocked and washed as described in section 2.7. In this assay, sera were diluted based on the results of the previous screening, as shown in Table 2.2. The initial dilution was placed in the first column in duplicate and the sera were double diluted down the plates 4 times in total. The diluted serum was mixed 6 times before moving down to the next titration wells. The washing and incubation steps were as described in section 2.7. The same procedure was carried out for all antigens.

2.7.4 Measurement of antibody specificity by inhibition ELISA

The inhibition experiments determining cross reactivity are described using the example of Pseudomonas antigen. Pseudomonas lysate antigen was diluted at 1in 2000 in PBS and added to each well of the 96-well ELISA plate. The plate was incubated overnight at 4 ºC. The plate was washed and blocked as described in section 2.7. Six microfuge tubes were

labelled for antigen inhibitors, including lysate of Pseudomonas (same as coated), Pseudomonas LPS, *Haemophilus influenzae* type b lysate, *Bordetella pertussis* lysate, *Burkholderia cenocepacia* lysate and *Streptococcus pneumonia* lysate. These were diluted in 2-fold dilutions from 1in10 up to 1in640 in 250 µl of diluent. Serum samples were diluted at 1in 250 in diluent. A high IgG responding serum to the Pseudomonas antigen was selected. 250 µl of diluted serum was added to each antigen inhibitor and incubated for 30 min at RT. Then, 100 µl of each sample was added to the plate and incubated at RT for 1 h. The plate was then washed and developed as described in section 2.7. The inhibition assay was shown graphically as an absorbance versus inhibitor concentration and expressed as the percentage inhibition of the non-inhibited serum. The inhibition assay was also carried out for the other antigens.

2.7.5 Ig subclass measurement in ELISA

Measurement of Ig subclasses was performed by ELISA as described in section 2.7. However, the secondary detector antibody (Sigma-Aldrich, UK) was substituted to allow detection of the subclasses, including IgG1, IgG2, IgG3 and IgG4, as well as the other classes, such as IgA and IgM. The dilutions of the secondary antibody used are shown in Table 2.3. In some instances, in which the secondary antibody was biotin-conjugated, an extra step was required, adding a dilution of Avidin–Peroxidase (Sigma-Aldrich, UK) at 1in 40000 and incubating for 1h at RT. After washing, the substrate was added and the reaction was stopped with 25 µl of 2M sulphuric acid as usual. Absorbance was read in the ELISA reader at 490 nm. Only the absorbance (minus blank) value at a fixed serum concentration of 1in 25 was used for the Ig subclasses.

Table 2.3. Antibody isotype detection.
Antibody conjugates for the detection of antibody isotypes were diluted in diluent and used at the dilutions indicated

Number	Antibody isotypes	Dilutions used	Details
1	IgG1	1:2000	Monoclonal Anti-Human IgG1-Biotin antibody produced in mouse
2	IgG2	1:8000	Monoclonal Anti-Human IgG2-Biotin antibody produced in mouse
3	IgG3	1:4000	Monoclonal Anti-Human IgG3- Biotin antibody produced in mouse
4	IgG4	1:10000	Monoclonal Anti-Human IgG4-Biotin antibody produced in mouse
5	IgA	1:5000	Anti-Human IgA (μ-chain specific) peroxide antibody produced in goat
6	IgM	1:5000	Anti-Human IgM (α-chain specific) peroxide, produced in the goat

2.7.6 Stability of the sera

Experiments were carried out to assess the effect of multiple freeze–thaw cycles on the antibody activity levels of human sera against bacterial antigens. A series of 11 aliquots of serum samples were included, comprising BR (n=5), COPD (n=4) and healthy subjects (n=2). These sera had been previously screened and titrated for antibody against *Moraxella catarrhalis.* The same sera were tested after 5 freeze–thaw cycles against *M. catarrhalis* using ELISA as described in section 2.7.

2.8 Determining T cell responses to panels of antigens

2.8.1 Dilution of the cells

To adjust the cell counts to the required concentration, an appropriate volume of R10 was added to cells to achieve the optimum concentration of 8×10^{6} PBMC/ml, thus allowing a sufficient cell response but avoiding high background or black out in the ELIspot results.

2.8.2 ELIspot protocol

Sterile 96-well ELIspot plates (Millipore, Watford.UK) were coated with 10 µl of capture anti-human IFNγ (Mabtech, Nacka Strand, Sweden) per ml of bicarbonate buffer, resulting in a concentration of 10 µg/ml. Coating with 50 µl of capture antibody was undertaken overnight at 4°C. The plate was washed 3 times with sterilized PBS and blocked with 100 µl of R10 medium (Appendix 6) for 1h at RT. Then, 50 µl of stimulus (Table 2.5) and 50 µl of 8×10^6/ml PBMCs (set up at $8x10^6$/ml) were added to the plate. The plate was incubated for 18–20 h at 37°C in a 5% CO_2 incubator. The plate was then washed 6 times with PBS before adding 50 µl of biotinylated-anti-IFNγ detector antibody (Mabtech, Nacka Strand, Sweden) diluted at 1in1000 in PBS. The plate was incubated for 2 h at RT. The plate was then washed 6 times and 50 µl of streptavidin ALP (Mabtech, Nacka Strand, Sweden) diluted at 1in1000 in PBS was added to the plate and incubated for 1h at RT. The plate was then washed 6 times and 50 µl of the substrate from AP Conjugate Substrate kit 170-6432 (Bio-Rad Laboratories Inc,) (Appendix 6) was added. The reaction was stopped by washing the plate thoroughly with tap water after approximately 10 min. The plate was dried overnight and spot-forming cells were counted using an Immuno-Spot reader (Auto-immun Diagnostika- AID, GmbH, Strassberg, Germany). The frequency of IFN-γ-producing cells was calculated by the number of spots in the wells after background subtraction and multiplied by the cell concentration factor (2.5 for $8x10^6$). The results are expressed as spot-forming cells (SFC)/10^6(M) PBMC.

2.8.3. Setting the ELIspot reader

Autoimmune diagnostic GMBH (AID) ELIspot reader takes high resolution images with 95 megapixel connected digital camera. Once the machine is switched on and a plate is inserted, the plate is adjusted in order to see the rim of the well before calibrating the stage in which wells are centred properly, thus the size of counting area of the spots is determined. The

properties of counted spots (Table2.4) is also determined. Once the calibration is saved, the read and counting function is then followed with speed of reading at maximum. Therefore, counts of spots are determined based on spot size, intensity and gradient of edge (Figure2.4).

Table 2.4. Thresholds for spot counting

	Intensity	Size	Gradient
MIN	4	3	1
MAX	255	500	90

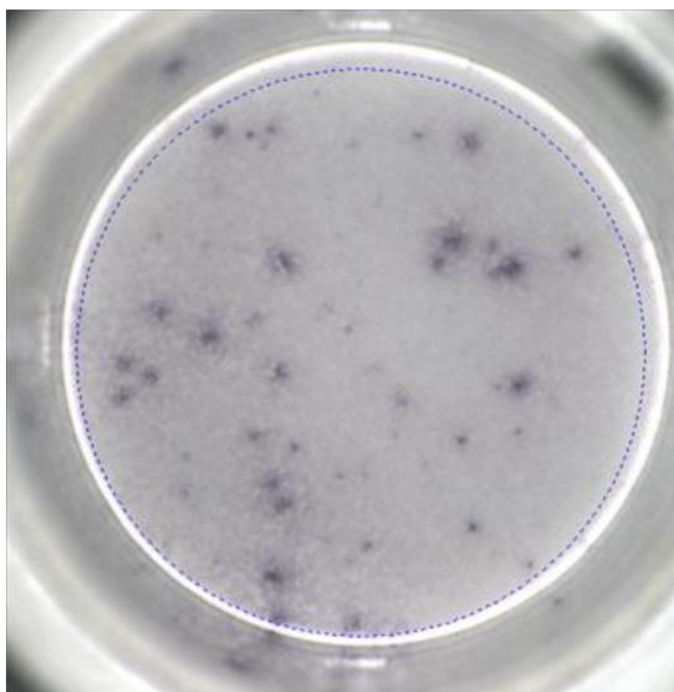


Figure 2.4: Example of a well of an ELIspot plate, showing positive spots.
Adopted from (Roche Innovatis AG CASY, 2002).

2.8.4 Depletion of $CD4^+$ T cells to confirm adaptive response

CD4 Dynabeads (Invitrogen, Paisley, UK) were pulse-vortexed for 30 seconds. 1×10^8 CD4 beads (25 µl) were transferred into a sterile FACS tube (Falcon, Corning, Sigma-Aldrich, UK). Then, 1 ml of R0 medium was added to wash the beads: the tube was placed in a magnet (Dynal) for 1 min and the supernatant was discarded. The tube was then removed from the magnet. 0.35 ml of cells (PBMCs) and 0.15 ml of R10 were added to the beads in the tube. The mixture was incubated for 30 min at 4 °C, mixing every 5 min. The tube was

placed in the magnet for 1 min and unbound cells were taken and transferred into another FACS tube. Subsequently, 0.7 ml of R10 medium was added to the cells. The cells were then used to investigate immune responses against a panel of antigens using ELIspot (see 2.8.2). Table 2.5 illustrates the stimuli used for stimulating PBMCs to produce cytokines such as IFNγ in ELIspot.

Table 2.5. Antigens and stimulus used for ELIspot and flow cytometry

Antigen and stimulants label	Antigen name	Details
F1	Medium (R10)	RPMI-1640 containing 5 ml penicillin-streptomycin, 10% of FBS and 5 ml of glutamine
F2	Positive control	Anti-CD3 monoclonal antibody (mAb) 1:200 (Mabtech)
F3	*B. pertussis*	Lysate + 1 peptide
F4	*P. aeruginosa* (NCTC)	48 µl Lysate + 30 µl peptides National collection of typed cultures
F5	*S. pneumoniae*	Lysate + 6 peptides
F6	*Mycobacterium tuberculosis (*TB*)*	Purified protein derived (PPD) + 6 peptides
F7	*H. influenzae* serotype b (HBo)	102 µl Lysate + 30 µl peptide, Oligosaccharide–human serum albumin conjugate NIBSC, Potters Bar, UK
F8	Respiratory Syncytial Virus (RSV)	Lysate + 4 peptides
F9	Influenza virus	55 peptides
F10	*Burkholderia cepacia complex*	2 lysates + 4 peptides, also termed (2)
F11	Mycoplasma pneumonia	Lysate (Native Antigen Company)
F12	*M. catarrhalis*	Lysate
F13	Cytomegalovirus (CMV)	Lysate + CD4 and CD8 epitope peptides Lysate from The Native Antigen Company (Upper Heyford, Oxfordshire, UK)
F14	*A. fumigatus*	Lysate (ALK-Abello Ltd, Reading, UK) + 1 peptide
PHA	Phytohaemagglutinin	5 ml of R10, 50 µl of PHA = 5 µl/ml
PMAI	Phorbol myristate acetate	50 µl of Phorbol myristate acetate, 1 µl of Ionomycin in 2 ml of R10 = 20 ng/ml and 1µg/ml, respectively
EBV	Epstein-Barr virus all	EBV-$CD8^+$ and EBV-$CD4^+$ antigens 2.5 ml
Candida	*Candida.* sp	Lysate (ALK-Abello Ltd, Reading, UK) and 1 peptide
***Burkholderia* (1)**	*B. cepacia complex* (1)	Lysates

Notes: ***** All other peptides found sequences of epitopes: Immune Epitope Database (IEDB) National Institute of Allergy and infection, Bethesda, USA. The peptides were synthesised by Mimotopes, Victoria, Australia**.** All lysates were produced in house unless otherwise indicated.

2.8.5 Frozen and fresh cells for ELIspot

Experiments were performed to investigate the differences between fresh and frozen cells in response to the same antigens. Here the ELIspot plate was divided into two parts: one part for the fresh cells and the other for frozen cells stimulated with the same antigens. The procedure was carried out as described in section 2.8.2.

2.8.6 Inhibition with anti-class II antibody to confirm adaptive response

Sterile 96-well ELIspot plates was coated with anti-human IFNγ as described in section 2.8.2. Following blocking, 25 µl of fresh cells were added, plus 25 µl of anti-class II antibody (L243, gift from Martin Glennie, Southampton) was also added to give a final concentration of 10 µg/ml. The plate was incubated for 30 min at 37 ºC, 5% CO_2. A mixture of 50 µl of appropriate antigens and stimuli were added to the plate. The experiment was then carried out as described in section 2.8.2.

2.9 Flow Cytometry

2.9.1 Optimizing the fluorescence compensation settings

Tubes were labelled for each fluorochrome as in Table 2.6. 2 µl of each antibody was added to the corresponding tubes. One drop of negative compensation beads and one drop of Anti-Mouse Ig, κ positive compensation beads – BD™ CompBeads (BD Biosciences, Oxford UK) (Appendix 7) were then added to each tube to optimize the fluorescence compensation settings and distinguish the positive and negative background for multicolour analysis. Two populations of micro particles were thus used: the BD™ CompBeads Anti-Mouse Ig, κ particles bind to any mouse κ light chain-bearing Ig and the BD™ CompBeads Negative Control has no binding capacity. As a negative control, one drop of negative beads was also

added to another empty tube. Tubes were then pulse-vortexed and incubated for 15 min in the dark at RT. Subsequently, 2 ml of 1X PBS was added to each tube and centrifuged at 1500 rpm for 5 min. The supernatants were discarded and the beads were re-suspended in 300 µl of 1X PBS. To calculate the spectral overlap for each fluorochrome, beads were acquired on a BD FACSCanto™ II flow cytometer (BD Biosciences, Oxford, UK), using the automated compensation program, which is part of the BD FACSDiva™ software.

Table 2.6. Fluorochrome labelled antibodies used in this study

Fluorochromes	Cell markers	Function	Clone	Laser (nm) Excitation	EM-max[a] (nm) (detection)
FITC	OX40[b]	Co-stimulation	ACT35	488	525
PE	CD49d[b]	Homing	9F10	488	575
PerCP-Cy5.5	CXCR3[c]	Chemokine receptor (Th1)	IC6/CXCR 3	488	695
PE-Cy7	CCR6[b]	Chemokine receptor (Th17)	B6H	488	785
APC	PD-1[b]	Energy marker	eBioJ105	633	660
APC-Cy7	CCR5[c]	Chemokine receptor (inflammatory)	2D7/CCR5	633	785
BV421	CD69[c]	Activation marker	KN50	405	421
BV510	CD4[c]	Th cell marker	SK3	405	510

Notes: [a] Maximum emission wavelength; [b] eBioscience (Affymetrix - eBioscience, Hatfield, UK); [c] BD Biosciences (Oxford, UK).

2.9.2 Cell activation and surface staining

Frozen PBMCs samples were thawed quickly in a 37°C water bath and washed by adding 10 ml of R0 prior to centrifuging at 1500 rpm for 5 min. The supernatants were discarded and cells re-suspended in 1ml of R10 and counted using a CASY counter as described in section 2.2.4. Then, 250 µl of 8×10^6 PBMCs was incubated with 250 µl of medium, polyclonal anti-CD3mAb, or lysates + peptides from *P. aeruginosa* or *H. influenzae* type b (Table 2.5). The cultures were incubated overnight at 37°C, 5% CO_2. The cells were then washed with 1X PBS and incubated (15 min, at RT, in the dark) with the antibody

combination shown in Table 2.7 and 50 µl of Brilliant Stain Buffer (BD Horizon™). The stained cells were washed with 2 ml of 1X PBS and re-suspended in 300 µl of 1X PBS prior to immediate acquisition on the flow cytometer. Data were analysed using BD FACSDiva™ software. For isotype control, stimulated cells were stained with isotype controls as in Table 2.7 and incubated for 15 min at RT. The stained cells were washed and re-suspended in PBS prior to immediate acquisition on the flow cytometer (BDFACSCantoII) and analysed using BD FACSDiva™ software.

2.9.3 Staining for intracellular molecules by flow cytometry

An experiment was performed to determine the number and phenotype of IFNγ–producing cells. To activate T cells, 250 µl of 8×10^6 PBMC was stimulated with 250 µl of medium, anti-CD3, or lysates of *P. aeruginosa*, or left unstimulated, for 20 h, the last 18 h of which were in the presence of 0.1 µg/ml Brefeldin A (Golgi plug, BD Bioscience, Oxford, UK) (Appendix 7) to allow the cytokines to accumulate inside the cells. The cells were then first stained for surface antigens, as described in section 2.9.2, prior to being fixed with 100 µl of IC Fixation Buffer (Affymetrix eBioscience, Hatfield, UK). Cells were incubated for 20 min in the dark at RT. Cells were then washed twice with 2 ml 1X Permeabilization Buffer (Affymetrix eBioscience, Hatfield, UK) and re-suspended in 100 ml 1X Permeabilization Buffer (Appendix 7). 2 µl of IFNγ PE (Affymetrix eBioscience) was added to the cells and incubated for 30 min in the dark at RT. The cells were washed twice: once in 1X Permeabilization Buffer and once in 1X PBS. The cells were then re-suspended in 1X PBS and acquired on the flow cytometer (BD FACSCanto™ II). Data were analysed using BD FACSDiva™ software.

Table 2.7. Antibody and isotype controls used in the flow cytometry protocol.

Antibody	Concentration (µg/ml)	Concentration/test (µg/2 ml)	Isotype	Concentration (µg/ml)	Amount isotype/ test
CD134 FITC[a]	100	0.2	IgG_1[a]	500	4 µl of 1:10 dil[n]
CD49d PE[a]	25	0.05	IgG_1[a]	200	2.5µl 0f 1in10 dil[n]
CXCR3 PerCP-Cy5.5[b]	100	0.2	IgG_1[b]	200	1 µl
CCR6 PE-Cy7[a]	25	0.05	IgG_1[b]	50	1 µl
PD-1 APC[a]	200	0.4	IgG_1[a]	200	2 µl
CCR5 APC-Cy7[b]	200	0.4	IgG_2[b]	200	2 µl
CD69 BV421[c]	12	0.024	IgG_1[d]	100	2.4 µl of 1in 10 dil[n]
CD3 BV510[c]	100	0.2	IgG_1[d]	100	2 µl

Notes: [a] eBioscience(affymetrix eBioscience, Hatfield, UK); [b] BDPharmingen (BD Bioscience, Oxford ,UK); [c] BDHorizon (BD Bioscience, Oxford, UK); [d] BioLegend (London, UK).

2.10 Measurement of cytokines for culture supernatant using multiplex ELISA

Cytokines in supernatants were measured in 10 BR patients and 4 healthy volunteers using the Mesoscale Scale Discovery (MSD) (Meso Scale Diagnostic, LLC, Gaithersburg, USA). Multiplex kit – pro-inflammatory panel 1 (IFNγ, IL-2, IL-4, IL-10, IL-12p70 and IL-13) and cytokine panel 1 (IL-5 and IL-17A) were used. For each panel, 1 ml of diluent 1 was added to 1 g of blocker B. Serial dilutions of 1in100 were made for each standard for both panels. Then, 25 µl of these standards were added to the appropriate 96-well plate (Meso Scale Diagnostic, LLC, Gaithersburg, USA). 25 µl of culture supernatant samples from previously stimulated PBMCs were added to each well of the 96-well plates. The plates were covered with an adhesive cover (Thermo Scientific, UK) and incubated for 2 h at RT on a plate shaker (100 rpm). A 25 µl of detector antibodies (Meso Scale Diagnostic, LLC, Gaithersburg, USA) were diluted in diluent 100 at 1X (MSD) and added in pro-inflammatory panel 1 (human calibrator blend) including anti-human IL-13, IFNγ- antibody, anti-human IL-10 antibody,

anti-human IL-2, anti-human IL-4 and anti-human IL-12P70. A 25 µl of detector antibodies also added to cytokine panel 1X (human calibrator blend), including anti-human IL-5 antibody and anti-human IL-17A, antibody (Sulfo-TAG). The plates were incubated for 2 h at RT on a plate shaker (100 rpm). The plates were washed 6 times with 150 µl of PBS −0.05% Tween-20. Then, 150 µl of read buffer 4X with surfactant (MSD) diluted in sterilized water (50:50) was added to each well of the plates. The plates were read on MSD sector imager. Data were analysed immediately in MSD Software and Excel.

2.11 Calculation and statistical analysis

ELISA data were always run in duplicate or triplicate and therefore the average absorbance for each set of replicate samples was calculated. The absorbance value of the blanks was then subtracted from the average values. These gave the screening values for determining the starting point for titration. Furthermore, antibody titres were expressed as the reciprocal of the highest dilution in which the absorbance value of a serum differed from zero. The calculation of the endpoint was obtained by plotting the mean absorbance on the x-axis versus dilutions on the y-axis in the standard curve and the end point was obtained from the equation $y=mx+c$, where c is the end point on the x-axis. In this project, Microsoft Excel was used for calculating both screening and titration values.

Statistical analysis was carried out using Graph Pad Prism5, Minitab17 and IBM SPSS-22. The data for antibody and T cells responses were found to be non-normally distributed, so the Mann-Whitney U-test was performed to compare the differences in antibody and T-cell levels between patients and healthy volunteers, as well as FEV_1% predicted and microbial colonization, in which $p<0.05$ was considered statistically significant. Correlations were analysed using Spearman’s test.

3 Chapter Three: Measurement of Anti-microbial Antibody Responses in Chronic Obstructive Lung Diseases

Abstract

Aim: This chapter investigates antibody responses in patients with chronic obstructive lung diseases, namely bronchiectasis (BR), chronic obstructive pulmonary disease (COPD) and asthma, against antigens derived from bacteria and fungi found within the lung microbiota. The responses were compared to those of healthy volunteers to determine if there were any significant differences between the clinical groups and healthy subjects.

Methods: Plasma was separated from the heparinised blood of stable BR patients (n = 119), COPD (n=58), and asthmatic (n=14) patients attending an outpatient clinic at the Freeman Hospital, Newcastle upon-Tyne, who were recruited in this study. Healthy volunteers (n=28) were recruited from the staff of Freeman Hospital. An indirect ELISA was setup and used to investigate the Igs against bacteria, virus and fungi-derived antigens. Anti-Pseudomonas Ig isotypes were also examined in BR (n=20) and COPD (n= 20) patients.

Results: Antibody titres in each clinical group were different from those of healthy volunteers. BR patients had significantly higher IgG titres against *P. aeruginosa, S. pneumoniae* polysaccharide, *H. influenzae*, *H. influenzae* polysaccharide and *S. maltophilia,* whereas patients with COPD had significantly higher IgG titre against *S. pneumoniae* polysaccharide. In contrast, reduced IgG titres were observed in patients with asthma in which healthy volunteers have significantly higher antibody response against *S.pneumoniae* and *M.catarrhalis*. Investigation of antibody Ig isotypes against *P. aeruginosa* showed that IgG1 was the predominant isotype in BR patients, whereas IgM was the main isotype detected in patients with COPD.

Conclusion: The increased magnitude of a specific antibody may be an indication of infection with *P. aeruginosa* and other pathogens in patients with BR and COPD. In contrast, the low level of IgG antibody responses in patients with asthma may be related to infrequent microbial infections or possibly the switching of antibody to IgE in this group. A variation in the antibody isotypes was seen among patients with BR and COPD, suggesting different exposure patterns.

3. 1 Background

A major cause of both morbidity and mortality for individuals worldwide are respiratory conditions such as bronchiectasis (BR), chronic obstructive pulmonary disease (COPD), cystic fibrosis (CF) and asthma (Cullen and McClean, 2015). BR, COPD and CF are associated with an intense inflammatory response as a result of recurrent microbial infections including bacteria and viruses (King, 2011). Antibody-mediated immunity is critical for host defence against pathogens, and individuals with underlying defects in humoral immunity are more susceptible to bronchiectasis and bacterial sinopulmonary infections. The process of antibody production in the respiratory tract in response to bacterial infection can occur rapidly via the activation of resident memory B cells and specific IgG secretion if there was previous exposure to the pathogens (Twigg, 2005). If the host is naïve to the pathogen, the antibody is produced more slowly, with IgM prominent followed by specific IgG and IgA in order to eliminate the infection. The production of antibody depends on the antigen exposure site. For instance, IgA responses occur in the upper airways, whereas the production of pathogen-specific IgG occurs once the pathogens reach the lower airways. Indeed, IgG utilises opsonizing properties for aiding phagocytic cells and for activating complements in the elimination of pathogens and the provision of effective immune responses against microorganisms (Twigg, 2005). T cells are also activated and contribute to antibody formation in response to cognate antigens of the pathogen. $CD4^+$ T cells are crucial in

antibody production where they promote the activation and differentiation of B cells to undergo class-switching from the production of IgM to the more efficient and avidly-binding IgG isotypes against protein antigens (Gadgil and Duncan, 2008). Isotype switching requires cytokine signals that are produced by T helper cells such as IFNγ, IL-4, IL-5, and signals by stimulatory molecules CD40L with CD40 on B cells (Garraud and Nutman, 1996).

However, the inflammatory cells also contribute to the inflammation and pathogenesis of chronic obstructive lung diseases. Neutrophils, macrophages and other innate and adaptive cells are recruited into the airways and release pro-inflammatory cytokines leading to the irreversible dilation of bronchial walls and the destruction of lung parenchyma resulting in impaired mucocilary clearance and promoting bacteria colonization (Whitters and Stockley, 2012). The predominant pathogenic bacteria isolated in such lung diseases are *P. aeruginosa, H. influenzae, S. aureus*, *S. pneumoniae* and *M. catarrhalis* (Kadowaki *et al.*, 2015). In contrast, asthma is an inflammatory disease of the airways not primarily associated with microbial infection and therefore these groups were used as a control to see if lung inflammation alone gives any antibody responses.

In this part of the study, the aim is first to prepare or collate the antigens derived from microbes associated with the lung disease to be utilised in antibody ELISA. Second, the measurement of antibody responses against lung organism antigens was carried out to determine whether or not variations in antibody levels between the clinical groups are a biomarker for colonization and if they reflect immune protection from infections. It is important to know that all patients were stable and no patients receiving steroids during the study.

3.2 Results

3.2.1 Demographic characteristics of participants

Patient cohorts consisting of 119 patients in the BR group (45 males, 74 female), 58 patients in the COPD group and 14 asthmatics were recruited in this study. A healthy volunteer group of 28 was recruited from the staff of the Freeman Hospital. Due to the cross-sectional and practical nature of the study, some clinical data sets of the COPD group, asthmatics and healthy volunteers were not available. A summary of the demographic and clinical characteristics of the patient cohorts is shown in Table 3.1. As shown, at the time of assessment the mean ages of patients were BR 65±1.08, COPD 69±1.23, asthmatics 57±3.45 and healthy volunteers 54±3.0 years cross-cohort. Symptoms scores were determined as described by Jones (2009)

Exacerbations were determined in the preceding 12 months, for which BR patients had more episodes over this period (4) followed by COPD patients (3) and asthmatics (2).

The mean value of smoking history (pack years) is higher among COPD patients, thus confirming that COPD is mostly caused by smoking thus, increased the rate of admission due to acute exacerbation. The mean of forced expiratory volume in one second (FEV_1 % predicted) was less than 50% in patients with COPD, demonstrating severe airflow obstruction, while BR patients achieved a mean value of less than 70% representing moderate airflow obstruction compared to a cohort of similar age. Asthmatics showed mean of FEV_1 71%, indicating better lung function test compared to BR and COPD. Healthy volunteers also underwent the lung function assessment, which showed a mean FEV_1 higher than 100% of the predicted value. The mean vital capacity (VC % predicted) in BR patients was 82%, whereas for COPD patients it was 77%. Asthmatics reported 90% and healthy volunteers showed a high level of 118%. Patients with COPD showed deterioration of the FEV_1/FVC ratio at 50% followed by BR patients (66%) and asthmatics (77%). However, healthy volunteers gave results of 83%. A summary of the patient demographic data is shown in Table 3.1.

Table 3.1. Demographics of the subjects included in this part of the study

Characteristics	BR n=119	COPD n= 58	Asthma n= 14	HV n=28
Sex (no.) Male/female)	45/74	Not av.	Not av.	Not av.
Age (y)	65 ± 1.08	69 ± 1.23	57 ±3.45	54±3.0
Symptom score	64 ± 2.19	68 ± 2.56	54 ± 6.4	Not app.
Exacerbations (per year)	4 ± 0.296	3 ± 0.38	2 ± 0.70	Not app.
Smoking history (pack years)	8 ± 1.33	47 ± 4.08	10 ± 3.92	Not av.
FEV_1 (% predicted)	68 ± 2.68	49 ± 2.70	71 ± 7.44	113±2.83
VC (% predicted)	82 ± 2.478	77 ± 2.40	90 ±7.63	118± 2.7
FEV_1/FVC ratio	66 ± 1.52	50 ± 2.17	77 ±5.05	83±1.75

Values are presented as means ± SEs, where chronic bronchitis is defined as the presence of chronic productive coughing for more than 6 months, and exacerbations represents the number per year. FEV_1 represent forced expiratory volume in the first second and FVC, forced vital capacity. Not av, indicates the data are not available whereas not app means that the category was not applicable.

3.2.2 SDS-PAGE (Sodium dodecyl sulfate polyacrylamide gel electrophoresis) analysis of bacterial protein

The bacterial lysates were prepared as described in section 2.3. The distributions of molecular weights of bacterial proteins were determined by SDS–PAGE, as described in section 2.5. The standard contains highly purified proteins of molecular masses of 15-250 kDa. The results shown in Figure 3.1 indicate the position of 9 distinct samples on SDS–PAGE alongside the standard molecular weight marked in lane 1. Lane 3 is *B. pertussis,* Lane 4 is *B. cenocepacia* and Lane 8 is *K. pneumoniae*, and these had higher protein intensity bands compared to the other samples. A further gel shown in Figure 3.2 also shows Lane 6 *P. aeruginosa* (NCTC) to have numerous protein bands with differing

molecular weights. Overall, the analyses of bacterial lysate showed multiple bands of protein.

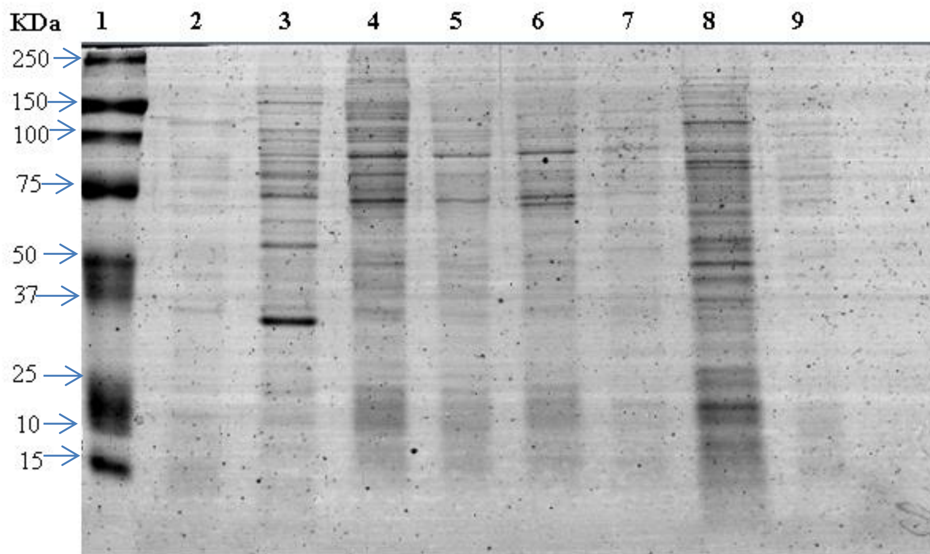


Figure 3.1: Visualisation of unknown protein bands with bacteria lysates by 12%SDS-PAGE. Lane1, the Mw ladder; Lane 2 *B. fragilis* controls; Lane 3 *B. pertussis*, Lane 4 *B. cenocepacia*, Lane 5 *C. difficile* control, Lane 6 *E. faecalis* control, Lane7 *E. coli*, Lane 8 *K. pneumoniae*, Lane 9 *B. multivorans*. Proteins were visualised by Coomassie blue and destained as described in section 2.5.

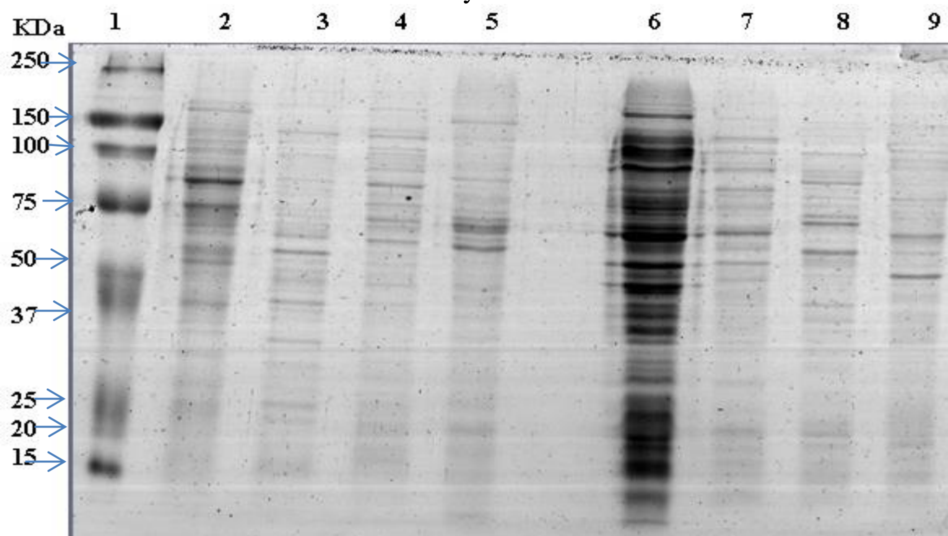


Figure 3.2: Visualisation of unknown protein bands within bacteria lysates by 12% SDS-PAGE.
Lane1, Mw ladder; Lane 2 *M. catarrhalis*, Lane 3 *P. intermedia*, Lane 4 *P. aeruginosa* (wild type), Lane 5 *P. aeruginosa* (wild type), Lane 6 *P. aeruginosa* (NCTC), Lane 7 *S. typhimurium* control, Lane 8 *S. aureus*, Lane 9 *S. pneumoniae*. Bacterial proteins visualised by Coomassie blue and destained as described in section 2.5.

3.2.3 Measurement of protein concentrations of lung bacteria antigens

The protein concentrations of each bacterial antigen were measured by Bradford assay as described in section 2.4. Figure 3.3 demonstrates the standard curve used to obtain the protein concentrations. The amount of protein in the unknown samples was calculated in

μg/ml from the standard curve equation. The concentration obtained from the curve was multiplied by the dilution factors of the samples.

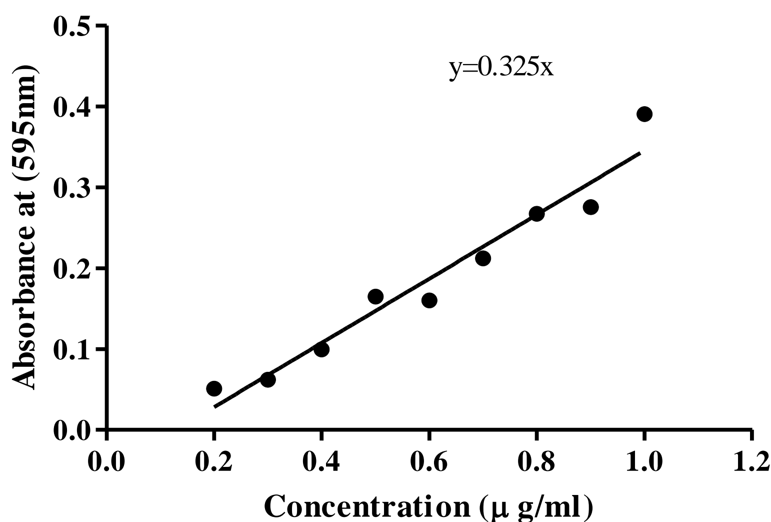


Figure 3.3: Standard curve for the Bio-Rad Bradford protein assay.
The curve is produced by the addition of 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 and 1 mg/ml BSA in 500 μl of PBS to 250 μl of Bradford dye reagents.

Table 3.2 shows the protein concentrations and lipopolysaccharide (LPS) content of each sample of the lung bacterial antigens as described in sections 2.4 and 2.6 respectively. *S. pneumoniae* lysate (SPN) has the highest protein concentration (33.06 mg/ml) whereas *B. pertussis* (BPT) has the lowest protein concentration (0.88 mg/ml**).** Furthermore, different strains of *P. aeruginosa* have differing protein concentrations. On the other hand, the content of LPS was low in most organisms except for *E. coli*, which contained the highest concentration of LPS (89.0 ng/ml).

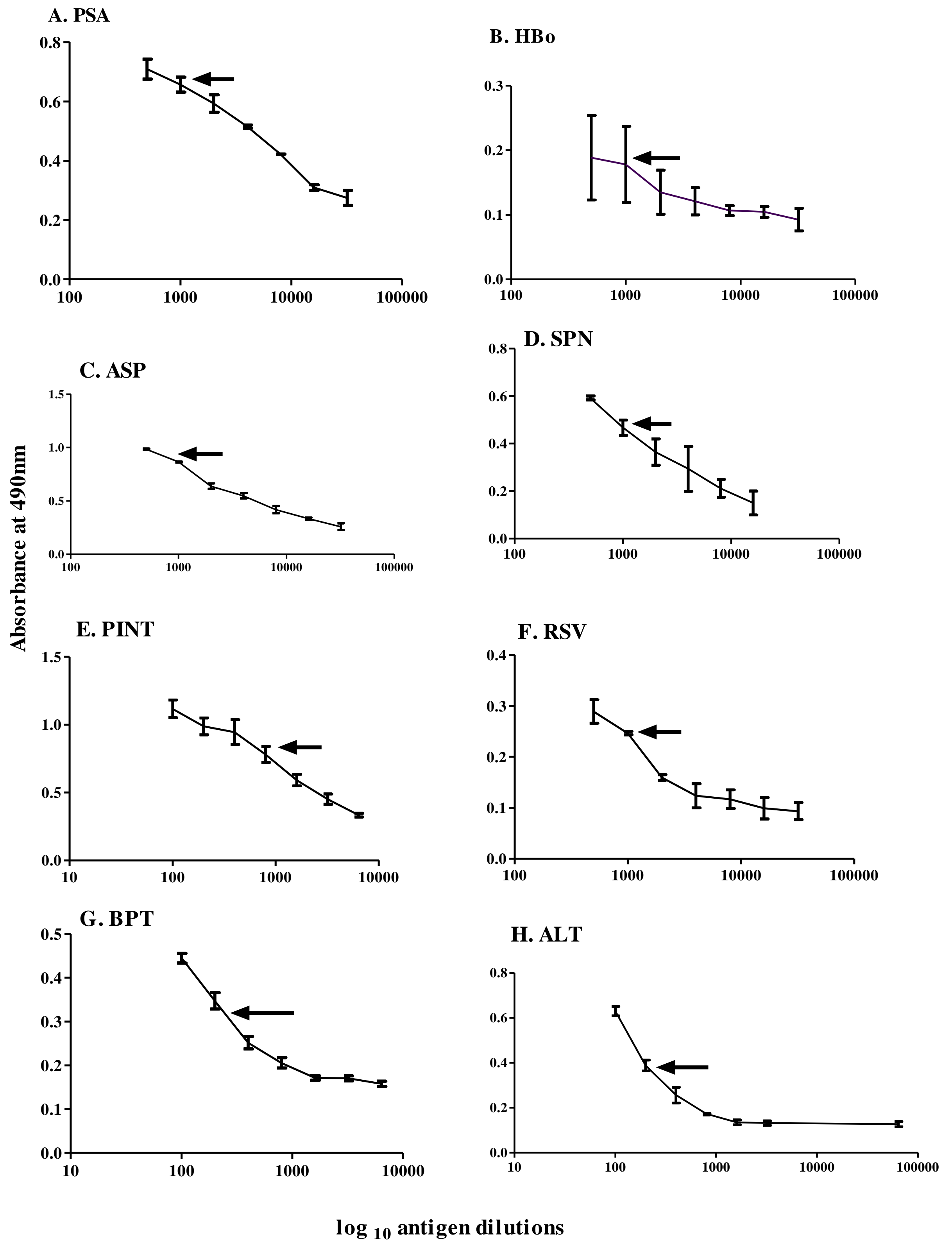
Table 3.2. Protein and lipopolysaccharide concentrations of microbe-derived antigens used in this study.

Number	Abbreviation	Species	Protein concentration (mg/ml)	LPS (ng/ml)
1	BPT	*Bordetella pertussis*	0.88	0
2	BCEN	*Burkholderia cenocepacia*	15.74	0.28
3	BMULTI	*Burkholderia multivorans*	16.54	0.48
4	MCAT	*Moraxella catarrhalis*	16.65	0.11
5	PINT	*Provetella intermedia*	1.21	0
6	CF2	*Pseudomonas aeruginosa*	13.63	0
7	CF3	*Pseudomonas aeruginosa*	22.01	0
8	PSA (NCTC)	*Pseudomonas aeruginosa*	16.2	0
9	SPN lysate	*Streptococcus pneumoniae*	33.06	0
10	NT Hi	*Non-typable Haemophilus influenzae*	56.6	Not av.
11	HBo	*Haemophilus influenzae* Type B polysaccharide	1.0	Not app
12	SM	*Stenotrophomonas maltophilia*	6.8	
13	ALT	*Alternaria alternata*	Not av.	Not app.
14	SPN poly	*Streptococcus pneumoniae*	Not app	Not app
15	ECO	*Escherichia coli*	3.05	89.0
16	Hib	*Haemophilus influenzae* lysate	3.7	Not app
17	ASP	*Aspergillus fumigatus*	Not av.	Not app

Note: in case of PSA one antigen for several different infections from different *P. aeruginosa* because lysate represents all strains of Pa and it contains vast majority of conserved antigens

3.2.4 Optimal coating concentration for antigens

In order to establish the optimal coating dilution of each lysate to carry out ELISAs, the titration of coating dilutions was performed as described in section 2.7.1. The results of coating dilution for each antigen for coating ELISA plates are shown in the Figure 3.4.



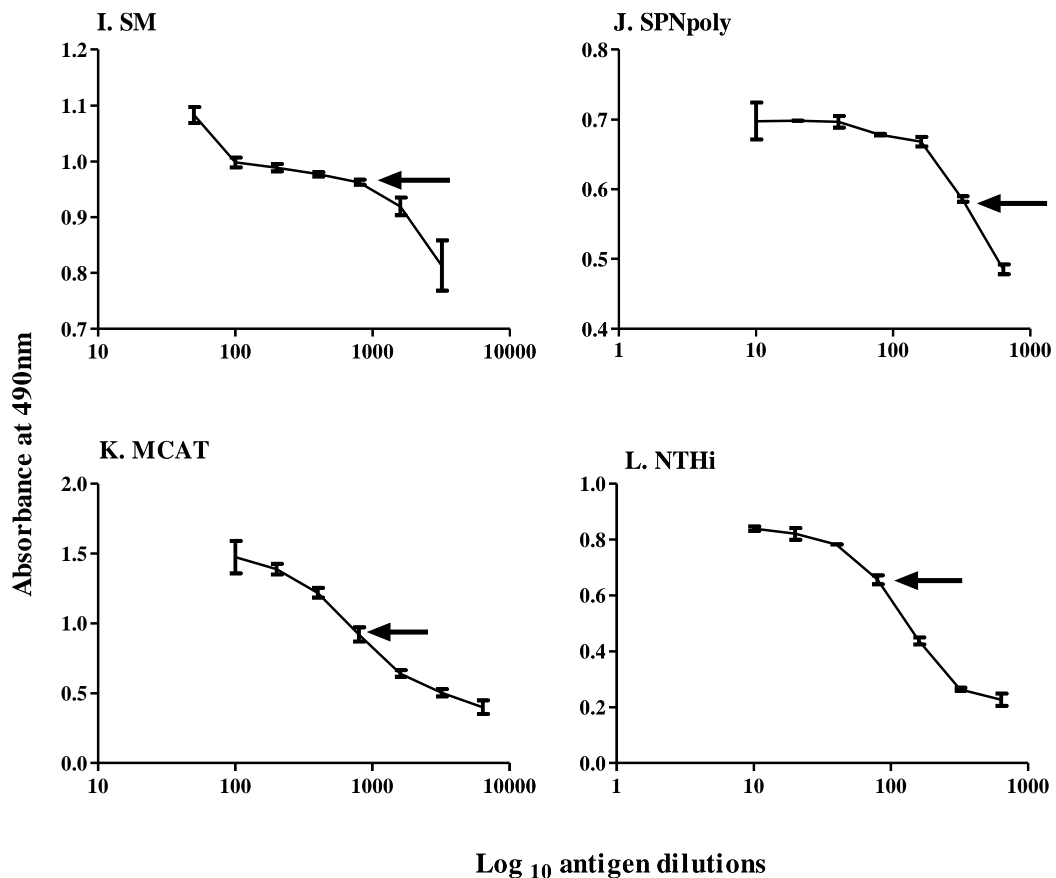


Figure 3.4(A-L): The optimal coating dilution of each antigen as determined by ELISA. The graphs showed the plate-coating concentrations for each antigen in the study. The x-axis represents the log_{10} antigen dilution while the y-axis represents the absorbance at 490nm. The result are represented by the mean ± SEM, (n =3) replicates per sample. (A*)* *P. aeruginosa*, (B) *H. influenzae* b polysaccharide (HbO), (C) *A. fumigatus*, (D) *S. pneumoniae*, (E) *P.intermedia* (F) RSV, (G) *B. pertussis*, (H) *A. alternata*, (I) *S. maltophilia*, (J) *S. pneumoniae* polysaccharide, (K) *M. catarrhalis*, and (L) NT*H. influenzae*. The arrows indicate the selected coating dilution.

Plate-coating concentration subsequently used was selected based on being on the plateau in the absorbance reading and on there being sufficient antigen for the entire project.

Table 3.3 shows the optimal coating dilutions of 15 microorganism species used as antigens in the ELISA technique to measure antibody response. These organisms were either bacteria lysates, polysaccharides or extracts from fungi or viruses. The results show that the optimal antigen dilutions differ between organisms, with *P. aeruginosa* being at a high dilution of 1in 2000, whereas, *H. influenzae* b, *S. pneumoniae*, *A. fumigatus*, *P. intermedia, M.*

catarrhalis, RSV*, B. cenocepacia, B. multivorans* and *S. maltophilia* were at 1 in 1000, *A. alternata* and *S. pneumoniae* polysaccharide were 1 in 500, *B. pertussis* was 1 in 250 and non-typeable *H. influenzae* was at the lowest dilution of 1 in 100.

Table 3.3. **Optimal coating dilutions of microbe-derived antigens.**

This determined by indirect ELISA for all antigens utilised in the study (with their sources indicated).

Organism number	Species	Abbreviation (Code)	Nature and source of antigens	Antigen coating dilutions
1	*P.aeruginosa*	PSA	Cell lysate (in-house)	1in 2000
2	*H.influenzae* b	HBO polysaccharide	Polysaccharide NIBSC, NIBSC, Potters Bar, UK	1in 1000
3	*S. pneumoniae*	SPN	Lysate (in-house)	1in 1000
4	*A. fumigatus*	ASP	Extract (Soluprick, Horsholm, Denmark)	1in 1000
5	*P. intermedia*	PINT	Lysate (in-house)	1in 1000
6	*M. catarrhalis*	MCAT	Lysate (in-house)	1in 1000
7	*B. pertussis*	BPT	Lysate (in-house)	1in 250
8	*A. alternata*	ALT	(Soluprick, ALK, Horsholm, (Denmark)	1in 500
9	Respiratory Syncytial Virus	RSV	Lysate (Gift from Geoff Toms, Newcastle University)	1in 1000
10	*S. pneumoniae*	SPN-Polysaccharide	Polysaccharide conjugate vaccine Prevenar 13(Wyeth pharmaceutical Inc, USA)	1in 500
11	*B. cenocepacia*	BCENO	Lysate (in-house)	1in 1000
12	*B. multivornas*	MULTI	Lysate (in-house)	1in 1000
13	*S.maltophilia*	SM	Lysate (in-house)	1in 1000
14	NT*H. influenzae*	NT-Hi	Lysate (in-house)	1in 100
15	*H. influenzae* b	Hib	Lysate (in-house)	

3.2.5 Inhibition ELISA to assess antibody specificity

Inhibition experiments were carried out to ascertain the IgG cross-reactivity and specificity against cognate antigen, as previously described in section 2.7.4, to ensure that the antibody is directed against the target antigen. The percentage of inhibition was calculated using the formula (No inhibition-max inhibition) $\div$ (no inhibition) $\times$100. Figure 3.5 shows the

inhibition of coated *P. aeruginosa* lysate (NCTC) by *P. aeruginosa* lysate (91%), LPS of *P. aeruginosa* (7%), *H. influenzae* b (6%), *B. pertussis* (3%), *B. cepacia* (0.8%) and *S. pneumoniae* (0.9%). Figure 3.6 displays the inhibition of coated NT*H. influenzae*, by NT*H. influenzae* (90%), *H. influenzae* lysate (88%) and *H. influenzae* polysaccharide (1%). The results indicate that cross-reactivity is unlikely.

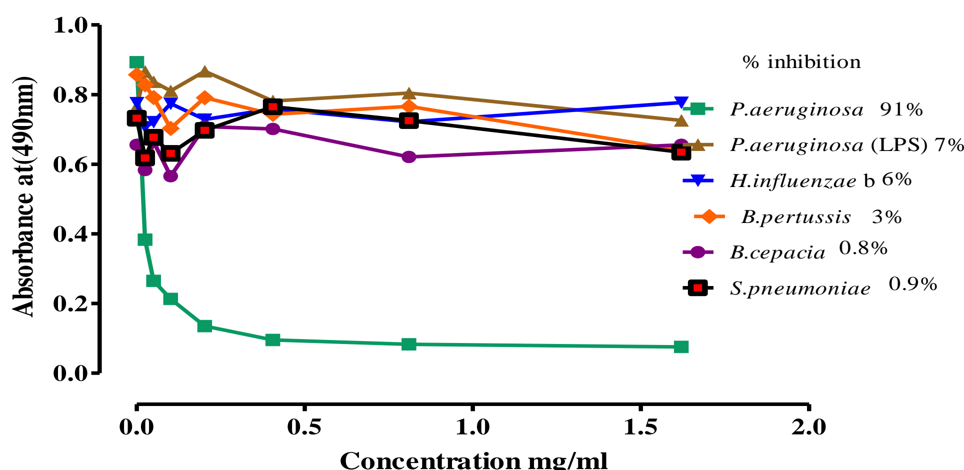


Figure 3.5: Inhibition ELISA for IgG against *P. aeruginosa*.
Six antigen inhibitors were tested, including *P. aeruginosa*, LPS of *P. aeruginosa*, *H. influenzae* b, *B. pertussis*, *B. cepacia* and *S. pneumoniae*. Absorbance at 490 nm was plotted against the protein concentration of the bacterial lysate (mg/ml). The percentages of inhibition of antibody binding for the antigens are shown in the key.

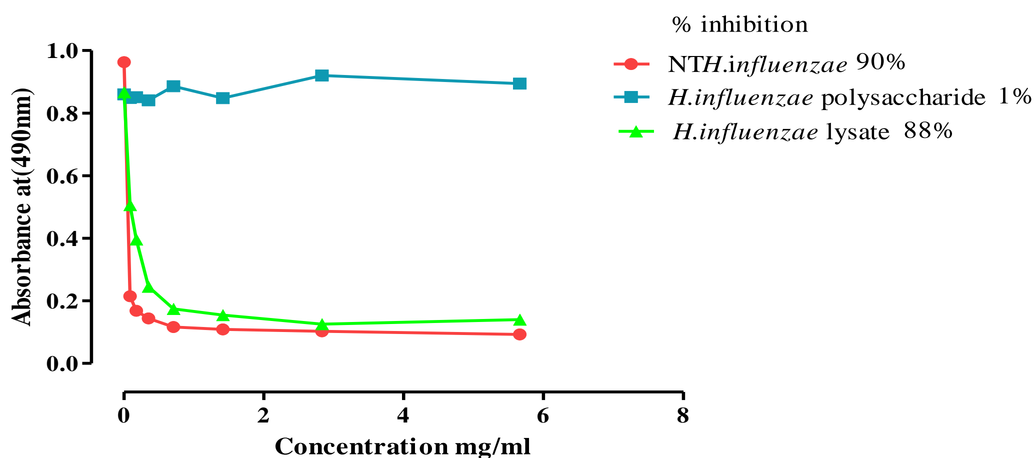


Figure 3.6: Inhibition ELISA for IgG against NT*H. influenzae*.
Three antigens were used as inhibitors, including NT*H. influenzae*, *H. influenzae* lysate and *H. influenzae* polysaccharide against coated NT *H. influenzae*. Absorbance at 490 nm was plotted against the protein concentration of NT*H. influenzae* (mg/ml). The percentages of inhibition of antibody binding for the antigens are shown in the key.

3.2.6 IgG ELISA for antibody screening in all groups against the antigens

Screening of the sera was performed to determine the magnitude of serum responses and to establish the starting dilution of antibody titre against the key antigens in the study, as shown in Table 3.3. BR patients (n=103/119) were investigated for antibody screening and titration, where 5 sera samples were not included (missing samples) and 11 samples were excluded from the analysis because the patients have immunodeficiency. COPD patients (n=57/58) were also investigated as well as asthmatics (n=14) and healthy volunteers (n=27/28). Each clinical group was initially screened at 1 in 25 dilutions in diluent for their responses as described in section 2.7.2. Absorbance was read at 490 nm for each patient serum. An example of the screening of each group against *P. aeruginosa* is shown in Figure 3.7(see Appendix 9 for more results of antibody screening). Figures 3.7-3.10 illustrate the IgG screening of sera against *P. aeruginosa* in patients with BR, COPD, and asthma as well as healthy volunteers. The results show that most patients and controls exhibited high antibody responses to *P. aeruginosa*. Similar patterns of IgG responsiveness were observed for the other antigens tested.

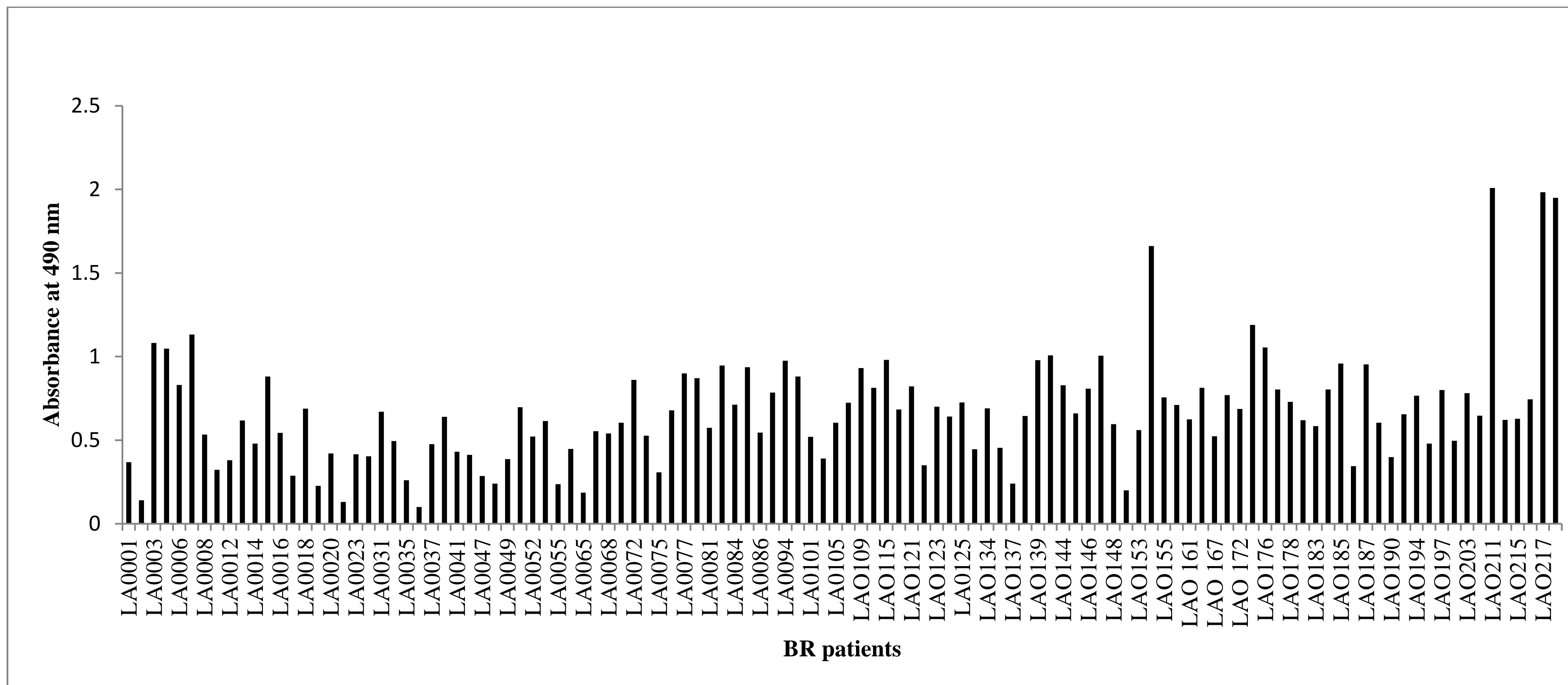


Figure 3.7: Screening profile of BR sera against *P. aeruginosa*.

Patient sera were initially screened at 1in 25 dilution for their response against the antigen to establish the starting point for antibody titration. Absorbance values for each BR patient are given.

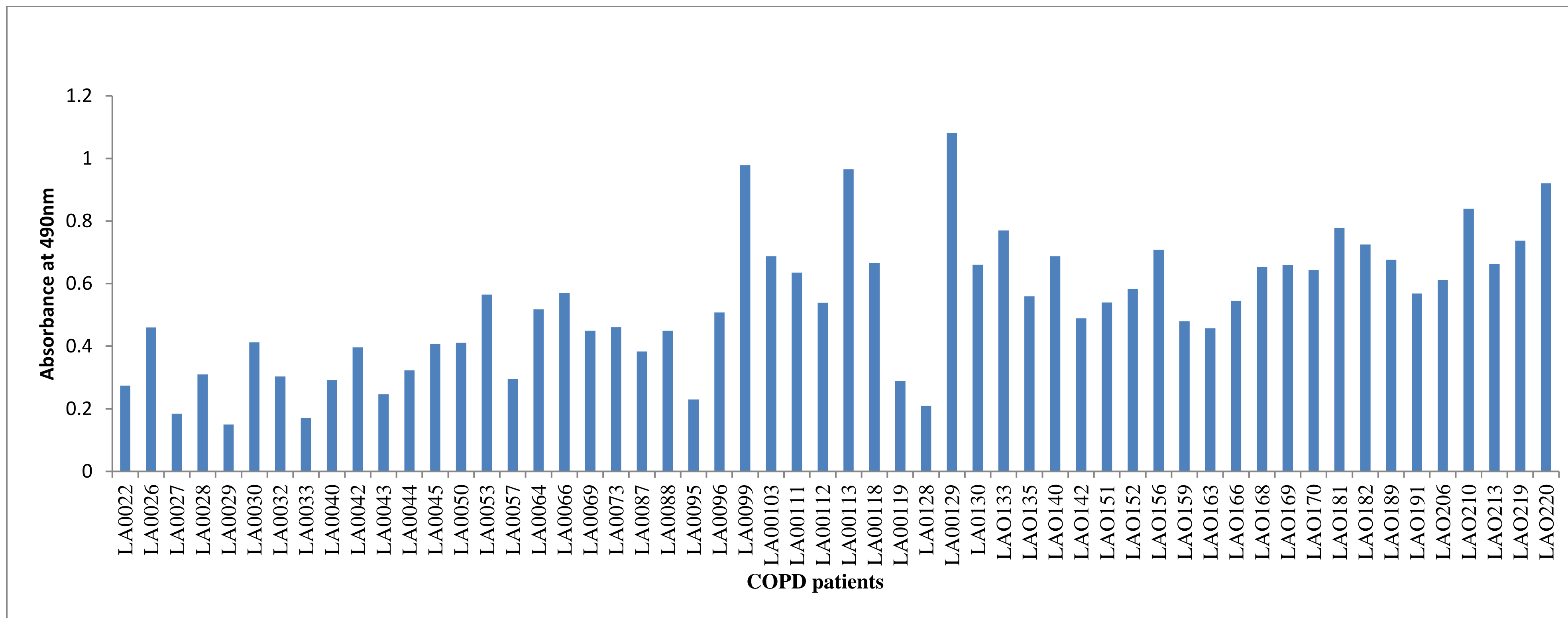


Figure 3.8: Screening profile of COPD sera against *P. aeruginosa*.

Patient sera were initially screened at 1in 25 dilution for their response against the antigen to establish the starting point for antibody titration. Absorbance values for each COPD patient are given.

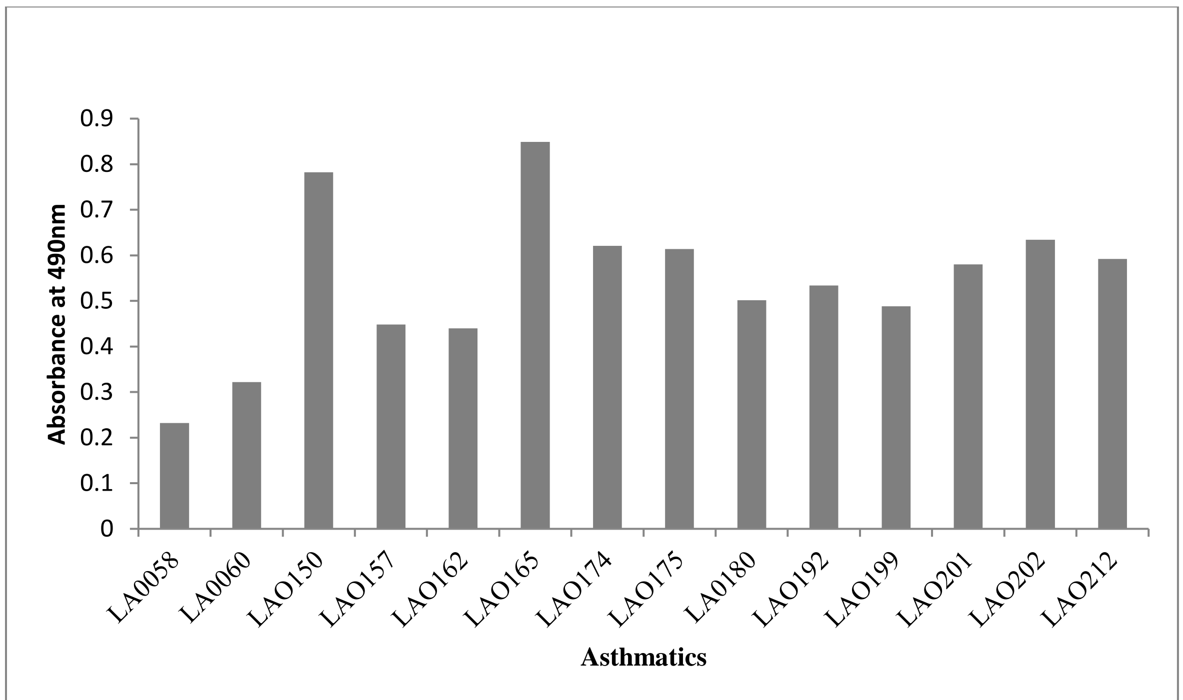


Figure 3.9: Screening of asthmatic sera against *P. aeruginosa*.
Patient sera were initially screened at 1in 25 dilution for their response. Absorbance values for each asthmatic are given.

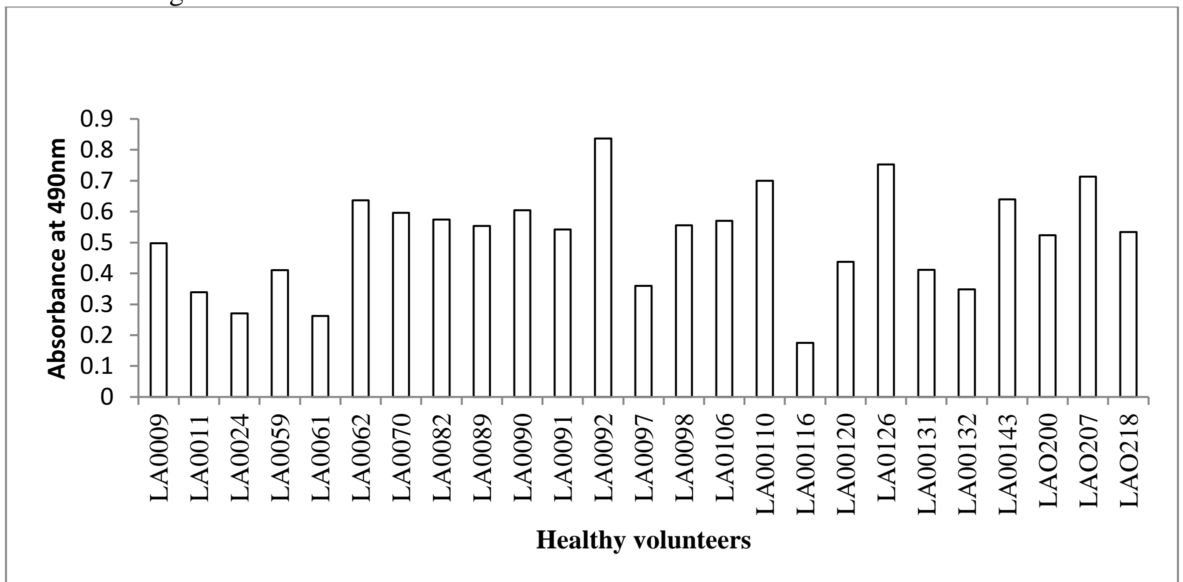


Figure 3.10: Healthy volunteers sera screened against *P. aeruginosa*.
Healthy volunteers sera were initially screened at 1in 25 dilution for their response. Absorbance values for each healthy volunteer are given.

Table 3.4 demonstrates the absorbance range at screening, indicating which subsequent starting sera dilution was used to obtain the end-point titre.

Table 3.4. Establishing initial dilution for titration.
Starting sera dilutions for titration were estimated from the mean optical density of sera with blank subtracted

Absorbance for 1 in 25 screening	Subsequent sera starting dilution
< 0.2	1:25
0.2-0.4	1:50
0.4-0.6	1:100
0.6-0.8	1:200
0.8-1.0	1:400
> 1.0	1:800

3.2.7 Strategy for obtaining end-point titre

A long series of dilutions of serum against antigen shows a typical S-shaped curve. Absorbance measurements in the upper plateau of the curve do not discern differences in antibody concentration. Hence, the linear parts (Figure 3.11) of the curve in this example between a dilution of approximately 1 in 100 to 1 in 1000, is used to extrapolate the dilution where the absorbance in zero. This is the end-point titre.

3.2.8 IgG antibody titre of patient's sera against antigens

Doubling dilution was carried out on patient sera, which were diluted according to the results of the first screening as shown in Table 3.4 and as described in section 2.7.3. Antibody titres were expressed as the reciprocal of the highest dilution in which the absorbance value of a serum differs from zero. Calculation of the end-point was obtained from the equation $y=mx+c$, where c, the intercept on the y- axis (dilution) is the extrapolated end-point titre Figure 3.12.

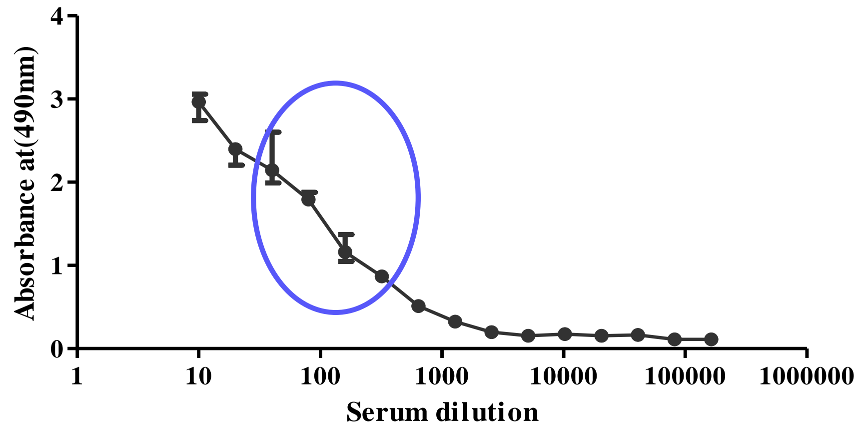


Figure 3.11: Long titration for IgG against *P. aeruginosa*.
Serum sample were used to assess the titration values against *P. aeruginosa*. Results were calculated and represented as the mean± SEM. The oval indicates linear part of serum dilution.

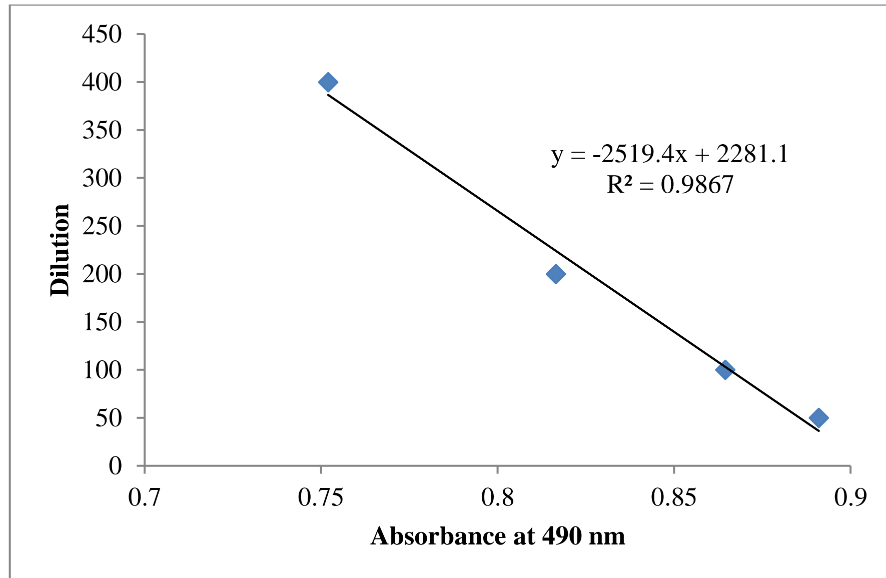


Figure 3.12: **Calculation of each patient end-point titre obtained from the graph using the equation y=mx+c.**
The dilution was plotted against absorbance and, in the example for PSA, the value of 2281 was found to be the end-point titre.

3.2.9 IgG titre against absorbance

Due to constraints on samples, reagents and time to perform serum dilution series for every sample, an analysis was performed to see if absorbance was a sufficient measurement of Ig levels rather than having to carry out titration for every test. Figure 3.13 below demonstrates the relationship between the screening and titration of anti-Pseudomonas IgG titres to see if screening absorbance is a sufficient measurement of antibody magnitude. The results showed a significant correlation between the titre and the screening absorbance (p=0.0001).

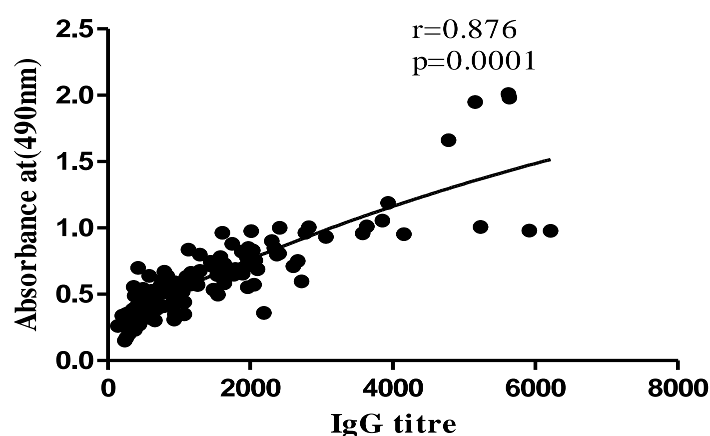


Figure 3.13: Titration versus absorbance to *P. aeruginosa* for the patients and healthy volunteers.

Anti-Pseudomonas IgG absorbance was plotted against the titration of the same cohort to see the association between them. The relationship was assessed using Spearman correlation. (n=147)

3.2.10 Stability of the sera

Since sera and plasma samples had to be kept frozen (and thawed when needed), an experiment was carried out to investigate the effect of repeating the freeze-thaw cycles of human sera on the activity of IgG antibody to a bacterial antigen. *M. catarrhalis* was selected as an example for testing antibody activity (Figure 3.14). Sera were used after 5 freeze-thaw cycles as described in section 2.7.6. The results showed no significant difference (p=0.947) between levels of antibody in first thawing sera and after 5 freeze/thaw cycles. These findings imply that the antibody levels are stable even after 5 freeze-thaw cycles.

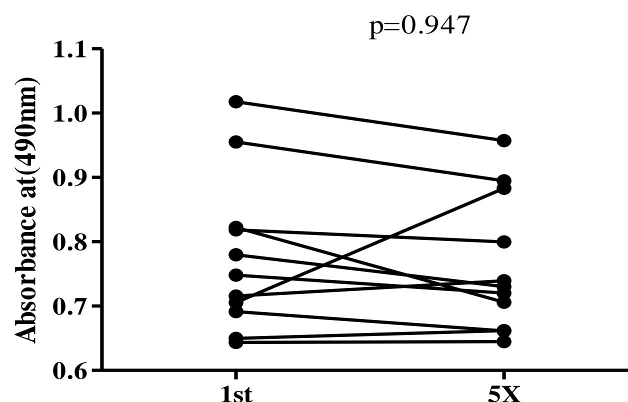


Figure 3.14: Activity of the sera before and after 5 freeze-thaw cycles.
Anti- *M. catarrhalis* IgG was used as an example to determine the effect of repeated freezing-thawing cycles on the antibody. Results represent the mean of absorbance. 1st represent the first use and 5X shows results after 5 freezing-thawing cycles. No significant difference was found (p=0.9476).

3.2.11 IgG titre in bronchiectasis patients (BR) compared to healthy volunteers (HV)

Indirect ELISA and end-point titration as described in section 2.7.3 was used to measure IgG titres against the key antigens shown in Table 3.3 for patients with bronchiectasis (BR) compared to healthy volunteers (HV). The findings showed significant differences between BR patients and healthy volunteers for *P. aeruginosa* (p=0.0002), *S. pneumoniae* polysaccharide, *H. influenzae* (p<0.0001*), H. influenzae* polysaccharide (p=0.05) and *S. maltophilia (*p=0.032*)*. Conversely, *S. pneumoniae* titres were significantly higher in healthy volunteers compared to BR patients (p=0.027). There were no significant differences between BR patients and healthy volunteers with respect to the other bacterial antigens, including NT*H. influenzae, M. catarrhalis,* and *A. alternata* (p>0.05). In addition, a similar response among patients with BR and healthy volunteers to *A. fumigatus* was seen. Overall, the antibody responses of BR patients against most antigens were higher than those of healthy volunteers.

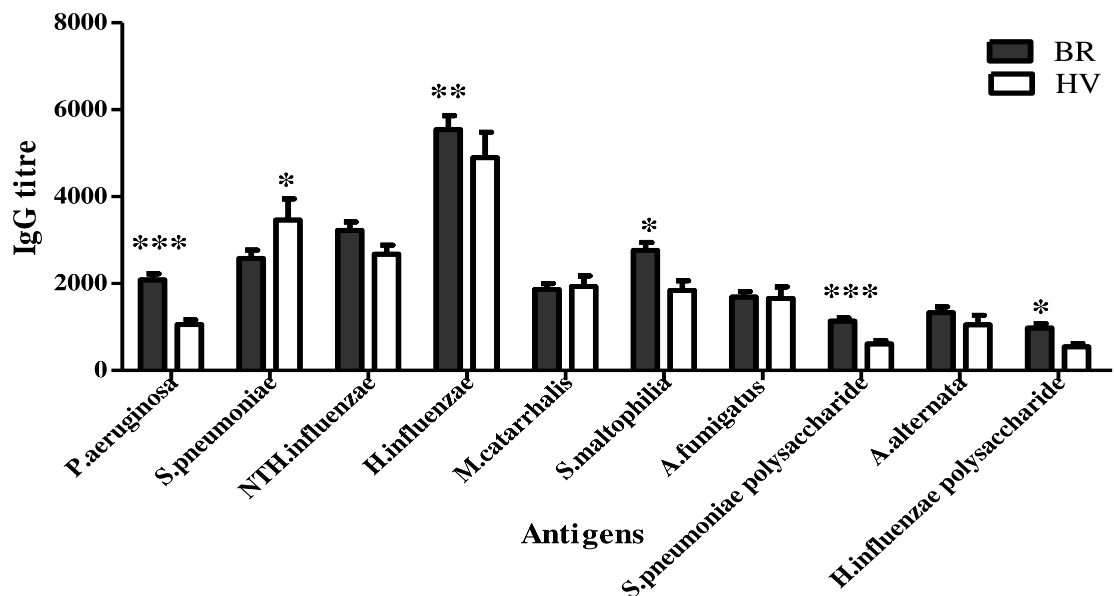


Figure 3.15: IgG titre against the key antigens included in the study in bronchiectasis patients and healthy volunteers.

Higher antibody titres were detected in BR patients compared to the healthy volunteers (HV). *** indicates highly significant p≤0.001. * indicate p ≤ 0.05. The data is represented by the mean ± SEM (n=103 for BR and 27 for HV).

3.2.12 IgG titre in COPD patients compared to healthy subjects

IgG titres of patients with COPD measured by indirect ELISA and end-point titre were compared to those of healthy volunteers as explained in section 2.7.3 to determine if there were any significant differences between these groups. The results showed that the COPD group had highly significant anti-*S. pneumoniae* polysaccharide (p= 0.0047) compared to healthy individuals. Meanwhile healthy volunteers had higher anti-*S. pneumoniae* IgG titres when compared to COPD patients (p=0.014). The results showed no significant differences between COPD patients and healthy subjects to *P. aeruginosa*, NT*H. influenzae*, *H. influenzae* b lysate, *M. catarrhalis*, *S. maltophilia*, *A. fumigatus*, *A. alternata* and *H. influenzae* b polysaccharide.

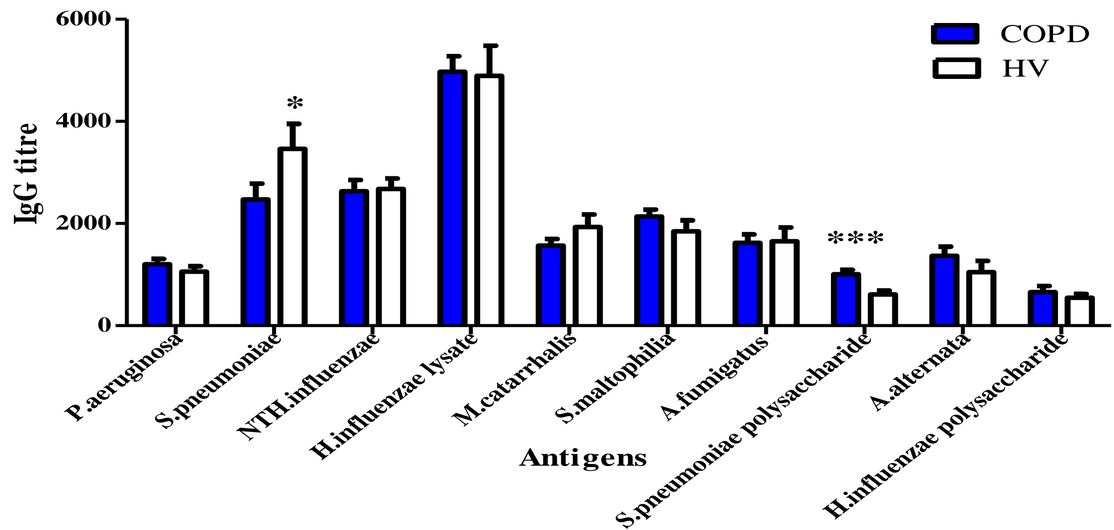


Figure 3.16: IgG titre against the key antigens in the study in COPD group and healthy volunteers.

*** indicates highly a significant difference between the COPD and HV (p=0.0047), and * indicates (p= 0.014). The results are represented by the mean ± SEM (n=57 for COPD and 27 for HV).

3.2.13 IgG titres in asthma patients compared to healthy volunteers

Specific IgG titres versus antigens included in the study were also measured as described in section 2.7.3 among asthmatics compared to healthy volunteers. The findings showed that asthmatics had significantly lower IgG titre against *S. pneumoniae* lysate (p=0.046) and *M. catarrhalis* (p=0.032). The results showed no significant differences in patients with asthma against *S. pneumoniae* polysaccharide, *A. alternata* and *H. influenzae* b compared to healthy controls.

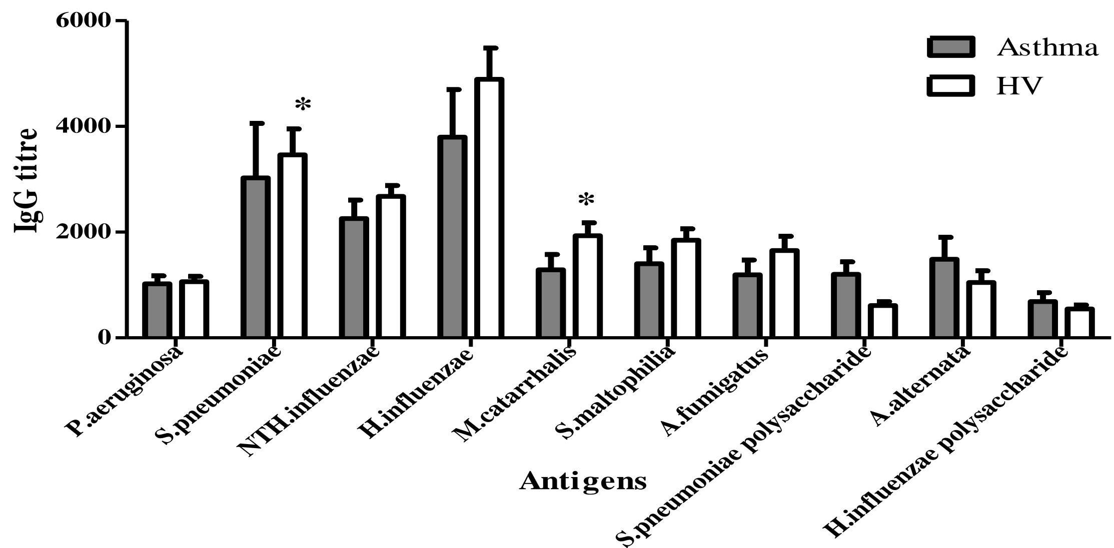


Figure 3.17: IgG titre against the key antigens in the study in asthmatics and healthy volunteers (HV).

* indicates $p \leq 0.05$. The results are represented by the mean ± SEM (n= 14 for asthma and 27 for HV).

3.2.14 IgG subclass

Sub groups of 20 BR patients and 20 COPD patients were tested for anti-*P. aeruginosa* Ig isotypes including IgG1, IgG2, IgG3, and IgG4, as well as the IgA and IgM classes. These samples were high IgG antibody titre responders to *P. aeruginosa* as determined previously in antibody screening and titration. Absorbance at 490 nm was plotted against the Ig isotype for serum dilution at 1 in 25. The isotype levels for BR and COPD patients are shown in Figures 3.18 and 3.19, respectively. IgG1 response was highest in BR patients followed by IgA and IgM. In contrast, COPD patients demonstrate higher IgM followed by IgG1. Other IgG isotypes were at low levels among patients with BR and COPD. Three healthy volunteers and three asthmatics were also measured for isotypes, IgA, and IgM against PSA. The results are shown in (Appendix 10).

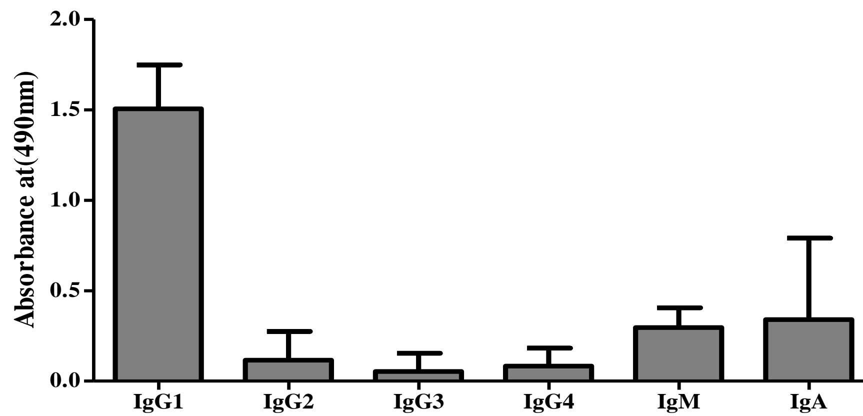


Figure 3.18: Antibody isotype levels against *P. aeruginosa* in BR patients.
Comparison of antibody isotypes in patients with BR showed that the predominant isotype was IgG1. Data are shown as mean ± SEM.

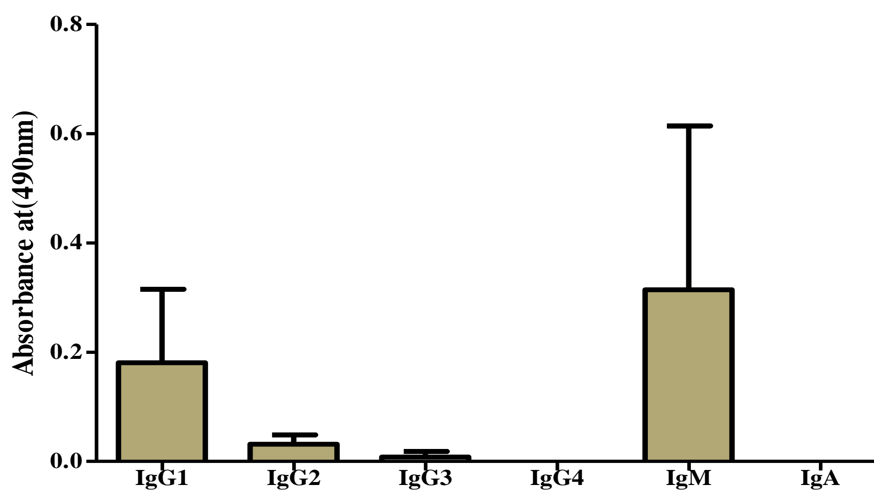


Figure 3.19: Antibody isotype levels against *P. aeruginosa* in the COPD group.
Comparison of antibody isotypes in patients with COPD showed that the predominant isotype in COPD was IgM. Data are shown as mean ± SEM**.**

3.3 Discussion

Bronchiectasis and chronic obstructive pulmonary disease are the most common conditions leading to progressive lung damage and loss of lung function. Both conditions are characterised by an abnormal dilation of airways that is not fully reversible. Bacterial infection and inflammatory response are the main determinants for promoting disease progression (Vestbo *et al.*, 2013, Whitters and Stockley, 2013). Recruited neutrophils are

found to be a major factor in the causation of airway inflammation, disruption of the local immune response and eventually damage to the bronchi (Shum *et al.*, 2000). It has been shown that high titres of antibody against *P. aeruginosa* are found in CF patients. The role of anti-Pseudomonas antibody is important for eliminating the bacteria and may provide an early indication of microbial colonization (Caballero *et al.*, 2001, Milagres *et al.*, 2009). In this study, it is hypothesised that microbial infection is the main cause of eliciting higher antibody titre in patients with obstructive lung disease. Therefore, the elevated antibody may be a sufficient marker for microbial colonization.

The aim of this section was to investigate the immune responses against different lung antigens among patients with chronic obstructive lung diseases (BR, COPD and asthma) compared to healthy volunteers, and to identify any significant differences between these groups. In this study, different forms of lung antigens were used, including a lysate of bacteria, polysaccharides, viruses and extracts of fungi, to ascertain the presence of antibodies specific to each antigen. The cross-reactivity of *P. aeruginosa* and NT*H. influenzae* were tested with other related bacteria to determine the specificity of IgG using an inhibition assay (see Figures 3.5 and 3.6). For *P. aeruginosa,* the inhibition assay showed minimal inhibition by the other antigens tested (Figure 3.5). The procedure of the inhibition assay is based on the hypothesis that the incubation of antigen and positive sera will allow the interaction between the antigen and the antibody and the formation of an antibody-antigen complex. Once the mixture is added to the ELISA plate coated with the same antigen, only free antibody will react with the restrained antigen in the plate. Therefore, the antibody specific to the soluble antigen cannot bind to the immobilized antigen and as a result will be washed away. The remaining antibodies that react with the immobilised antigen can then be determined by the assay (Bergmann *et al.*, 2006). Therefore, the results show that no cross-reaction was observed in *P. aeruginosa* with other Gram-negative bacteria, indicating that

the antibody is specific for the cognate antigen. However, a cross-reactivity was observed between *H.influenzae* lysate and NT*H.influenzae.*

In this study, the stability of the sera was also examined after multiple freeze-thawing to monitor the sustained quality of sera during the study. The results demonstrated that the antibody activity was not affected even after multiple freeze/thaw cycles. This finding supports those of a previous study conducted to measure the antibody activity of IgG, IgA and IgM, which showed the antibody to be stable even after 30 freeze-thaw cycles (Rastawicki *et al.*, 2012).

3.3.1 Antibody response in patients with BR and HV

Antibody analysis revealed that patients with BR had significantly higher IgG titres compared to healthy volunteers against *P. aeruginosa* ($p=0.0002$)*, S. maltophilia* ($p=0.032$), *H. influenzae* polysaccharide ($p=0.05$), *H. influenzae* and *S. pneumoniae* polysaccharide ($p<0.001$). These patients are more susceptible to persistent microbial infections. The most common pathogens isolated from the respiratory tract of non-cystic fibrosis bronchiectasis (nCFBR) patients include *H. influenzae*, *P. aeruginosa*, *S. pneumoniae*, and *M.catarrhalis* (Angrill *et al.*, 2002, Pasteur *et al.*, 2000). Therefore, increased antibody levels against these organisms in BR patients are expected to be due to multiple boosting. Furthermore, higher antibody titres were also observed in BR patients than in healthy volunteers against NT*H. influenzae*, and *A. alternata*. However, the differences were not significant ($p>0.05$). The reason for these responses may be the immunological stimulus from the antigens in the airways in combination with the PAMPs (such as LPS) leading to inflammatory responses and increased magnitude of antibody (Caballero *et al.*, 2001, Stead *et al.*, 2002). It has been suggested that detectable levels of IgG against NT*H. influenzae* in both bronchiectasis and healthy controls may be due to healthy volunteers having a Th_1 response and protective immunity against these bacteria, whereas patients with bronchiectasis develop a Th_2

response with associated antibody production but weaker immunity against infection (King *et al.*, 2003, Murphy, 2003). The data also shows a similar magnitude of response of the antibody between BR and healthy volunteers against *A. fumigatus* and this may be because both groups have a similar degree of exposure to *A. fumigatus*. A study has demonstrated that the culture of sputum from patients with CF underestimates colonization by *A. fumigatus,* and no correlation was found between the antibody titre and *A. fumigatus* colonization (Máiz *et al.*, 2008).

A previous study has shown decreased antibody response to Pneumococcus polysaccharide in patients with BR (Stead *et al.*, 2002). This result is contrary to our finding, which showed significantly higher antibody titres against *S. pneumoniae* polysaccharide in BR and COPD patients. This may be because the patients responded to natural infections or they had previous immunisation against Pneumococcus, which is boosted by infection. Interestingly, healthy volunteers showed significantly higher IgG titres against *S. pneumoniae* lysate, suggesting that the protective immune response among healthy volunteers arises from exposure to this bacterium rather than by vaccination (King *et al.*, 2003).

3.3.2 Antibody response in patients with COPD and HV

The COPD group contained 57 patients compared to 27 healthy volunteers. Similarly to BR patients, a significant difference was observed in COPD patients against *S. pneumoniae* polysaccharide (p=0.0047). This implies that these patients may have had previous immunization or exposure to the bacteria as discussed above for BR patients. However, only slightly increased antibody titres were observed in COPD patients against *P. aeruginosa*, *H. influenzae b*, *S. maltophilia* and *A. alternata*, but the differences were not significant, suggesting that levels of bacterial infection eliciting inflammatory and antibody responses among patients with COPD, were less than in BR (Millares *et al.*, 2014). In contrast, a lower antibody titre was found in COPD subjects against *S. pneumoniae* lysate, NT*H. influenzae*

and *M. catarrhalis*. Decreases in IgG antibody titre in COPD compared to BR may be influenced by smoking in COPD patients. Smoking is thought to inhibit the activation of B cells and immunoglobulin secretion, leading to the impaired ability of the host to clear infection (Al-Ghamdi and Anil, 2007, Voss *et al.*, 2015). Furthermore, as a component of cigarette smoke nicotine promotes bacteria replication and decreases the production of cytokines, including IL-6, IL-12, TNFα and IFNγ (Matsunaga *et al.*, 2001). A similar response to *A. fumigatus* between COPD patients and healthy subjects was observed, as seen among patients with BR.

3.3.3 Antibody response in patients with asthma and HV

Fourteen patients with asthma were included in the antibody analysis. The findings demonstrated that asthmatics had lower antibody responses against *S. pneumoniae* lysate, NT. *H. influenzae*, *M. catarrhalis*, *S. maltophilia* and *A. fumigatus* and the differences for both *S. pneumoniae* lysate and *M. catarrhalis* were significant ($p= 0.046$ and $p=0.032$ respectively) compared to healthy controls. Reduced antibody levels observed among asthmatics may be related to rare microbial infections that are necessary to activate humoral responses. In contrast, the antibody levels against *S. pneumoniae* polysaccharide, *A. alternata* and *H. influenzae* b were slightly higher among patients with asthma. However, no statistical significance was found ($p>0.05$). This contrasts to a previous study, which showed that antibodies against *S. pneumoniae* polysaccharide were lower in patients with asthma compared to non-asthmatics. The reason behind this may be the limitations of statistical power as reported in the previous study (Zhao *et al.*, 2013). The lower levels of IgG antibody in patients with asthma against most antigens may be explained by the fact that the presentation of allergen peptides to TCR via MHC II in combination with costimulatory molecules and a high level of IL-4 may lead to Th_2 cell differentiation and B cell isotype switching to IgE (Holgate, 2012).

3.3.4 IgA, IgM and IgG subclasses in COPD and BR

The study sought to examine the IgG sub classes and other classes including, IgM and IgA in BR and COPD groups against *P. aeruginosa* as described in section 2.7.5. A sub group of 20 patients was selected from each clinical group; these patients had higher total anti-Pseudomonas IgG. The findings showed that the level of IgG1 was greater among BR patients compared to the other sub classes (IgG2, IgG3 and IgG4) and classes (IgM and IgA). Increased IgG1 in patients with BR seems to be associated with the biochemical nature of the Pseudomonas antigens itself, since the protein antigens are associated with the T cell-dependent elicitation of IgG1, whereas the polysaccharide increases the response of IgG2 (Obukhanych and Nussenzweig, 2006, Twigg, 2005).

Additionally, the level of anti-Pseudomonas IgA was slightly higher compared to the other sub classes. IgA is the main humoral immune response against bacterial infection at the mucosal surface of the lungs. Its role is to inhibit the organism binding to the mucosal surface, neutralising extracellular pathogens and clearing infections (Blutt *et al.*, 2012, Brett *et al.*, 1988, Twigg, 2005). Therefore, as BR is associated with *P. aeruginosa* infections, increased IgA production is expected for the clearing of the Pseudomonas and to protect against re-infection. It has been reported that increased anti-Pseudomonas IgA may occur before the anti-Pseudomonas IgG response in patients with cystic fibrosis colonized by *P. aeruginosa* (Brett *et al.*, 1988). Specific IgA against exotoxin A, lipopolysaccharide, alginate and whole cells of *P. aeruginosa* have been previously demonstrated in the serum of CF patients (Cukor *et al.*, 1983, Pedersen *et al.*, 1990). Here the elevated level of anti-Pseudomonas IgA in patients with BR may provide an indication of *P. aeruginosa* colonization in the lung mucosa.

In contrast to BR, the results showed that patients with COPD had higher anti-Pseudomonas IgM responses. This may be due to a disruption in T cell responses that would promote IgM

to IgG class switching. A previous study revealed that increased IgM antibody against NT*H. influenzae* in both sera and saliva reduced infections and chronic respiratory disease in patients with hypogammaglobulinemia (Micol *et al.*, 2012). Anti-Pseudomonas IgG1 levels also increased compared to other sub classes in patients with COPD, indicating a role of the T cell-dependent antibody response.

Low levels of IgG sub classes, including IgG2, IgG3 and IgG4, in patients with BR and COPD have been found in this study. Bacterial whole cell lysates were used in which the protein is the most antigenic component which therefore provides cognate antigen for T cell help. High levels of IgG2 and IgG3 against Pseudomonas in patients with cystic fibrosis have been associated with worsening clinical condition (Pressler *et al.*, 1990). In the same study, the authors reported that the investigation of antibody sub classes should be restricted to certain antigen components, and that IgG sub class production occurs in response to the particular protein or polysaccharide (Pressler *et al.*, 1990). Low levels of anti-Pseudomonas IgA in patients with COPD may be due to the low extent of Pseudomonas colonization in the lungs of COPD patients compared to BR, which is investigated in the next chapter.

4 Conclusion

The study described in this chapter demonstrated that increased IgG antibody titres in BR and COPD patients in comparison to healthy volunteers may be the result of microbial exposure and infections. In contrast, patients with asthma have lower IgG antibodies. This suggests that elevated IgG levels are associated with bacterial infection. IgA may also provide an indication of pulmonary infections among patients with BR and COPD. Investigation of IgG isotypes against further specific antigenic components in patients with BR and COPD is needed.

4 Chapter Four: Relating Antibody Responses to Microbial Colonization and Disease Severity in Patients with Bronchiectasis (BR) and Chronic Obstructive Pulmonary Disease (COPD).

Abstract

Aim: The aim of this chapter is to compare the microbiological results from sputum, and disease severity, with antibody responses against corresponding antigens in BR and COPD. In the whole cohort, 119 patients with BR and 58 patients with COPD were recruited into this study. Out of these, 116 BR and 48 COPD subjects contributed to the sputum analysis. Asthmatics were excluded from the microbial analysis.

Methods: IgG titres against microbial derived-antigens were measured by indirect ELISA end point titre. Lung function assessment was performed on patients and healthy volunteers. Culture-based microbiology was carried out to detect the presence of pathogens in the sputum of patients with BR and COPD.

Results: Culture-positive isolates were obtained from 108 and 27 patients for BR and COPD, respectively. The remaining BR (n=8) and COPD (n=21) samples were classified as no pathogen isolated. Non-typeable *H. influenzae* was the predominant bacterial species isolated in both groups, followed in abundance by *P. aeruginosa* detected in BR and *S. pneumoniae* in COPD patients. Increased magnitude of antibody was associated with the degree of microbial colonization when it was categorised according to standardised clinical criteria. Lung function (FEV_1% predicted) was significantly reduced in patients who had chronic infection compared to those for whom it was intermittent. By contrast, no relationship between antibody titre and lung function was observed.

A significant difference was observed in antibody titres against *P. aeruginosa, M. catarrhalis* and *S. maltophilia* at different scores of exacerbations.

Conclusion: Polymicrobial colonization was detected among patients with BR and COPD. These pathogens induce humoral immune responses and elevated levels of IgG which are in part dictated by levels of exposure. Bacterial infections also promote lung function deteriorations and exacerbations, but these are not predicted by level of specific antibody.

4.1 Background

Ever since the Human Microbiome Project (HMP) omitted the lung from the list of priority organ systems, much work has focused on the communities of micro-organisms in healthy and diseased conditions, shedding light on the pathogenesis of lung infections (Beck *et al.*, 2012, Dickson *et al.*, 2013). Recent studies have found microbial communities in the airway of healthy humans, confirming that the lung is not a sterile organ (Yonker *et al.*, 2015).

Bacterial colonization is associated with reduced mucociliary clearance in the lower respiratory tract, and it promotes the hyper-inflammatory response, airway destruction, further supporting microbial colonization. This phenomenon is termed the "vicious cycle hypothesis" as described by Cole (1986).

Bronchiectasis is strongly associated with chronic airway infection leading to progressive damage to the bronchial wall and impaired lung function. Previous studies investigating the pathogenic microorganisms of BR from sputum samples have identified *H. influenzae* and *P. aeruginosa* as the most common pathogens isolated, and further analysis of *H. influenzae* found it to be the non-typeable (NT) strain (King *et al.*, 2007). Other lung isolates include *S. pneumoniae*, *M. catarrhalis* and non-tuberculous mycobacteria (NTM). *S. aureus* is less frequently isolated and its isolation suggests the possibility of cystic fibrosis. *P. aeruginosa*

has antibiotic resistance particularly due to the formation of mucoid biofilm, which results in severe damage to the underlying airway. It has been shown that *H. influenzae* is isolated from patients with moderately declining lung functions, whereas *P. aeruginosa* is implicated in worsening lung function, exacerbation, and hospitalization (King, 2011).

Microorganisms are the main aetiological factors contributing to the pathogenesis of COPD in both stable and exacerbation conditions. In stable conditions, the chronic infections promote airway inflammation, leading to increased frequency of exacerbation and impaired lung function. Furthermore, studies have shown that 70% of COPD exacerbation is associated with respiratory infections, the most frequently isolated bacteria from COPD patients being NT*H. influenza, S. pneumoniae* and *M. catarrhalis.* Other Gram-negative bacteria, including *P. aeruginosa* and *S. maltophilia*, are also present in patients with severe exacerbation (Matkovic and Miravitlles, 2013, Sethi and Murphy, 2001).

Culture-independent approaches are becoming an important technique in the clinical field and in conjunction with routine culturing. The technique has shown that, for example, patients with cystic fibrosis have more diverse polymicrobial communities than is found using culture-dependent methods (Tunney *et al.*, 2013).

In response to microbial infection, serum antibody titre typically rapidly elevates after infection followed by stabilization then decreases towards a baseline over the following years. For several infections, such as HIV and CMV, this process is termed "seroconversion", which is used to ascertain the level of infection. Thus, the antibody level is boosted by re-exposure or repeated contact with the organisms (Teunis *et al.*, 2012, Teunis *et al.*, 2002). Considering the role of the immune response against infection, the aim in this study is to investigate antibody levels in relation to infection status by pathogenic microorganisms. The frequency of microbial colonization and specific antibody levels are

also compared to lung function to see if the acquisition of bacteria is associated with lung function decline and disease exacerbation. Ultimately, the aim is to ascertain if antibody responses serve as biomarkers for lung disease.

4.2 Results

4.2.1 Microbial community detected in bronchiectasis and chronic obstructive lung disease patients

From the whole cohort of sputum samples, 108 BR and 27 COPD patients were culture-positive for pathogenic microorganisms. The remaining BR (n=8) and COPD (n=21) samples were classified as having no pathogens isolated. The following Figure 4.1 and Table 4.1 show the abundance of microorganisms in the sputum of patients with BR and COPD patients. The results demonstrated that the most common pathogenic organisms isolated from the sputa of BR and COPD patients were *H. influenzae*, found in 65.5% and 37.5% of patients respectively, followed by *P. aeruginosa* found in 57.7% and 14.5%. *S. pneumoniae* was isolated from 45.6% of BR and 20.8% of COPD patients. *M. catarrhalis* was found in 38.7% of BR and 29% of COPD patients. The percentage of patients with no pathogen isolated was 6.8% in BR and 43.7% in COPD. Multiple infections, in which more than two or more pathogens were isolated, were observed among many of these patients, particularly BR, and hence the percentages add to greater than 100.

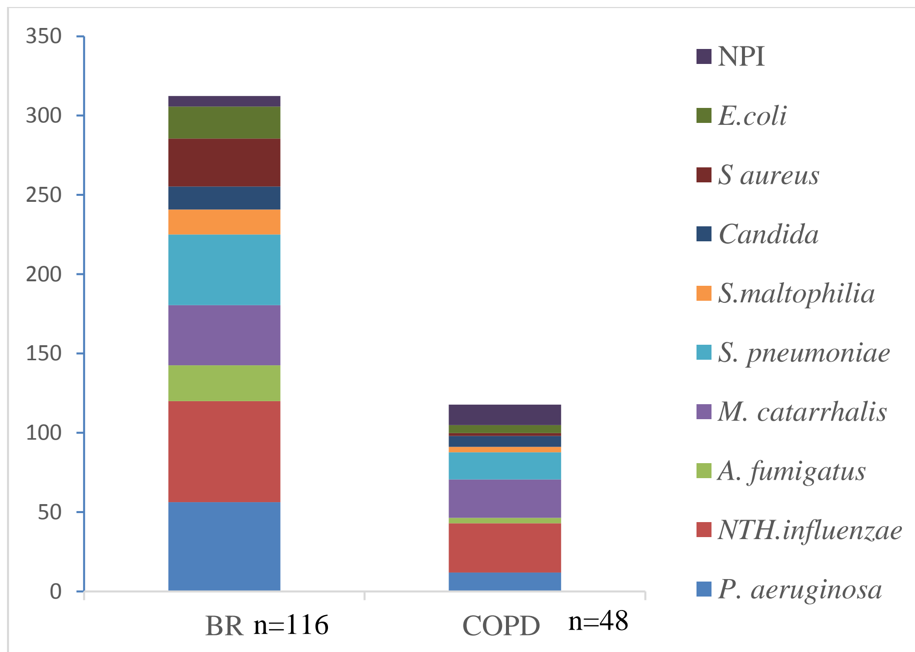


Figure 4.1: The abundance of identified microorganisms in sputum samples collected from clinically stable patients with BR and COPD.

The culture-dependent technique was performed to examine the sputum for pathogenic microorganisms. The most common pathogenic organisms isolated were NT*H. influenzae* in BR and COPD followed by *P. aeruginosa* in BR and *M. catarrhalis* in COPD. NPI indicates the percentage of no pathogens isolated.

.

Table 4.1. Shows the abundance of microorganisms isolated from the sputum of patients with BR and COPD.

Microorganism	Bronchiectasis patients (%)	Chronic obstructive pulmonary disease patients (%)
NT. *H.influenzae*	65.5	37.5
P. aeruginosa	57.7	14.5
S. pneumoniae	45.6	20.8
M. catarrhalis	38.7	29
A. fumigatus	23.2	4
S. maltophilia	16.3	4.1
***Candida.* sp**	14.6	8.3
S aureus	31	2
E.coli	20.6	6.2
NPI	6.8	43.7

4.2.2 Classification of patients according to frequency of infections

Patients were classified into four groups based on the frequency of isolation of microorganisms from the sputum samples. Total of 116 of the BR patients and 48 COPD patients were either culture-negative or culture-positive for pathogenic microorganisms. The negative samples for BR (n=8) and COPD (n=21) were categorized as no pathogen isolated (NPI). The culture-positive BR (n=108) and COPD (n=27) samples were classified into three groups. Occasionally isolated, where they had only one microbial isolate per year. Chronic infection is defined by the isolation of pathogenic microorganisms in the culture on two or more occasions in one year, and this category is further subdivided into chronic previously and chronic currently based on the date of isolation (being the same as date of sample) (see Table 4.2).

Table 4.2. Classification of patients based on microbiological results

0	**No pathogen isolated (NPI)**	
1	Occasional	1 isolation in a year
	Chronic, divided into 2 and 3	Chronic colonization is defined by the isolation of potentially pathogenic bacteria in sputum culture on 2 or more occasions, at least 3 months apart in a 1 year period
2	Chronic previously	A period of 1997-2013
3	Chronic currently	All of 2013 and 2014

4.2.3 Antibody response associated with microbial colonization in bronchiectasis (BR) patients

IgG titres against microbe-derived antigens, including *P. aeruginosa, S. pneumoniae, A. fumigatus, M. catarrhalis,* NT*H. influenzae* and *S. maltophilia*, were measured by ELISA for bronchiectasis patients. IgG titre was compared to the results of the incidence of

colonization as classified in Table 4.2. *P. aeruginosa* showed a significant difference when compared to non-colonized and currently colonized ($p<0.001$) and between occasionally and currently colonized ($p<0.001$). Significant differences were also seen between non-colonized and chronic previously ($p=0.001$) as well as between occasional and chronic previously ($p=0.003$). Similarly, a significant difference was seen between those who were non-colonized compared to currently colonized by *S. maltophilia* ($p= 0.036$). Conversely, no significant difference was found between the groups for other pathogens in the study, including *S. pneumoniae, A. fumigatus, M. catarrhalis* and NT*H. influenzae*, although there was a trend for increased titre with increased exposure.

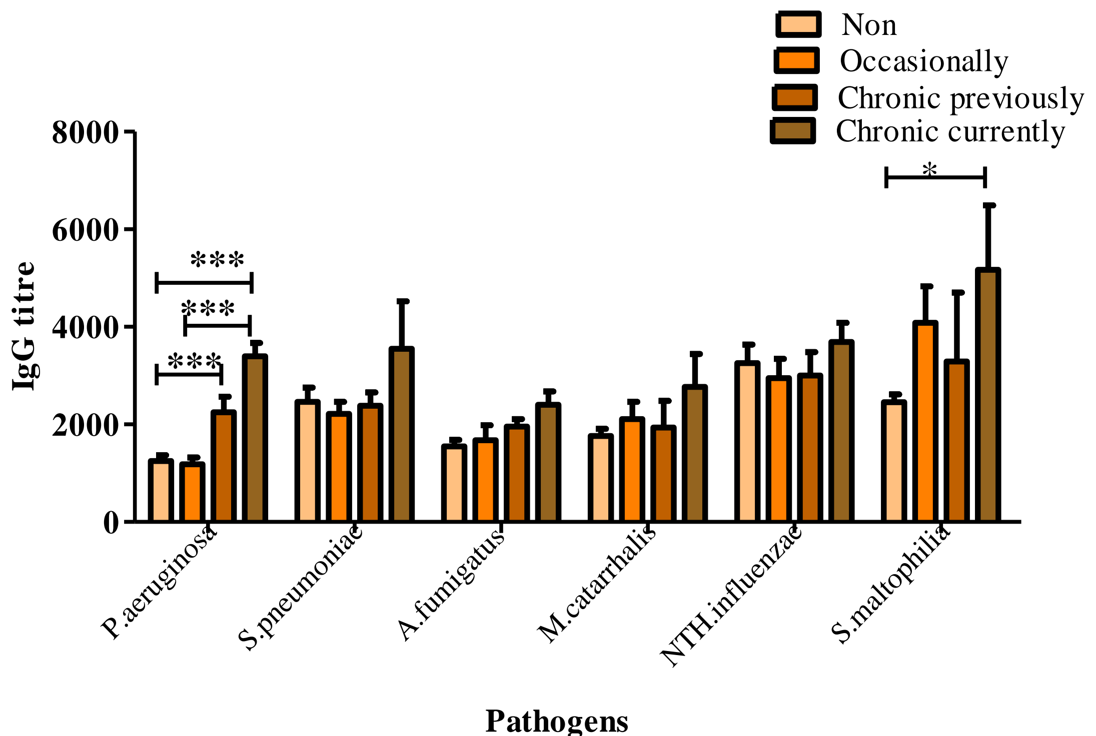


Figure 4.2: Antibody titres compared to the results for pathogens microbiologically isolated from the sputum of BR patients.

The serum IgG responses against six microbe-derived antigens were compared to the results of incidence of colonization. The colours match with the frequency of colonization as indicated in the chart. *** indicates highly significant differences were found between no pathogen isolated, occasionally group and chronic colonization category for *P. aeruginosa* ($p < 0.001$). * indicates significant difference $p<0.05$ *S. maltophilia*. Data are represented by mean ± SEM.

4.2.4 Antibody response associated with microbial colonization in chronic obstructive pulmonary disease (COPD) patients.

IgG titres against lung antigens in the study were also compared to incidence of microbial colonization for COPD; patients showed a significant difference (p=0.04) between non-colonized and currently colonized patients for *M. catarrahlis.* Meanwhile, patients who had no NT*H. influenzae* isolation (non-colonized) had significantly higher antibody titres compared to those with occasional colonization (p= 0.04). Similarly, for *S. pneumoniae*, non-colonized patients displayed higher but not significant antibody titre compared to those chronically colonized by this bacterium. Furthermore, patients who were occasionally colonized by *P. aeruginosa* had higher antibody compared to patients with no isolation of these bacteria. In contrast, no obvious difference was found between non-colonized patients and patients with chronic isolation for *A. fumigatus*.

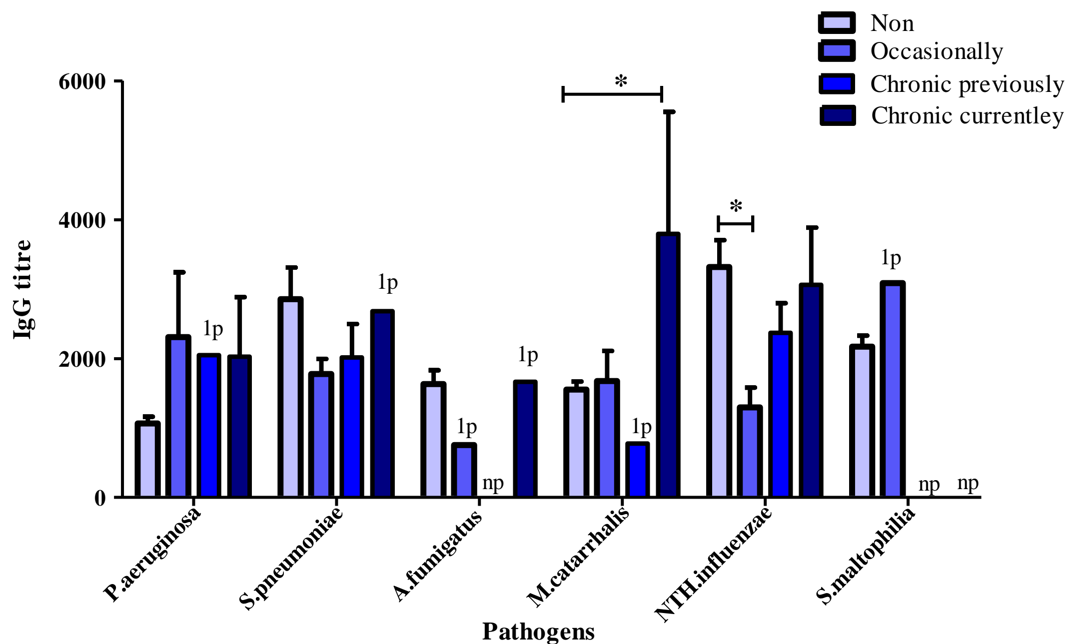


Figure 4.3: Antibody titres compared to the result of pathogens isolated from the sputum of COPD patients.

The serum IgG responses against six microbe-derived antigens were compared to the results of incidence of colonization. The colours match the frequency of colonization as indicated in the chart. Significant differences were found between the non-pathogen isolated category and chronic colonization in *M. catarrhalis* and between non-pathogen isolated and occasionally in NT*H. influenzae* with p=0.04 for both. Data are represented by the mean ± SEM. (np that indicates no

culture+ patients were identified, 1p indicates only 1 patient was identified, and so no error bars are shown).

4.2.5 Lung function associated with colonization in BR and COPD

Patients with BR and COPD underwent spirometry testing during their clinical assessment (see chapter 2). Lung function as measured by FEV_1% predicted were assessed in order to determine the severity of the disease in each patient. It was seen that both clinical groups had progressive lung function decline and loss of FEV_1% overtime.

Figure 4.4 shows the relationship between FEV_1% predicted and the incidence of microbial colonization in patients with bronchiectasis. Patients with no-pathogen colonization were compared to those with current microbial colonization. An inverse relationship was broadly observed between microbial colonization and lung function. The results show a significant reduction in FEV_1% predicted in BR patients curently colonized by *P. aeruginosa,* and *M. catarrhalis (*p=0.02) compared to the non-colonized group. A significant decrease was also observed between those with chronic current isolation and those where *S. maltophilia* had not been colonizing (p=0.006). No significant reductions were found between the groups who were non-colonized or colonized by *S. pneumoniae, A. fumigatus*, and NT*H. Influenza*.

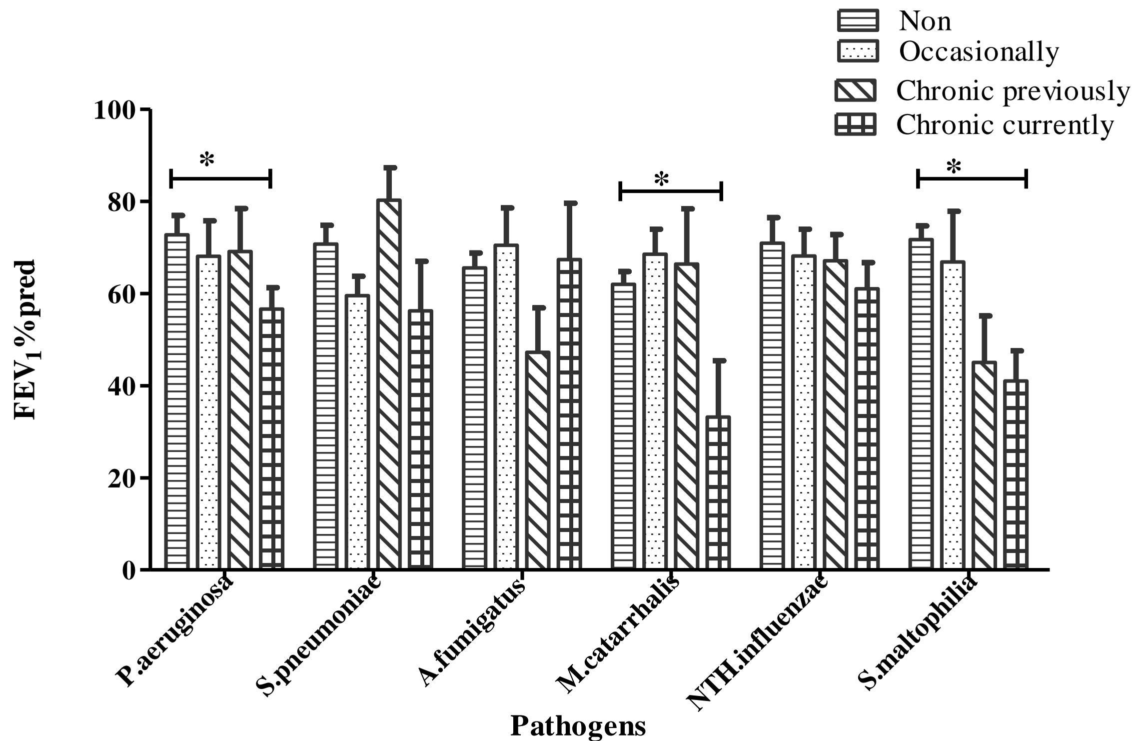


Figure 4.4: Lung function assessment compared to the results of microbial colonization in BR.

The results of FEV_1% predicted were compared to the incidence of colonization for each potentially pathogenic microbial species. The data are represented by the mean± SEM. Significant differences between non-pathogen isolated and chronic currently colonised, with $p \leq 0.05$.

Figure 4.5 shows the relationship between the presence of bacterial infection and FEV_1% predicted in patients with COPD. The results demonstrate a significant reduction in FEV_1% predicted between patients who are non-colonized and currently colonized by *P. aeruginosa* (p=0.019). However, significantly decreased FEV_1% predicted (p=0.02) was observed among patients who had negative culture for *S. pneumoniae* compared to those with previous colonization. The data did not show any significant reduction in FEV_1% for *M. catarrhalis*, NT*H. influenzae* and *A.fumigatus* colonization.

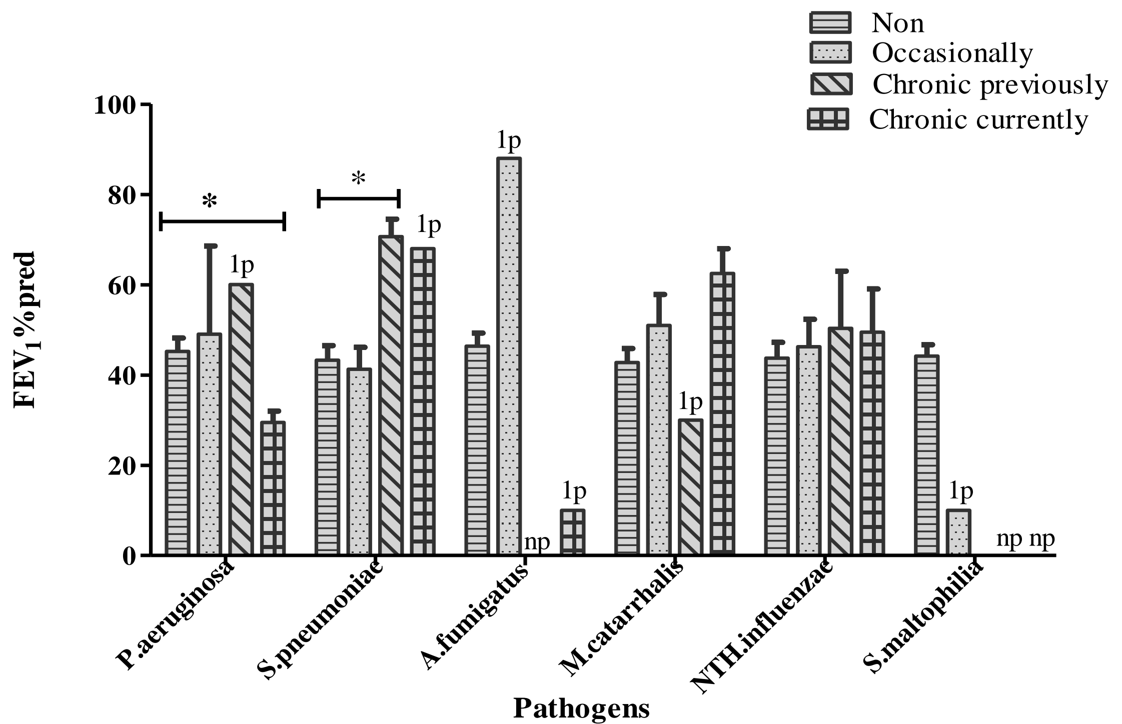


Figure 4.5: Lung function assessment compared to the results of microbial colonization in COPD.

The results of FEV_1% predicted were compared to the incidence of colonization for each potentially pathogenic microbial species. The data are represented by the mean± SEM. Significant differences were found between non-pathogen isolated, chronic and occasionally infected categories. (np indicates no patients were analysed,1p indicates only 1 patient was analysed and no error bars are shown).

4.2.6 Lung function in BR exacerbated patients and healthy volunteers

BR exacerbating patients were classified into three subgroups according to the Bronchiectasis Severity Index (BSI) (see Appendix11) as either <3 exacerbations or >3 exacerbations per year, or hospitalised. The FEV_1% predicted was assessed in these exacerbation groups and compared to healthy volunteers (Figure 4.6). Significant lung function decline was observed among patients with different scores for exacerbation compared to healthy volunteers ($p=0.006$). In addition, the one-way ANOVA showed high statistical significance ($p<0.0001$).

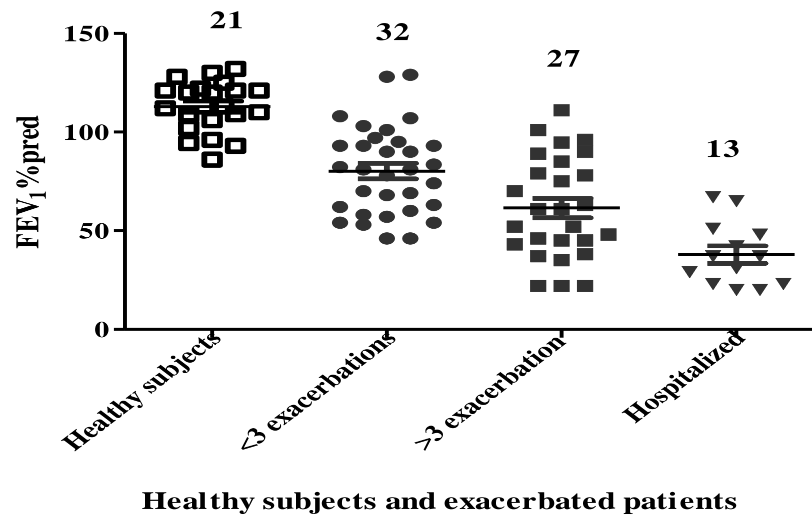


Figure 4.6: FEV_1% predicted assessment was tested in variously-exacerbated BR patients compared to healthy subjects.
Data are represented by mean± SEM. (The number in each group is indicated).

4.2.7 Antibody response in exacerbated patients with bronchiectasis

IgG titres against the main bacteria associated with lung function decline, including *P. aeruginosa*, *S. maltophilia* and *M. catarrhalis*, were compared for BR patients with different exacerbation scores in order to see if there is a significant difference in the antibody titres between these groups and whether or not antibody responses may associate with disease severity and exacerbation. Figure 4.7 shows that significant differences between the >3 exacerbation and hospitalised (p=0.037) and between the >3 and <3 groups (p=0.040) against *P. aeruginosa* were seen. Similarly, significant differences were found between >3 and <3 exacerbations (p=0.010 and p=0.040) against *M. catarrhalis* and *S. maltophilia* respectively. The results indicate that the antibody could be a biomarker for disease severity and exacerbation.

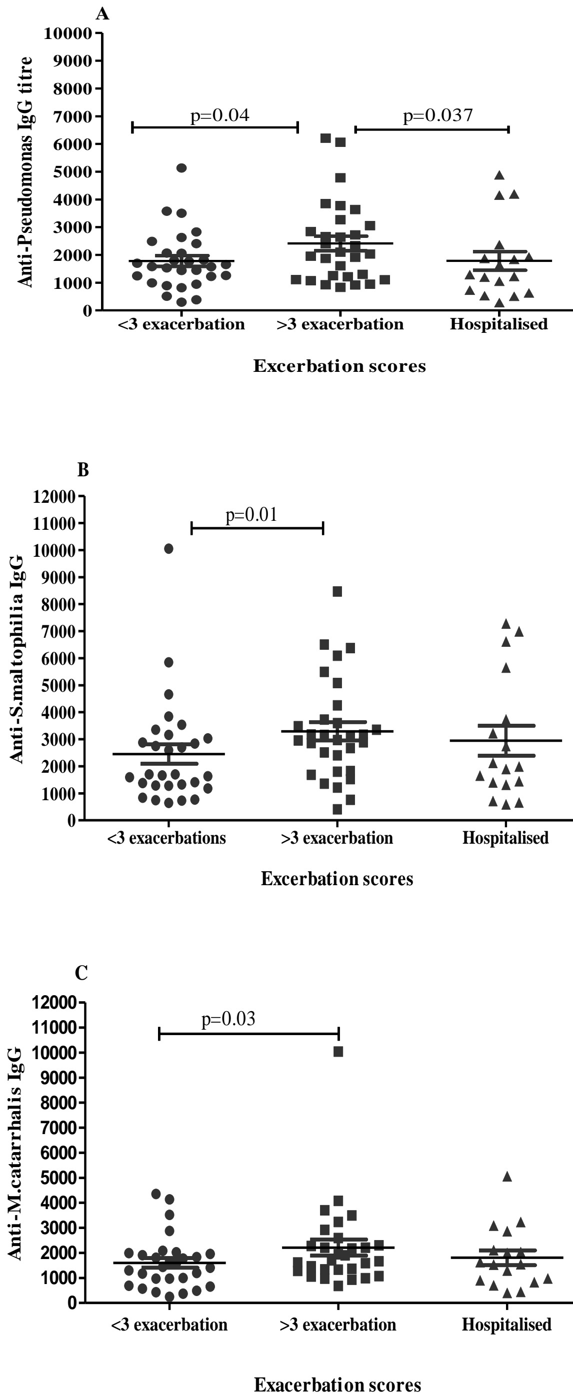


Figure 4.7 (A-C): IgG titres against (A) *P. aeruginosa*; (B) *S. maltophilia* and (C) *M. catarrhalis* compared for each exacerbation score.

A significant difference was found between > 3 and < 3 exacerbations against these antigens. A significant difference was also found between >3 and hospitalised against *P. aeruginosa*. The Mann-Whitney test was used to obtain p-values. Data are represented by mean ±SEM.

4.2.8 Correlation between antibody responses and lung functions in BR and COPD patients

IgG titre against *P. aeruginosa*, *S. maltophilia* and *M. catarrhalis* was plotted against $FEV_1\%$ predicted to see if there is a significant correlation between antibody response and deterioration of lung function, and whether or not immune response can be a biomarker to predict lung function decline. The results show no significant correlation between antibody response and lung functions in the BR and COPD groups (Figures 4.8 and 4.9 (A-C)).

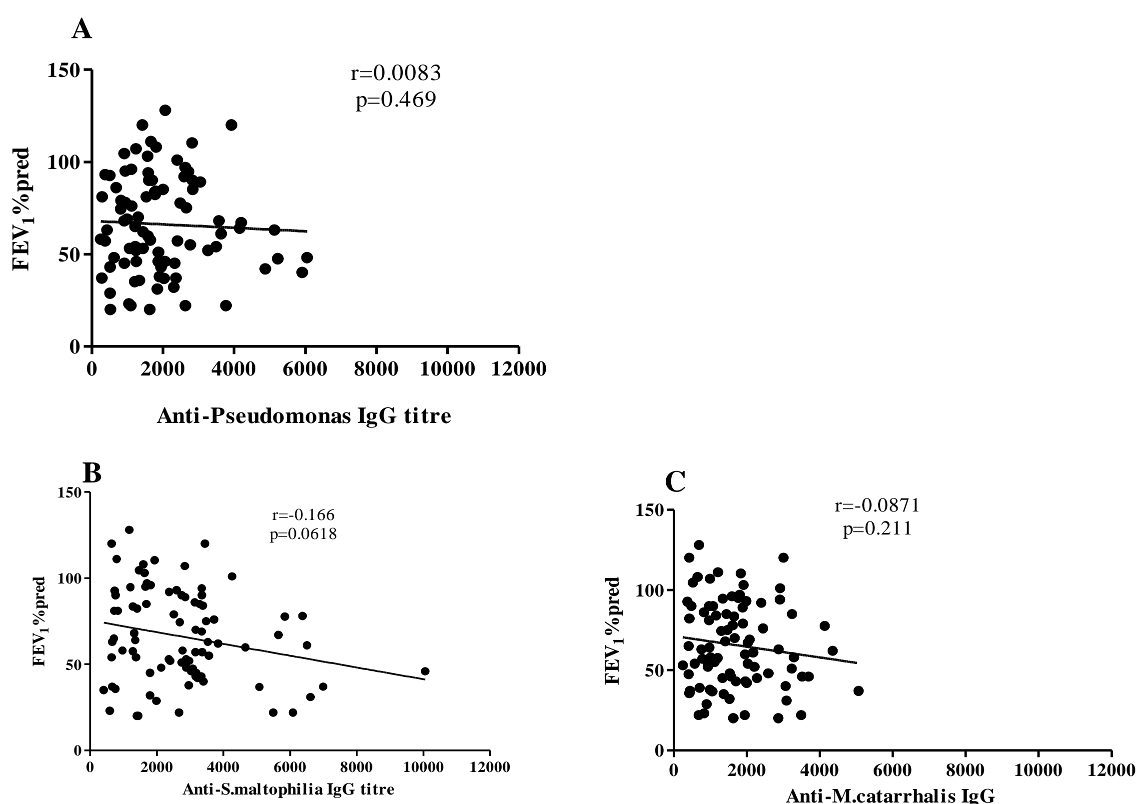


Figure 4.8 (A-C): Spearman correlation analysis for BR patients of IgG titre and $FEV_1\%$ predicted.

(A) IgG response against *P. aeruginosa*; (B) IgG titre against *S. maltophilia* and (C) IgG titre against *M. catarrhalis*. No significant differences were found ($p>0.05$).

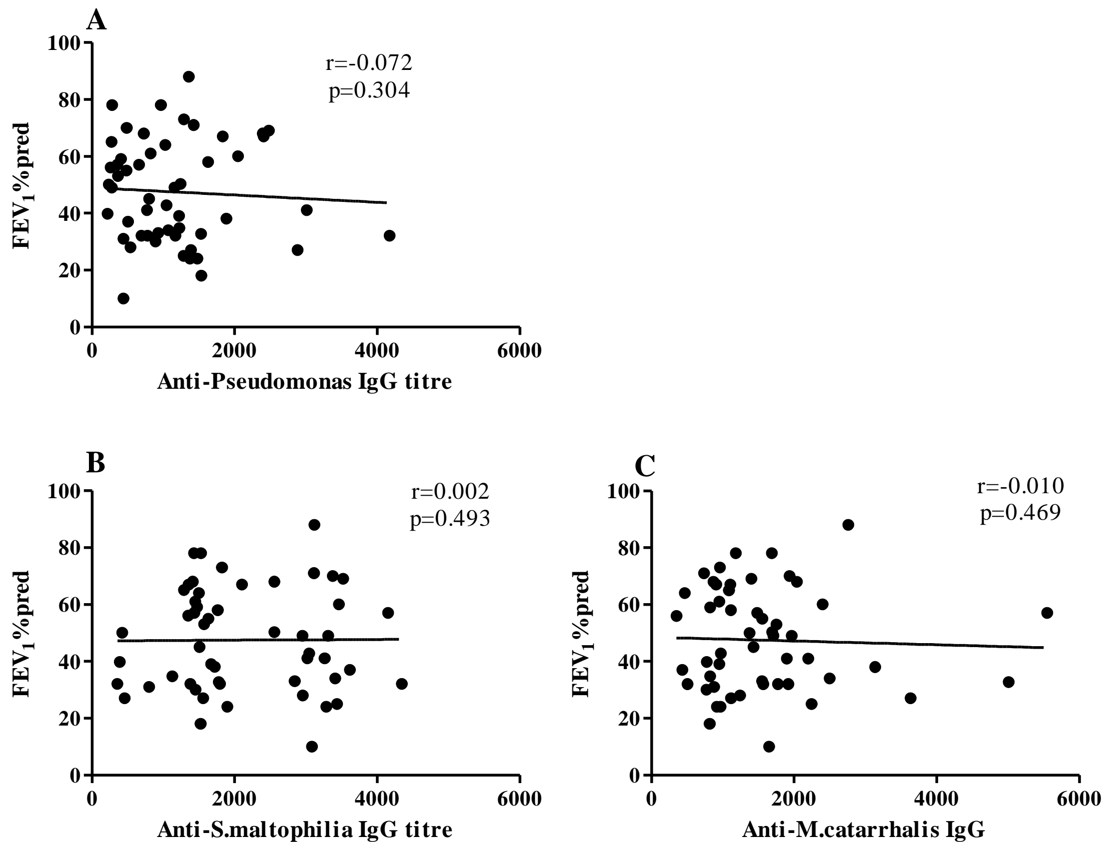


Figure 4.2(A-C): Spearman correlation analysis for COPD patients of IgG titre and FEV_1% predicted.

(A) IgG response against *P. aeruginosa*; (B) IgG titre against S. maltophilia; and (C) IgG titre against M. catarrhalis. No significant differences were found (p>0.05).

4.3 Discussion

In this study, it was found that NT*H. influenzae* is the most common pathogen isolated from BR and COPD patients (Figure 4.1). This finding agrees with those of previous studies found that it is the most common pathogen isolated from patients with bronchiectasis (Angrill *et al.*, 2002, King *et al.*, 2007, Pasteur *et al.*, 2000), followed in frequency by *P. aeruginosa* for patients with BR and *M. catarrhalis* of patients with COPD. *H. influenzae* can stimulate mucus production, adhere in the submucosa, invade cells and damage the epithelium of the airway. This bacterium may produce biofilms for protection from antibody and immune cells, resulting in colonization in the airways (King, 2011). A previous study of children with CF bronchiectasis showed that there was an inverse correlation between *P. aeruginosa* and

H. influenzae in microbial analysis suggesting that *P. aeruginosa* may inhibit the growth of *H. influenzae* (Klepac-Ceraj *et al.*, 2010). Therefore, the interaction between these microorganisms needs more investigation in patients with lung disease.

4.3.1 Microbial infections and IgG titre

In this study, IgG titres were investigated in BR and COPD patients and analysed based on microbial isolation as shown in Table 4.2. Those currently chronically colonized by different pathogens showed higher antibody titre among patients with bronchiectasis. More specifically, there were significantly higher antibody titres in patients who had current colonization compared to being non-colonized for *P. aeruginosa* and *S. maltophilia* ($p<0.001$ and 0.036 respectively). Significant differences were also observed between non-colonized patients and those with chronic previously category ($p=0.001$) as well as patients occasionally colonized and those with previous chronic colonization ($p=0.003$) for *P. aeruginosa*.

It has been shown that increased antibody titres are correlated with *P. aeruginosa* colonization in patients with cystic fibrosis (Brett *et al.*, 1987, Brett *et al.*, 1986, Cordon *et al.*, 1992). Bacterial infections promote the inflammatory response and increase the specific antibody response, which may be unable to clear the infections but may form immune complexes and activate inflammatory mediators that lead to lung damage (Caballero *et al.*, 2001). Increased antibody titre may perhaps be useful as an indicator of microbial infections, especially for diagnosing young children who are unable to expectorate sputum, and for determining the need for antibiotic treatment (Cordon *et al.*, 1992). These findings support those of a previous study conducted to detect anti-Pseudomonas antibodies in patients with non-cystic fibrosis bronchiectasis by Western blot (Caballero *et al.*, 2001). Significant differences were also observed among COPD patients between non-colonized and those who

were currently colonised by *M. catarrhalis* ($p=0.04$). Interestingly, COPD patients who had no NT*H. Influenzae* isolation had significantly higher antibody titre ($p=0.04$) compared to those with occasional isolation, although the antibody titre was also higher in patients chronically colonized by this bacterium. Likewise, detectable antibody against *S. pneumoniae* was also observed among the patients with no isolation of this bacterium in the corresponding sputum. Increased antibody titre with the negative culture isolation seen in patients with COPD may be due to the specific antibiotic treatment, especially if these patients had chronic exacerbations and worsening lung functions (Cordon *et al.*, 1992). In addition, existing antibodies may be providing immune protection in this group. An elevated level of antibody observed in patients with COPD against *P. aeruginosa* and *S. maltophilia* in those who had occasional isolation of these bacteria may be an indication of the degree of bacterial exposure.

4.3.2 Microbial infections and deterioration of lung function

Concerning the relationship between bacterial infection and the deterioration of pulmonary functions in both BR and COPD clinical groups, the results demonstrated significantly lower lung function ($p= 0.02$) with those currently colonized by *P. aeruginosa* and *M. catarrhalis* than non-colonized in BR patients. Similarly, a significant reduction was seen in FEV_1% ($p=0.006$) in patients chronically colonized by *S. maltophilia* than non-colonised patients. Lower FEV_1% predicted was also observed among BR patients at different categories of chronic colonization by *S. pneumoniae*, *A. fumigatus* and NT*H. influenzae*, but no significant differences could be detected. A significant decrease in FEV_1 % predicted was seen in COPD patients chronically colonized by *P. aeruginosa* ($p= 0.019$). In contrast, a significant decrease in FEV_1% predicted was found in COPD patients who had no *S. pneumoniae* isolation in the corresponding sputum sample, suggesting that other factors such as quality

of life, a previous history of exacerbation, and childhood pneumonia may be associated with lung function decline (Kim *et al.*, 2016).

These findings corroborate those of previous studies conducted on patients with bronchiectasis and the role of Pseudomonas in progressive lung function decline (Davies *et al.*, 2006, King *et al.*, 2007, Ma *et al.*, 2015, Martinez-Garcia *et al.*, 2007). Colonization with *P. aeruginosa* in non-cystic fibrosis bronchiectasis is strongly associated with an accelerated decline of lung function, and even antibiotic treatment for an exacerbation shows no improvement in FEV_1 and FVC compared to other microorganisms (Murray *et al.*, 2009). Impairment of lung functioning is associated with pathogenic bacterial load and species changes, and the best-preserved lung function is most likely to have no pathogenic bacteria isolated (King, 2009, Marin *et al.*, 2010). The Global Initiative for Chronic Obstructive Lung Disease (GOLD) stated that the severity of airflow obstruction should be categorised as follows: patients with stage I: mild 80% or above, stage II: moderate 50-79%, stage III: severe 30-49%, and stage IV: very severe below 30% (Decramer *et al.*, 2012). Thus, at the time of sampling in this study, BR patients colonized by *P. aeruginosa*, *S. pneumoniae*, *A. fumigatus*, and NT*H. influenze* presented with GOLD stage II, whereas patients who had current chronic colonisation by *M. catarrhalis* and *S. maltophilia* presented at GOLD stage III.

Furthermore, COPD patients who had current colonization by *P. aeruginosa* and *A. fumigatus* as well as occasional colonization by *S. maltophilia* and previous colonization by *M. catarrhalis* presented with GOLD stage IV (i.e. very severe below 30%). The data suggests that bacterial infections in patients with BR and COPD are the main factor associated with airflow obstruction.

4.3.3 Lung function and exacerbated BR patients

When lung function (FEV_1 % predicted) was assessed in the exacerbated patients with different scores, the deterioration of lung function was associated with exacerbation frequency and a considerable variation in FEV_1 was observed during the course of exacerbation. It has been shown that recurrent exacerbation strongly contributes to lung function decline in patients with CF (Amadori *et al.*, 2009, Sanders *et al.*, 2011).

4.3.4 Antibody responses and exacerbations

IgG titre was also investigated in exacerbated patients at different scores to find out if the higher antibody titres correlated with exacerbation. The findings showed that patients with >3 exacerbations have significantly higher antibody titre against *P. aeruginosa*, *M. catarrhalis* and *S. maltophilia* compared to patients with <3 exacerbations (p=0.04, 0.03 and 0.01 respectively). A significant difference was also seen versus *P. aeruginosa* in >3 exacerbations and hospitalised patients (p=0.037). It is likely that microbial infections stimulate the inflammatory and antibody responses resulting in destruction of lung tissues, and subsequent deterioration of lung function and induction of exacerbation. Thus, occurrence of exacerbation is a result of the vicious cycle of inflammation rather than the microbe itself (Perera *et al.*, 2007). Nevertheless, data in non-CFBR patients remain inadequate (Brett *et al.*, 1986). Thus, further studies to understand the mechanism of microbial infection and inflammatory response (particularly T cell responses) and their role in inducing exacerbations are required. In contrast, no correlation between IgG titre and lung function was observed in patients with BR and COPD. T cell responsiveness is the subject of the next chapter.

5 Conclusion

In this study, culture-based methods enabled the characterization of the lung microbiome in the lower airways of BR and COPD cohorts. The findings reveal that the lower airways of BR and COPD patients are colonized by poly-microbial communities that perpetuate immunological responses. In agreement with previous studies, it has been show that NT. *H. influenzae* was the main organism isolated from the sputum of patients with BR and COPD, followed by *P. aeruginosa* in BR and *M. catarrhalis* in COPD. Measurement of antibody against lung pathogens may be helpful in differentiating between early infection and chronic colonization as well as being useful in monitoring the progress of infection. The results demonstrated that microbial infection induces airflow obstruction and is associated with recurrent exacerbations. In addition, anti-microbial antibody titres are also increased in patients with persistent exacerbation. Hence, antibody titre can be a useful indication of disease severity and exacerbations in patients with BR but not sufficient for FEV_1% predicted.

5 Chapter Five: Measurement and Significance of Anti-microbial T Cell Responses in Chronic Obstructive Lung Diseases

Abstract

Aim: T cell responses against microbial infections are thought to contribute to the pathogenesis of chronic obstructive pulmonary diseases, since infiltrating T cells have been found in the lamina propria and lung epithelium of patients with BR and COPD. Nevertheless, they remain poorly understood. The aim of this chapter is to investigate the nature of T cell responses against lung microbes as a potential clinical biomarker of disease progression.

Methods: Peripheral blood mononuclear cells (PBMCs) were separated from heparinised blood collected from BR (n=119), COPD (n=58) and asthmatic (n=14) patients. Healthy volunteers (n=28) were also included in this study. PBMCs were used in ELIspot to investigate the magnitude of T cells in the form of IFNγ responding to lysates and peptides obtained from bacteria, viruses and fungi.

Results: Lower T cell responses were observed among patients with BR, COPD and asthma compared to healthy volunteers against most antigens derived from bacteria, viruses and fungi that colonize the lung. T cell responses were not associated with microbial infections or lung function, but it is associated with exacerbation for *M. catarrhalis* in patients with BR.

Conclusion: This study demonstrated the reduced response of IFNγ–producing T cells to lung microbial antigens in patients with chronic obstructive lung diseases. This is likely to be a result of T cell polarization or anergic from the burden of microbial infections. The measurement of T cell responses may therefore have minimal use as a biomarker in these lung diseases.

5.1 Background

Patients with underlying pulmonary diseases such as BR and COPD are characterized by persistent intense bacterial infection which may accelerate their disease severity (Seemungal *et al.*, 2001, Tan *et al.*, 2003). The process of adaptive host defence and active cell-mediated immunity in health and disease conditions against many such pathogens occurs mainly through antigen-specific $CD4^+$ T cells. These cells are important in the adaptive immune response by providing help for B cells to produce antibody and switch isotype and to support virus-specific $CD8^+$ cytotoxic T lymphocytes differentiation to combat intracellular infections, as well as having different effector functions; for example, via IFNγ secretion (de Bree *et al.*, 2007). Therefore, the generation of robust Th_1-type $CD4^+$ T cell responses are an essential process to provide an effective immune response against bacterial infection (Kim *et al.*, 2016). After acute infection, memory T cells are generated that can rapidly proliferate and perform effector functions upon reinfection. However, in some circumstances during chronic infections, T cells become exhausted, which is defined by the ineffective function and expression of various inhibitory receptors (Crawford *et al.*, 2014). A great number of T cell infiltrates, mainly $CD4^+$ and $CD8^+$, within the lung mucosa of COPD and BR as a result of microbial stimulations, have been observed (Boyton *et al.*, 2013, Gaga *et al.*, 1998). Several studies focusing on the protective role of T cell responses against bacterial and viral infections have found that IFNγ is crucial but not sufficient for protection against infection (Caccamo *et al.*, 2010). Furthermore, IFN-γ and IL-2 production, as well as the capacity for $CD4^+$and $CD8^+$ proliferation, is central to immune protection. Indeed, multifunctional $CD4^+$ and $CD8^+$ T cells promote an effective immune response for microbial clearance (Caccamo *et al.*, 2010).

The aims of this chapter are to evaluate CD4$^+$ T cell responses against lung microbial antigens, to investigate the magnitude of T cell responses in patients with BR, COPD and asthmatics compared to healthy volunteers, and to determine whether or not T cell responses correlate with microbial colonization, lung functions and exacerbation status.

5.2 Results

5.2.1 Characterization of T cell responses to antigens in the study

In order to establish that the response of T cells in the form of IFNγ is specifically made by CD4$^+$ T cells and not innate cells, experiments were carried out to determine the effects of depleting CD4$^+$ cells on IFNγ ELIspot responses against selected antigens, as described in section 2.8.4. The findings showed decreased responses against *P. aeruginosa, H. influenzae*, *M. tuberculosis, A.fumigatus* and *M. pneumoniae* antigens following the depletion of CD4$^+$T cells, indicating that CD4$^+$ T cells are the predominant cell type responding to these antigens. Responses against RSV were only minimally reduced.

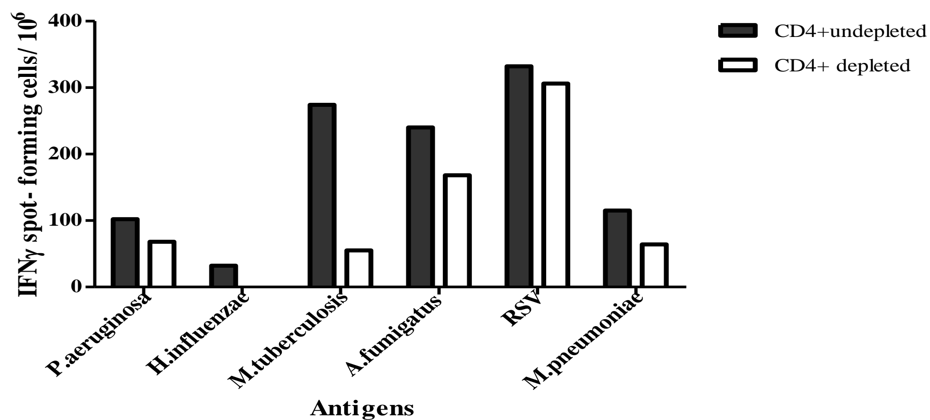


Figure 5.1: IFNγ ELIspot response of T cells to a panel of antigens in healthy controls. Data is presented as spot-forming cells per 10^6 PBMC with the medium only background subtracted. Decreased response of T cells was found following CD4$^+$ depletion. The experiment was repeated three times with similar results obtained.

5.2.2 Comparison of IFNγ ELIspot between fresh and frozen cells

Since the ELIspot assays were carried out on frozen cells, we wished to see if the freezing itself affects cell viability and functions, therefore, fresh PBMCs were compared to frozen cells obtained from a healthy control as described in section 2.8.5. The findings demonstrated that fresh and frozen cells gave mainly comparable levels of IFNγ ELIspot response, indicating that the PBMCs were stable after freezing. Notable exceptions were for *M. tuberculosis* and *H. influenza,* but even these responses were not lost entirely due to freezing.

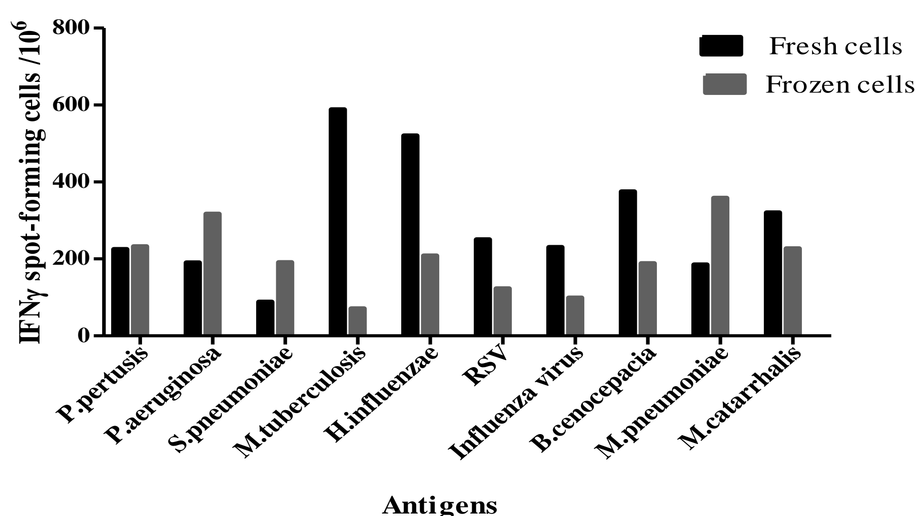


Figure 5.2: IFNγ ELIspot of fresh and frozen cells. Fresh PBMC from a healthy volunteer was compared to frozen cells.

Data are presented as spot-forming cells in 10^6 with the medium only background subtracted. The experiment was repeated twice with similar results obtained.

5.2.3 Inhibition with Anti-class II antibody

To further show the $CD4^+$ T cell dependence of ELIspot responses, anti-class II antibody was utilised to inhibit antigen presentation to $CD4^+$ T cells as described in section 2.8.6. The results showed an obvious inhibition of T cell responses against most of the antigens, which means that anti-class II antibody blocked the presentation of antigen to $CD4^+$ T cells by antigen-presenting cells. The exceptions were RSV, *B. cenocepacia* and *M. pneumoniae*. It

is likely that $CD4^+$ was therefore the main T cell responding to these antigens in the form of IFNγ.

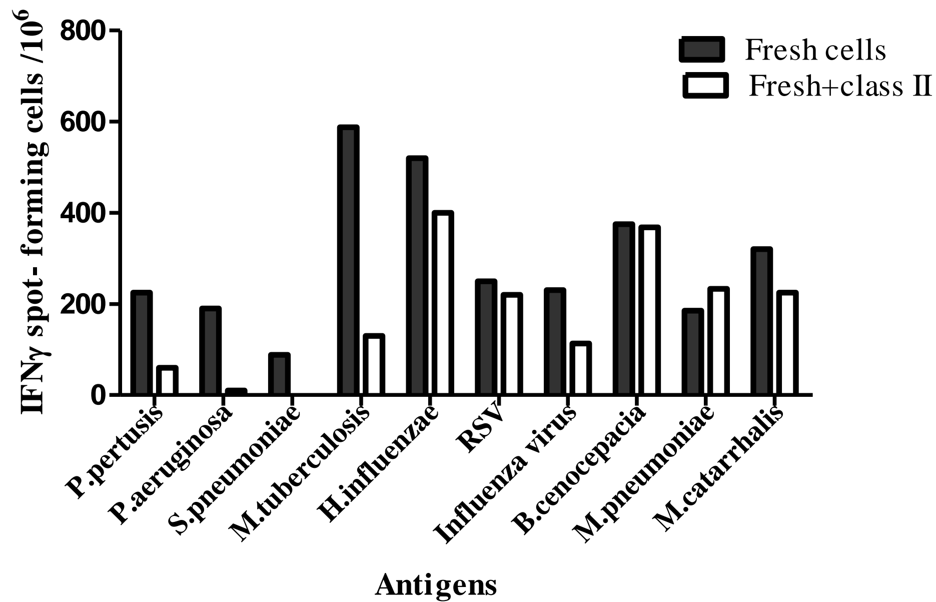


Figure 5.3: IFNγ ELIspot with class II inhibition of T cells.
Class II antibody was used to inhibit antigen presentation to $CD4^+$ T cells. Data are presented as spot-forming cells in 10^6 with the medium only background subtracted. The experiment was repeated twice with similar results obtained.

5.2.4 T cell responses in bronchiectasis (BR) patients compared to healthy volunteers (HV)

T cell responses were tested against the panel of antigens comprising lysates, extracts and peptides of bacteria, viruses or fungi, as well as anti-CD3 and PHA utilised as positive controls (see Table 2.5). The responses were measured by IFNγ ELIspot in BR patients and HV as described in section 2.8.2. The results (Figure5.4) showed significantly lower levels of T cell responses indicated by IFNγ production in BR patients compared to HV against *P. aeruginosa* (p=0.002), *S. pneumoniae* (p=0.001), *A. fumigatus* (p=0.03), *B. pertussis* (p=0.01) and *M. tuberculosis* (p=0.002). Similarly, the responses of T cells were also lower for RSV, *B. cepacia*, and *M. pneumoniae* but no significant differences were found here. Conversely, patients with BR displayed higher levels of IFNγ responses compared to HV

against Cytomegalovirus, but the increase was not significant (p=0.055). Responses to the 2 positive controls, anti-CD3 and PHA, were comparable between the BR patients and the HV. These findings suggest that chronic infections may reduce the specific response of T cells in patients with BR.

In this analysis, 11 BR patients having immunodeficiency (ID) were excluded (resulting group termed BR-ID). Comparison of the IFNγ response of the BR-ID group with the overall BR group including those with immunodeficiency, (BR+ID) and with healthy volunteers, (Figure 5.5) showed a significant difference between BR+ID and healthy volunteers (p=0.001) and a significant difference when comparing BR-ID and healthy volunteers (p=0.002). No significance differences were found between BR+ID and BR-ID (p=0.399).

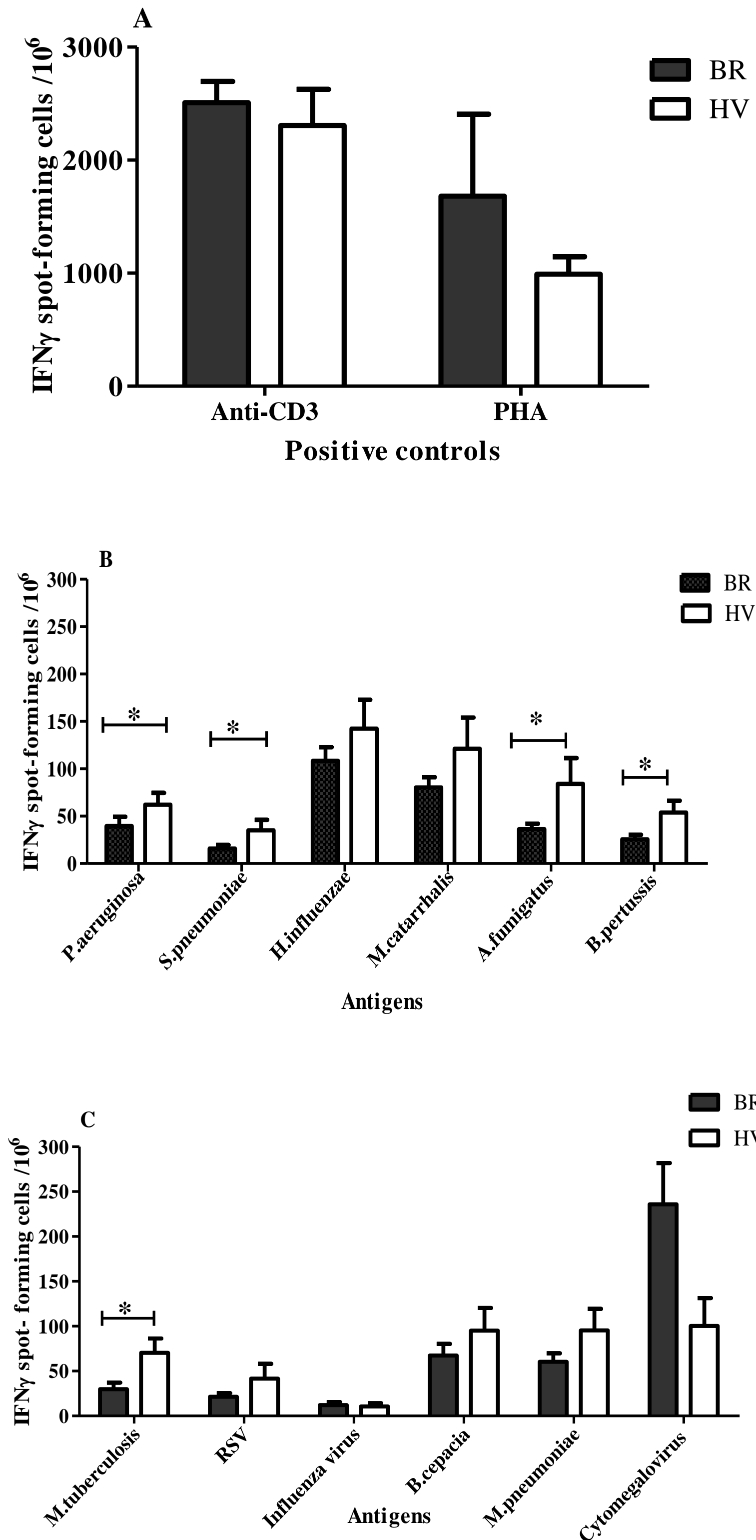


Figure 5.4(A-C): Measurement of IFNγ production followed stimulation with antigens in both BR patients and healthy volunteer (HV).

(A) positive controls stimulus, (B) and (C) microbial antigens. Data are shown as mean ± SEM. Statistical significance of these differences was analysed using the Mann-Whitney test. * $p<0.05$. n=103 for BR and n=27 for HV.

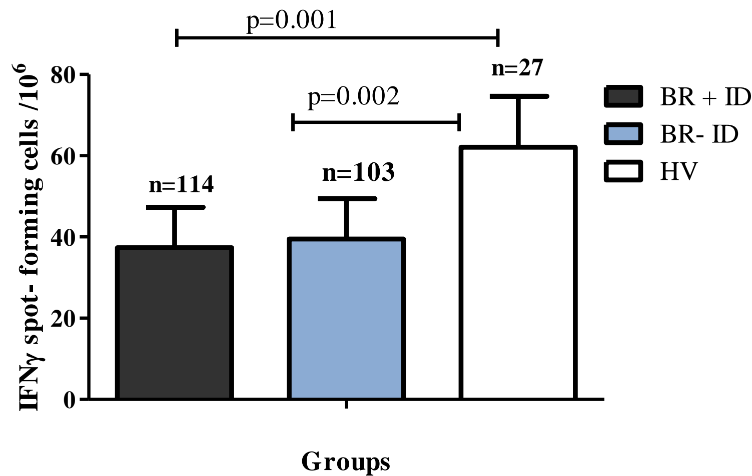


Figure 5.5: IFNγ production in response to *P. aeruginosa* in different groups of BR and healthy volunteer (HV).

All BR patients (BR+ID patients) and those with immunodeficiency excluded (BR-ID) were compared to healthy volunteers. Significant differences were observed between +/- immunodeficiency compared to healthy volunteers. Data are shown as mean ± SEM.

5.2.5 T cell responses in patients with chronic obstructive pulmonary disease (COPD) compared to healthy volunteers (HV)

The responses of T cells were also measured in COPD patients against the same antigens and positive controls as in the previous section (Figure5.6). Patients with COPD had significantly lower levels of IFNγ in response to *P. aeruginosa* (p=0.014) and *S. pneumoniae* (p=0.031) compared to healthy volunteers. Although the responses were lower against *H. influenzae*, *M. catarrhalis*, *A. fumigatus*, *B. pertussis*, RSV, *B. cepacia* and *M. pneumoniae* there were no significant differences. In contrast, higher levels of IFNγ versus *M. tuberculosis* and Cytomegalovirus were seen in COPD, but without statistical significance. Furthermore, positive controls anti-CD3 and PHA show no significant differences.

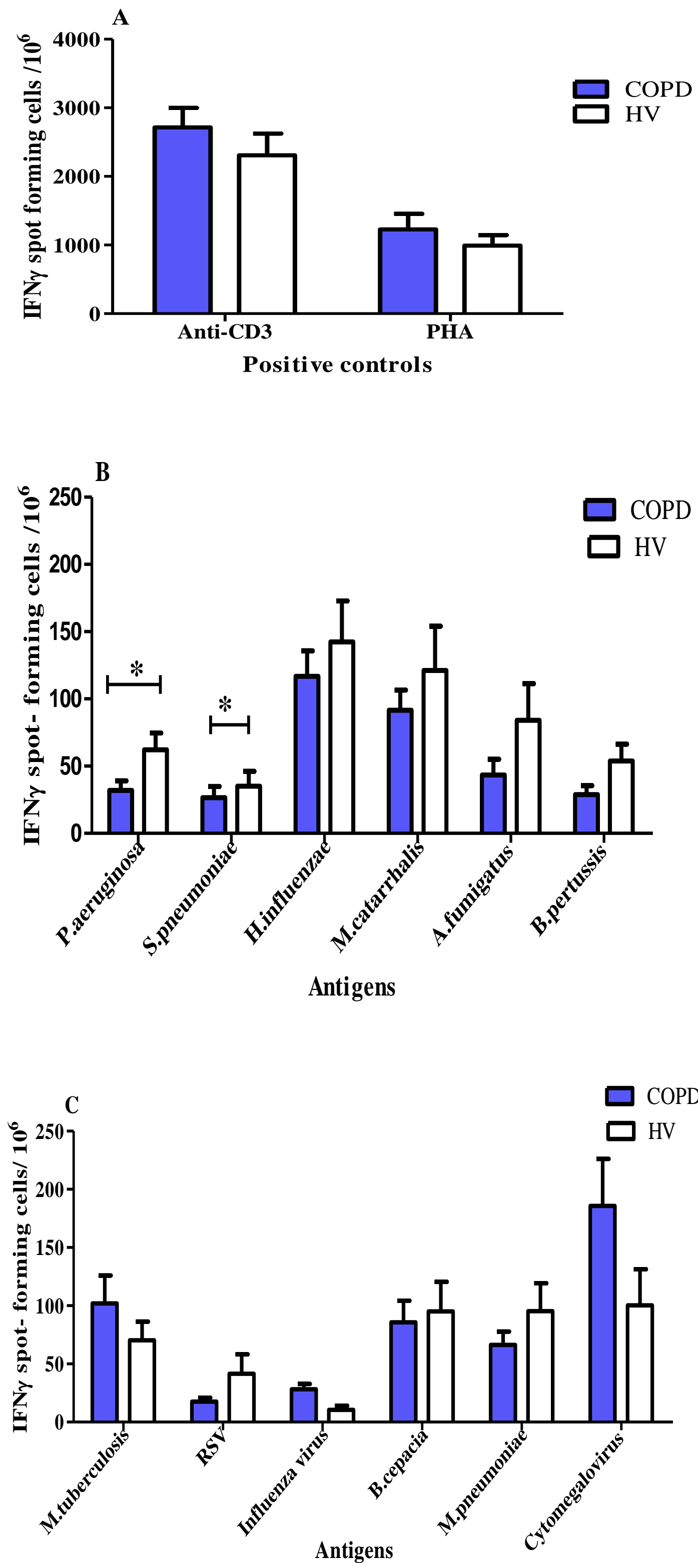
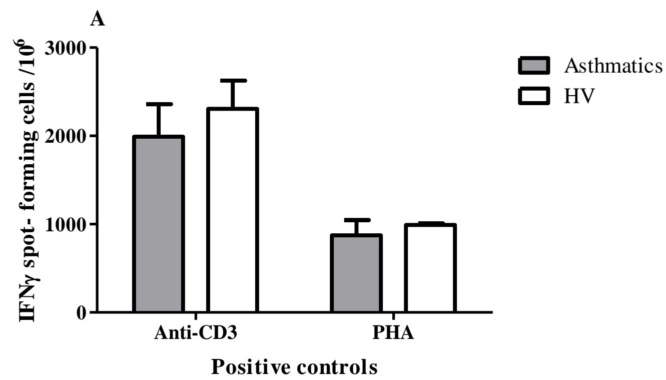


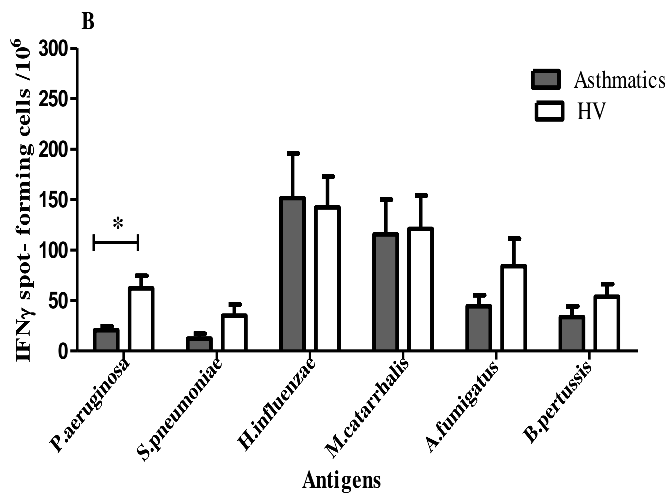
Figure 5.6(A-C): T cell responses in the form of IFNγ in COPD patients and healthy volunteers (HV).

Measurement of IFNγ production after PBMCs stimulation with lung antigens in COPD patients and HV. (A) positive controls stimulus; (B) and (C) microbial antigens. Data are shown as mean ± SEM. Statistical significance of these differences was analysed using the Mann-Whitney test. * $p<0.05$. n=57 for COPD and n= 27 for HV.

5.2.6 T cell responses in asthmatics compared to healthy volunteers (HV)

Investigation of T cell responses against the panel of lung antigens from bacteria, fungi and viruses as well as positive controls were also carried out in asthmatics and compared to healthy volunteers. The result showed that (Figure 5.7) asthmatics had significantly lower levels of IFNγ compared to HV against *P. aeruginosa* (p=0.040). No significant differences for other antigens were seen in this analysis.





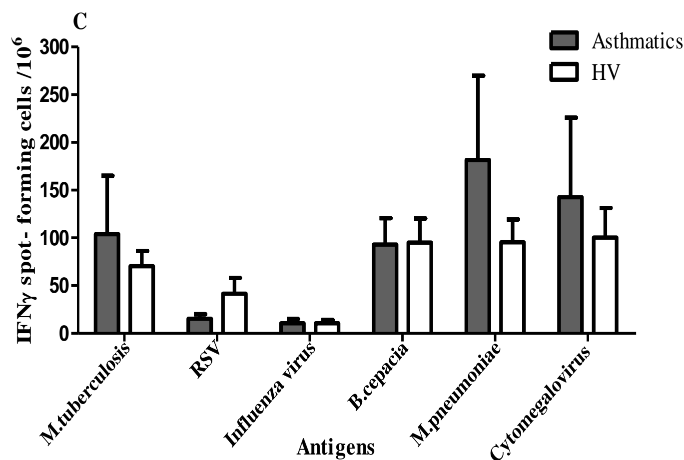


Figure 5.7(A-C): T cell responses in the form of IFNγ in asthma patients and healthy volunteers (HV).

Measurement of IFNγ production followed stimulation of PBMC with the panel of lung antigens in both asthmatics and HV :(A) positive controls stimulus, (B) and (C) microbial antigens. Data are shown as mean ± SEM. * $p<0.05$. n=14 for asthma and n=27 for HV.

5.2.7 T cell responses versus microbial colonization in BR and COPD

To investigate whether or not the responses of T cells are associated with microbial infections, IFNγ responses against each antigen were compared to the results of the incidence of microbial colonization, as classified in Table 4.2, for patients with BR and COPD. Figures 5.8 and 5.9 show that no significant differences were found in IFNγ T cell response for BR or COPD patients between groups who were non-colonized or colonized to differing degrees and by different pathogens: *P. aeruginosa*, *S. pneumoniae*, *H. influenzae*, *M. catarrhalis* and *A. fumigatus* ($p>0.05$ for each). The results suggest that occasional colonization resulted in larger T cell responses than non- or chronic colonization in patients with BR.

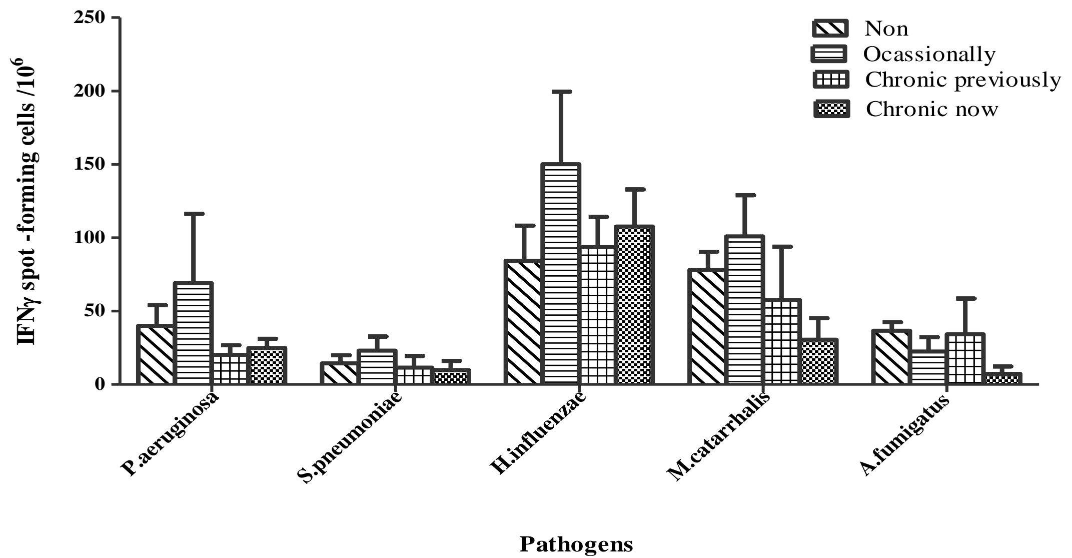


Figure 5.8: IFNγ T cell responses of BR patients compared to microbial colonization.
IFNγ response against each antigen was compared to the result of microbial colonization. Data are shown as mean± SEM. No significant differences were found.

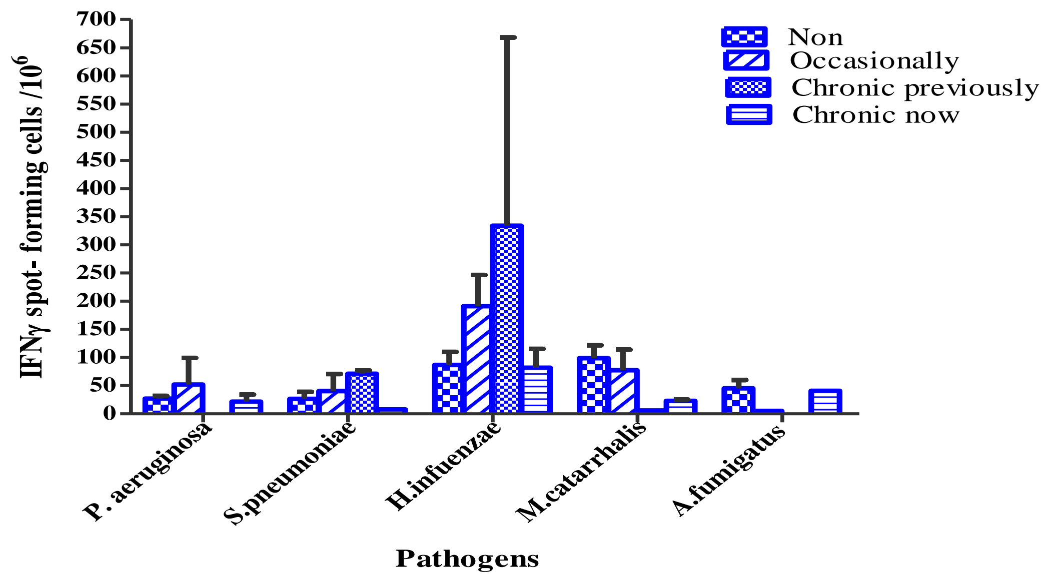


Figure 5.9: IFNγ T cell responses of COPD patients compared to microbial colonization.

IFNγ response against each antigen was compared to the result of microbial colonization. Data are shown as mean ± SEM. No significant differences were found.

5.2.8 T cell responses in exacerbated patients with bronchiectasis

IFNγ T cell responses against *P. aeruginosa* and *M. catarrhalis* were investigated in patients with various degrees of exacerbation (Figure 5.10). Only 75 out 86 patients were measured for T cell responses because seven ID patients were excluded and there were four missing samples. These patients were classified into three sub groups according to Bronchiectasis Severity Index (BSI): <3 exacerbations, or >3 exacerbations per year, or hospitalised. Significant differences were found when <3 and >3 were compared to hospitalised patients (p=0.021 and p= 0.049 respectively) in response to *M. catarrhalis*. However, no significant differences were observed between these groups against *P. aeruginosa*.

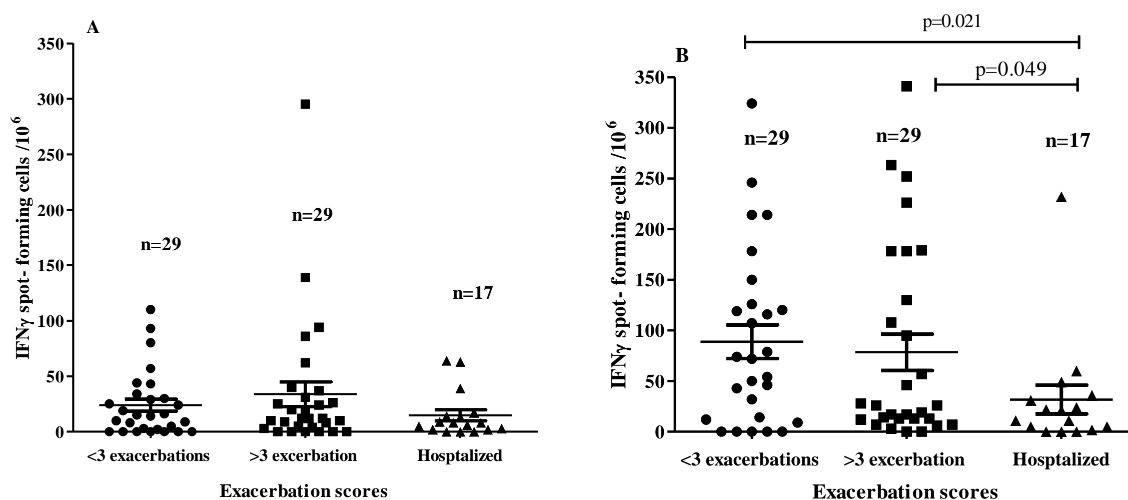


Figure 5.10(A-B): IFNγ ELIspot against (A) *P. aeruginosa* and (B) *M. catarrhalis* was compared for each exacerbation score.
Significant differences were observed in T cell responses among these categories to *M. catarrhalis*. Data are presented as mean± SEM. The number in each group is indicated.

5.2.9 Correlation between T cell responses and lung function in BR and COPD patients

T cell responses to *P. aeruginosa* and *M. catarrhalis* were plotted against FEV_1% predicted to find out if they are significantly correlated and to determine if the responses of T cells can be a prognostic marker for lung function deterioration in patients with BR and COPD. The

results in Figures 5.11 and 5.12 show no significant correlation between the T cell responsiveness and lung function decline. This indicates that T cell response is not a sufficient marker for lung function decline.

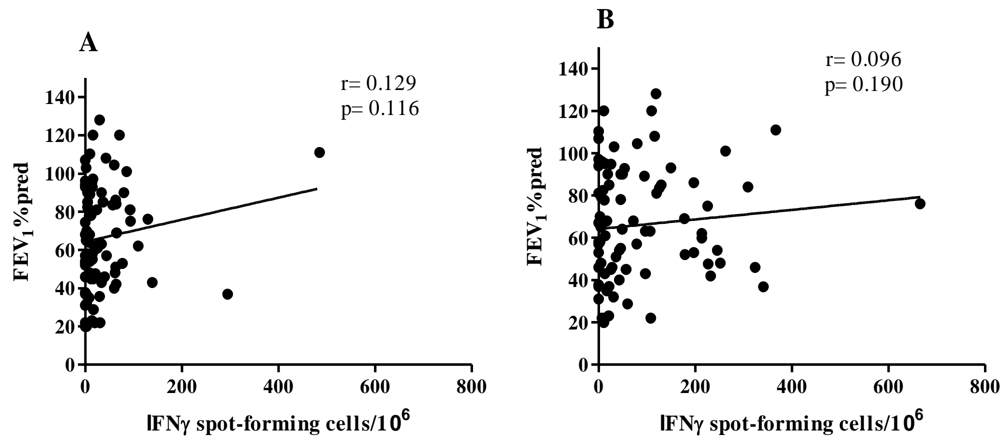


Figure 5.11(A-B): Relationship between lung function and T cell responses in patients with BR.

(A)*P. aeruginosa*; (B) *M. catarrhalis*. No significant correlations were found (Spearman correlation and Mann-Whitney analysis was used).

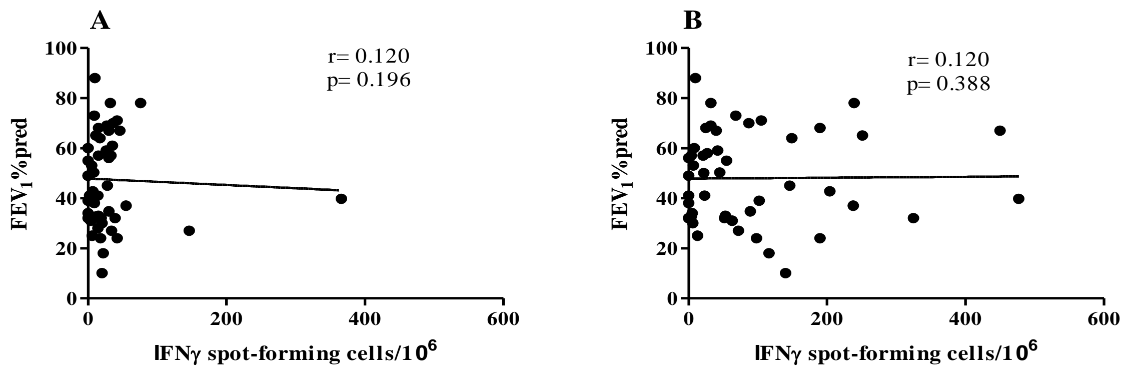


Figure 5.12(A-B): Relationship between lung function and T cell responses in patients with COPD.

(A) *P. aeruginosa*; (B*) M. catarrhalis*. No significant correlations were found (Spearman correlation and Mann-Whitney analysis was used).

5.2.10 Relationship between antibody and T cell responses

To determine whether the responses of antibody and T cells is correlated, Spearman corelation analysis was also carried out on data for patients and healthy volunteers included in the study. The results shown in Table 5.1 demonstrate a significant negative correlation between antibody and T cell responsiveness in COPD patients against *M. catarrhalis* (p=0.005). Similarly, significant negative correlations were found among healthy volunteers in response to *M. catarrhalis* (p=0.030) and *H. influenzae* (p=0.017). Only negative correlations, but without significance, were seen in patients with COPD and BR against *S. pneumoniae*. Healthy volunteers also showed a negative but insignificant correlation against *P. aeruginosa*.

Table 5.1. Spearman correlation analyses of antibody and T cell responses against selected antigens among patients and healthy volunteers included in the study.

	BR		**COPD**		**Asthma**		**HV**	
Antigens	**r**	**P-value**	**r**	**P-value**	**r**	**P-value**	**r**	**P-value**
P.aeruginosa	0.0687	0.385	0.039	0.772	0.00	1.000	-0.135	0.502
S.pneumoniae	-0.118	0.244	-0.221	0.099	0.047	0.874	0.359	0.066
A.fumigatus	0.015	0.895	0.005	0.715	0.136	0.689	0.012	0.957
M.catarrhalis	0.064	0.531	-0.378	**0.005**	0.056	0.863	-0.419	**0.030**
H.influenzae	0.139	0.165	-0.166	0.221	0.152	0.604	-0.456	**0.017**

5.2.11 Measurement of cytokines by MSD of T cell-stimulation culture supernatants

An analysis was conducted to assess the concentrations of various cytokines in the culture supernatant of antigen-stimulated T cells, in BR patients and healthy volunteers, including IFNγ, IL-2, IL-4, IL-5, IL-10, IL-13, IL-12 and-IL-17. PBMCs were stimulated with *P. aeruginosa* (PSA) or *H. influenzae* (Hi) antigens overnight and the supernatants were collected as described in section 2.10. The level of each cytokine produced (ng/ml) in

response to both stimuli are shown in Figure 5.13. No significant differences in IFNγ production were observed between patients and healthy volunteers in response to PSA (p=0.226) and Hi (p=0.419). The other cytokine levels were weak and no significant differences were detected between patients and healthy volunteers. Moreover, IFNγ levels were plotted against ELIspot IFNγ spot-forming cells/10^6 from the same individuals to see if they are significantly correlated (Figure 5.14). A significant negative correlation was observed for BR patients with respect to IFNγ spot-forming cells and MSD IFNγ in response to *H. influenzae* antigen (p=0.044). IFNγ spot-forming cells were also analysed against the other cytokines measured in this experiment and the result are shown in Table 5.2. A significant negative correlation was also observed for IFNγ spot- forming cells and MSD IL-4 in response to *P. aeruginosa* in BR patients (p=0.049). Furthermore, a significant negative correlation was seen in BR patients against *H. influenzae* for IL-2 (p=0.003) and IL-12 (p=0.035).

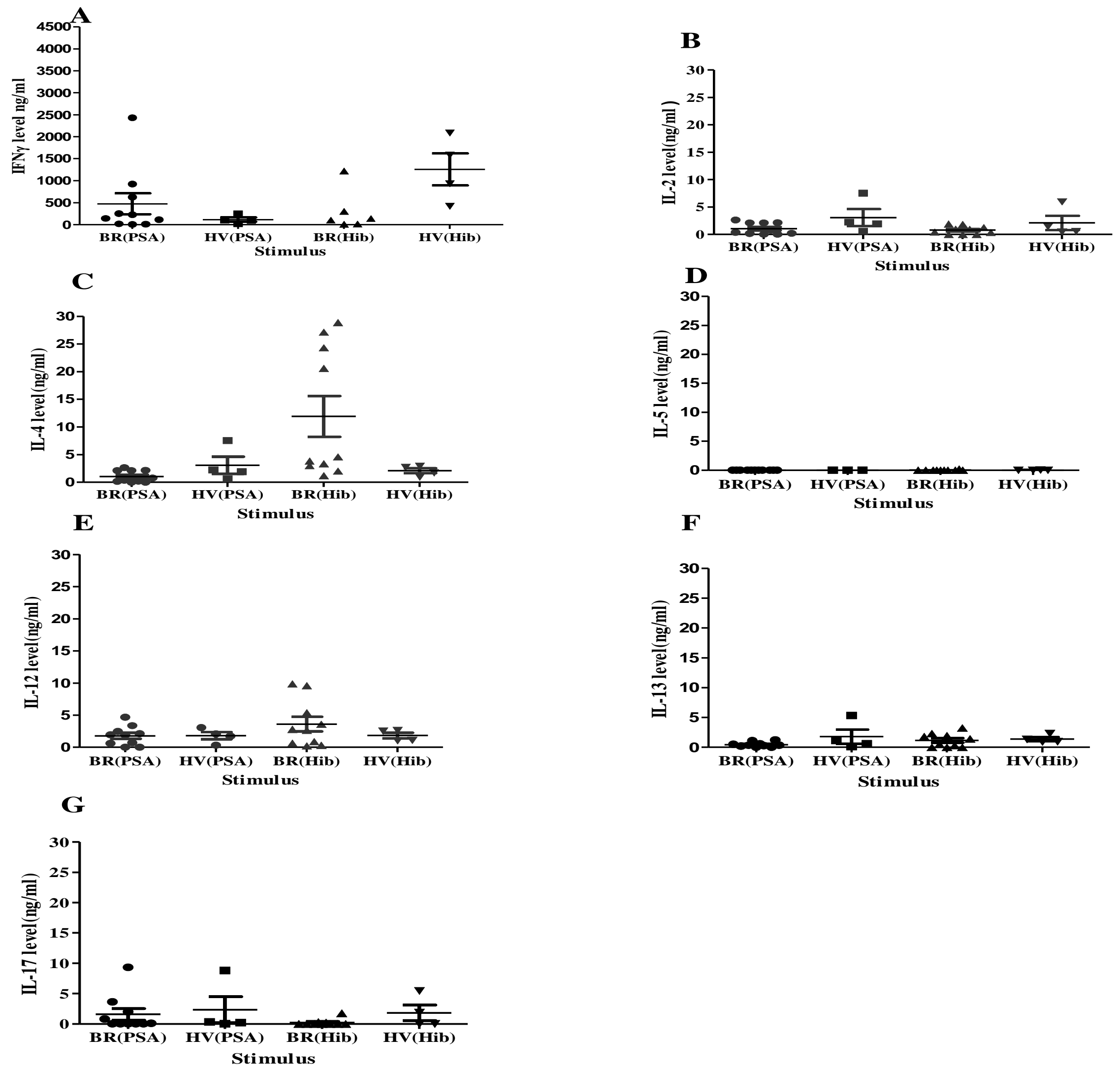


Figure 5.13(A.G): Cytokines levels (ng/ml) in BR patients and HV following stimulation with *P.aeruginosa* and *H.influenzae.*

(A) Production of IFNγ;(B) IL–2;(C) IL-4 ; (D) IL-5 ; (E) IL-12 ; (F) IL-13 ; and (G) IL-17. Data are represented as mean± SEM. n=10 for BR and n=4 for HV.

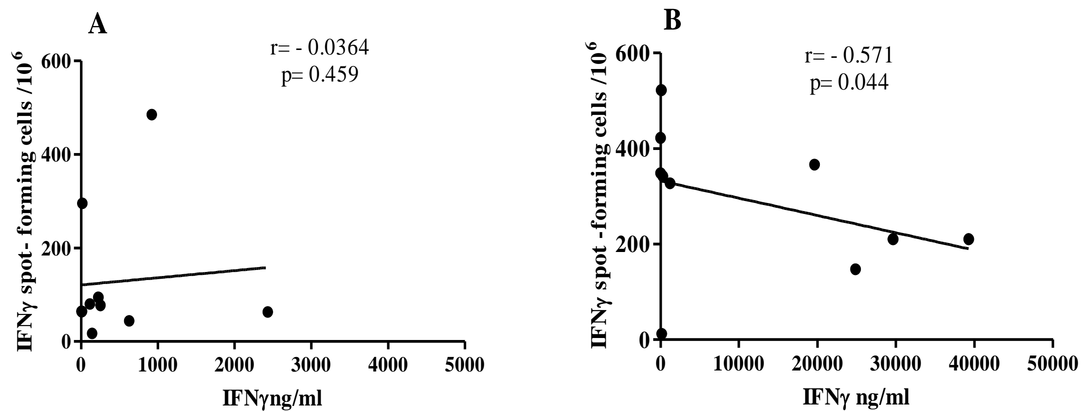


Figure 5.14 (A-B): Spearman correlation analysis for BR patients of IFNγ spot-forming cells per 106 PBMC and IFNγ level measured by MSD.

(A) *P. aeruginosa*; and (B) *H. influenzae*. A significant negative correlation was found between IFNγ ELIspot and the level of IFNγ measured by MSD in response to the *H. influenzae* antigen. (p=0.044).

Table 5.2. Spearman correlation analysis for BR patients of IFNγ spot -forming cells per 10^6 PBMC and MSD cytokines (ng/ml).

The cells were stimulated with *P. aeruginosa* and *H. influenzae* * indicates that all values are = 0

Stimulation with *P. aeruginosa*			**Stimulation with *H.influenzae***		
Cytokines	P-value	Spearman r	Cytokines	P-value	Spearman r
IL-2	0.77	-0.103	IL-2	**0.003**	-0.837
IL-4	**0.049**	-0. 669	IL-4	0.413	-0.292
IL-10	0.213	-0.432	IL-10	0.137	-0.505
IL-12p20	0.136	-0.506	IL-12p20	**0.035**	-0.669
IL-13	0.705	-0.137	IL-13	0.397	-0.302
IL-17	0.901	-0.045	IL-17	0.620	-0.180
IL-5	*	*	IL-5	0.179	-0.464

5.2.12 Determining the phenotypes of T cells responding to antigenic stimulation

Experiments were performed to determine whether or not IFNγ$^+$ cells were also CD69$^+$ following antigen stimulation, because CD69$^+$ was used in the flow cytometry to identify activated cells in response to antigen stimulation in the next section. PBMCs were obtained from a healthy donor and stimulated with medium only, anti-CD3, or *P. aeruginosa* overnight at 37°C in a 5% CO_2 incubator. The target cells were surface-stained for CD4 and

CD69 and intracellularly stained for IFNγ following fixation and permeabilization as described in section 2.9.2 and 2.9.3. The results show that within the responding CD4^{+} T cells most of the lymphocyte cells expressing IFNγ also expressed CD69.

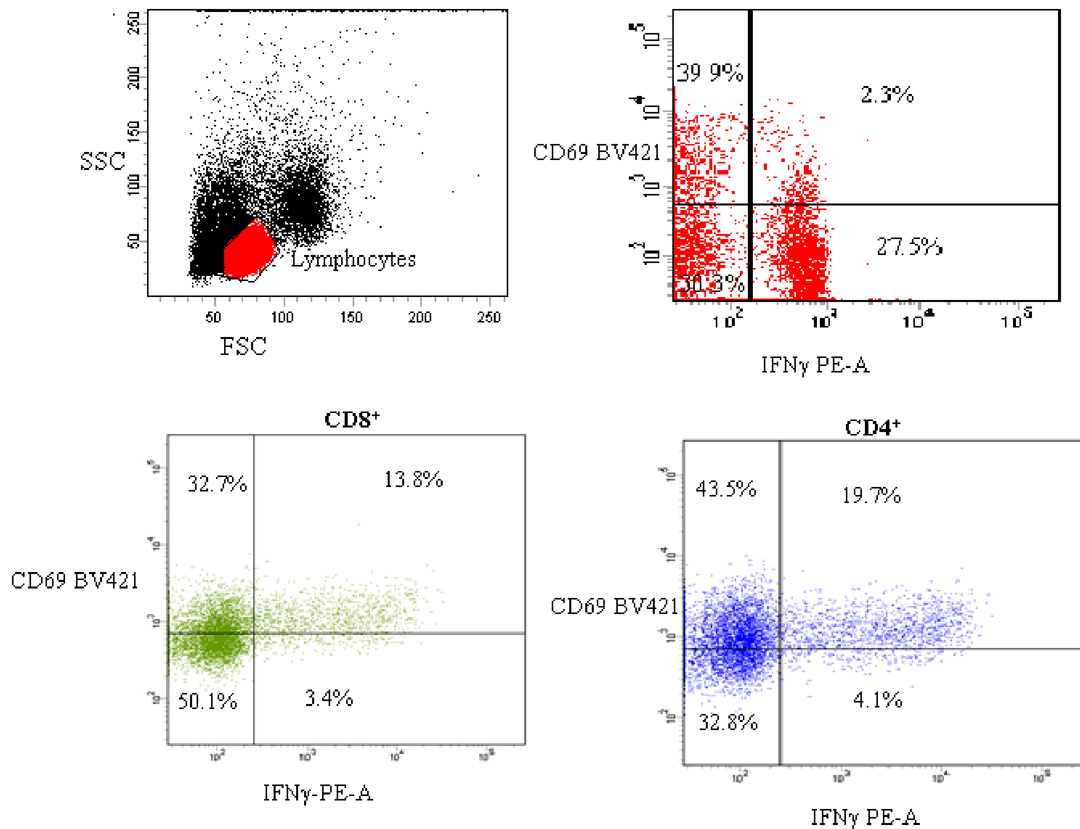


Figure 5.15: IFNγ T cells positive for CD69 on CD4^{+} and CD8^{+} following anti-CD3 stimulation.

The cells were gated within the lymphocyte population. The percentage of the lymphocytes positive and negative for IFNγ are shown. Eight colour flow cytometer were used**.**

5.2.13 Expression of CD4^{+}CD69^{+} in response to different stimulus in BR patients and healthy volunteers by Flow cytometric analysis

PBMCs were stimulated with either anti-CD3, *P. aeruginosa* or *H. influenzae,* or with medium as a negative control, as described in section 2.9.2. Typical results are shown in Figures 5.16 – 5.19. The stimulated cells were stained for a range of activation and homing markers as described in section2.9.2. The results show that incubation with anti-CD3 significantly increased the expression of CD69^{+} on CD4^{+} T cells in both patients ($p<0.001$)

and healthy volunteers (p<0.001) (Figure 5.20). The proportion of $CD4^+$ cells co-expressing $CD69^+$ also significantly increased when patients PBMCs were incubated with *P. aeruginosa* (p=0.003). However, there was no significant difference from healthy volunteers (p=0.1285). Significant differences were also observed when comparing resting cells and cells stimulated with *H. influenzae* for BR patients (p=0.0025) and for healthy volunteers (p=0.035) (Figure 5.21).

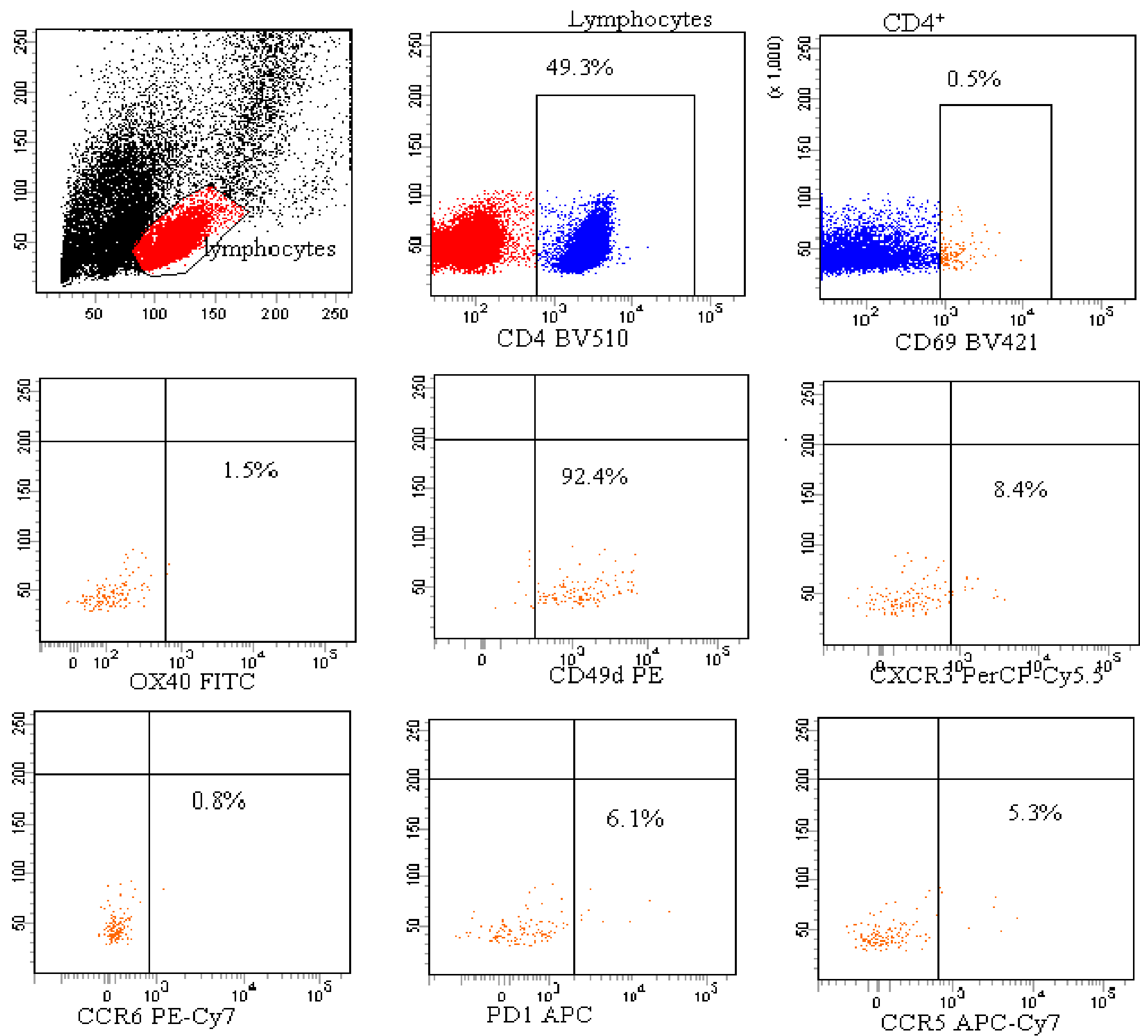


Figure 5.16: Expression of phenotype markers on $CD4^+CD69^+$ cells following the stimulation of PBMCs with medium.

The lymphocytes are gated on $CD4^+$ and the percentages of each $CD4^+CD69^+$ are indicated for each candidate marker. Eight colour flow cytometry was used to analyse the data.

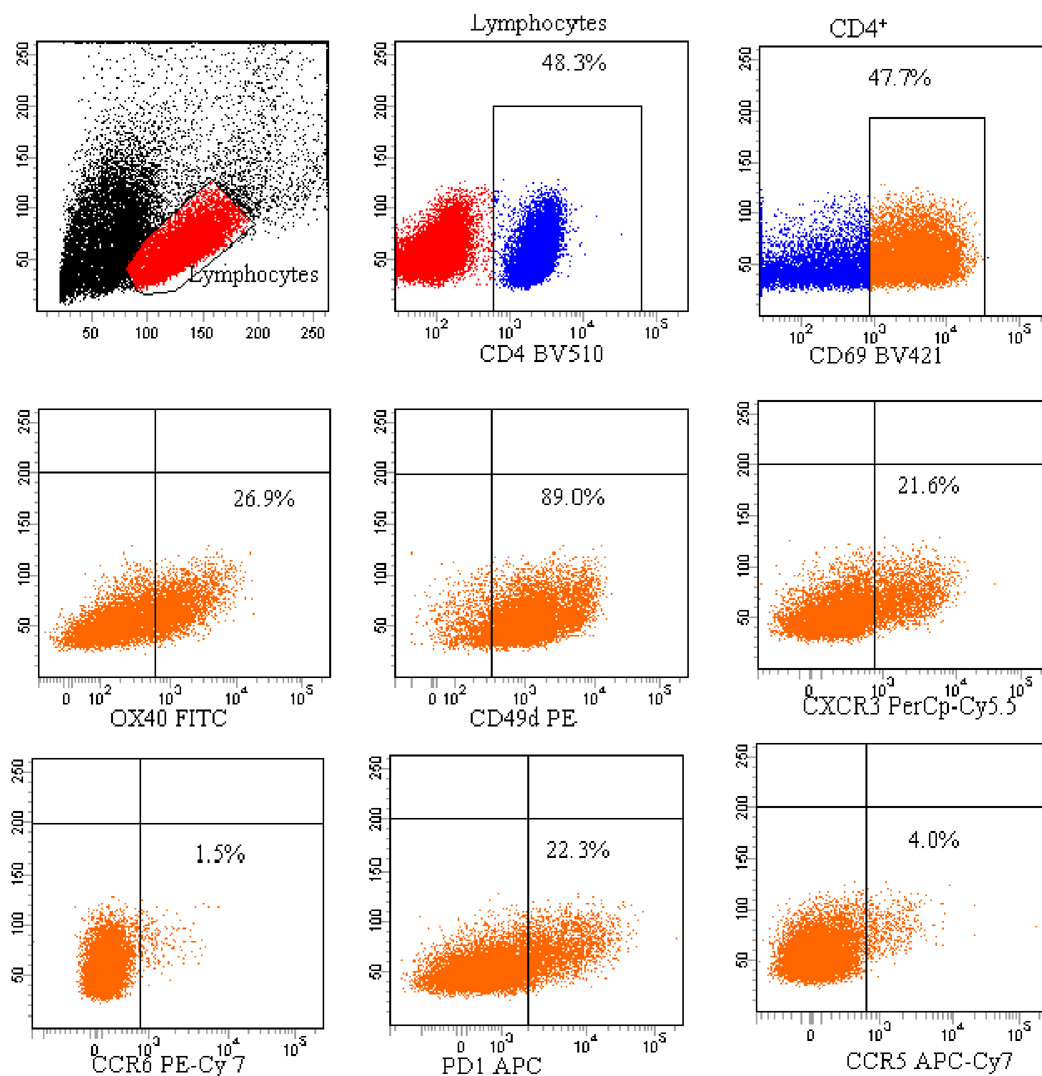


Figure 5.17: Expression of phenotype markers on CD4⁺CD69⁺ cells following stimulation of PBMCs with anti-CD3.

The lymphocytes are gated on CD4⁺and the percentages of each CD4⁺CD69⁺ are indicated for each candidate marker. Eight colour flow cytometry was used to analyse the data.

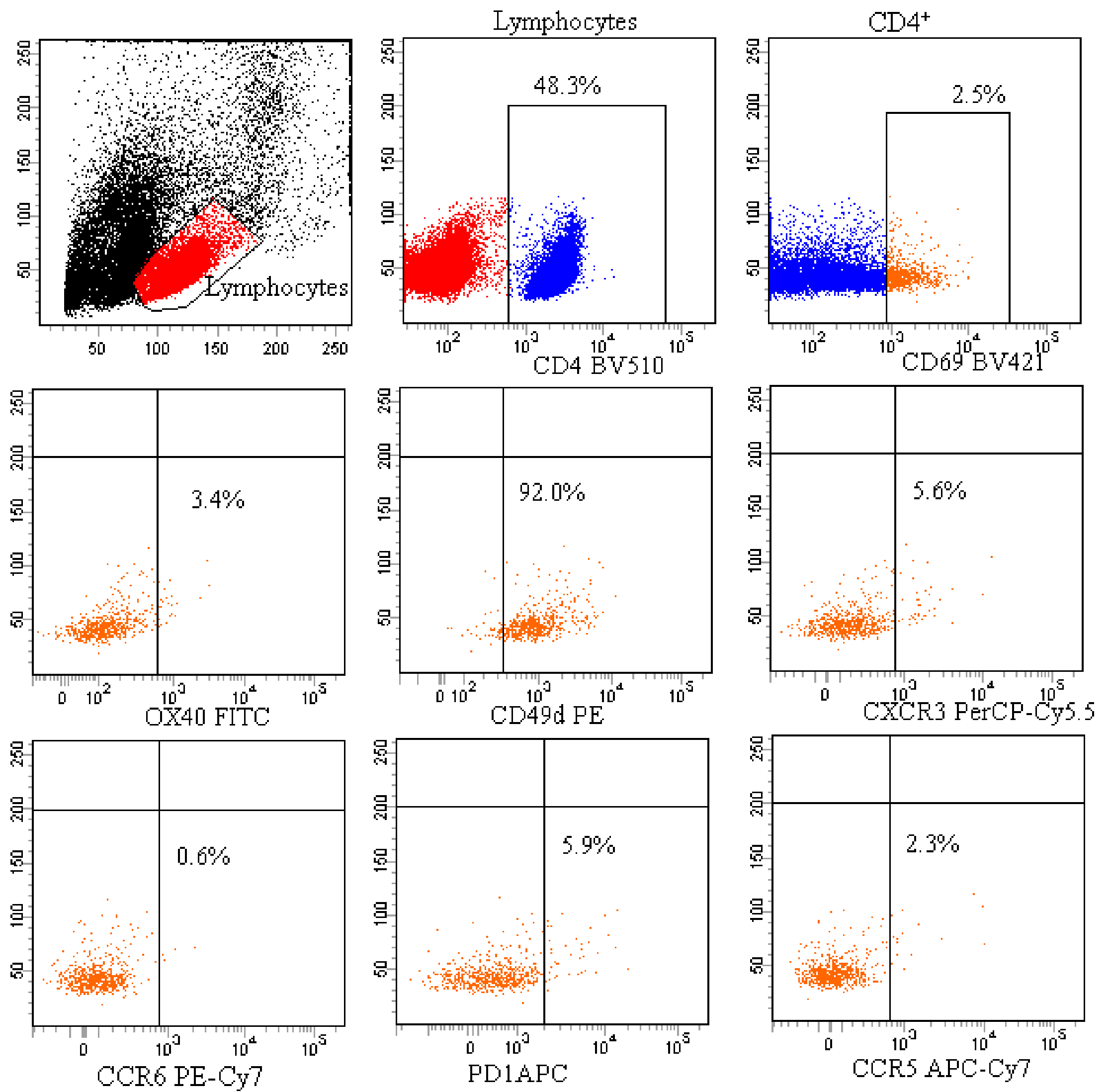


Figure 5.18: Expression of phenotype markers on CD4⁺CD69⁺ cells following stimulation of PBMCs with *P. aeruginosa* antigen.

The lymphocytes are gated on CD4⁺and the percentage of each CD4⁺CD69⁺ are indicated for each candidate marker. Eight colour flow cytometry was used to analyse the data.

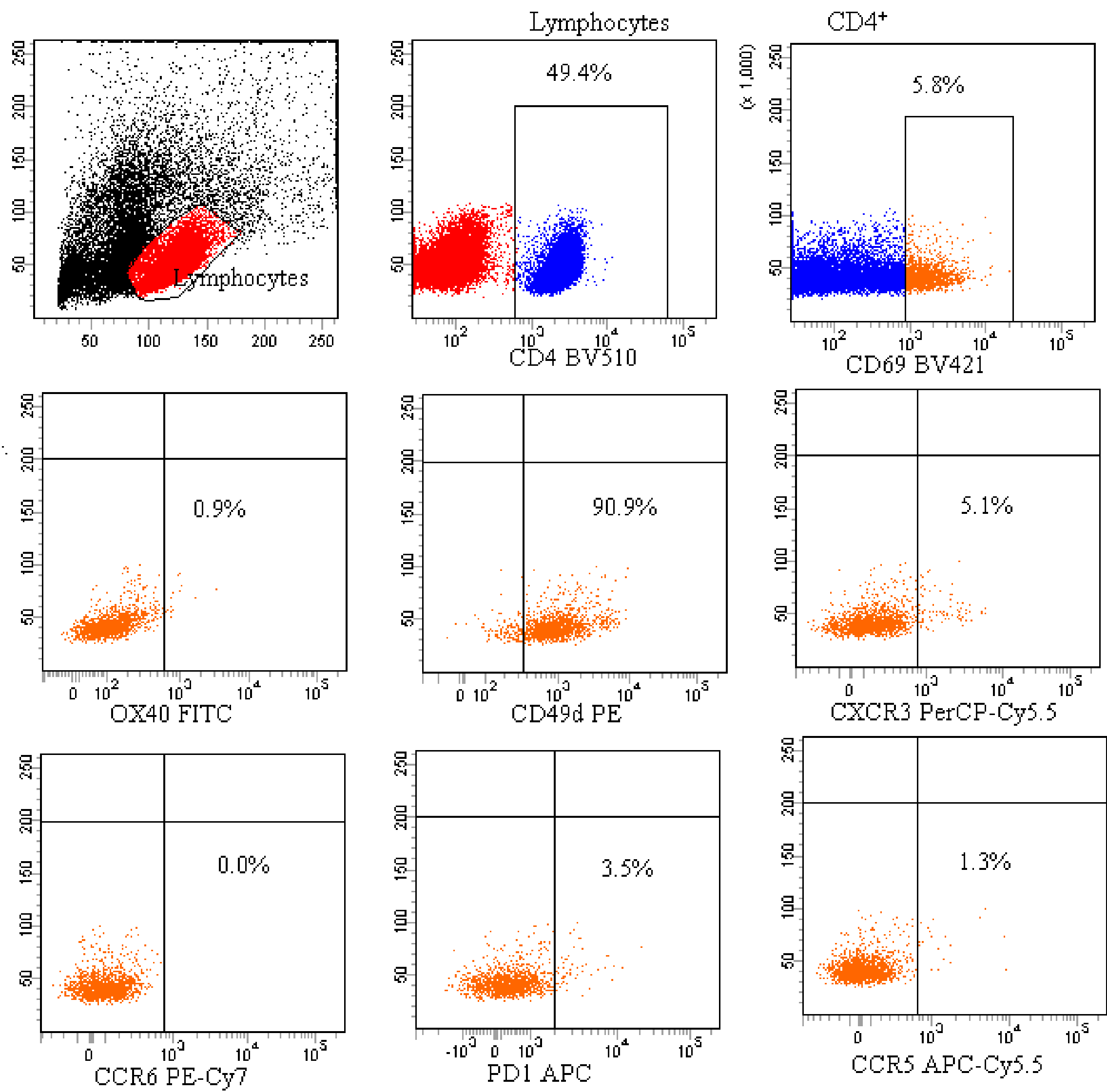


Figure 5.19: Expression of phenotype marker on CD4$^+$CD69$^+$ cells following stimulate of PBMC with *H.influenzae* antigen.

The lymphocytes are gated on CD4$^+$ and the percentages of each CD4$^+$CD69$^+$ are indicated for each candidate marker. Eight colour flow cytometry was used to analyse the data.

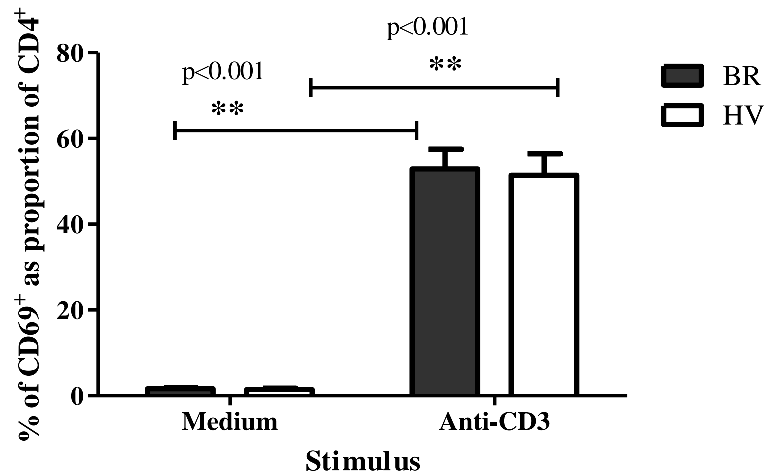


Figure 5.20: Expression of CD69⁺ as a proportion of CD4⁺ in BR and HV on resting cells and after stimulation with anti-CD3.
Data are presented as mean± SEM. ** indicates statistical significance with ($p < 0.001$) calculated by Mann-Whitney test. BR n= 10 and HV n=8.

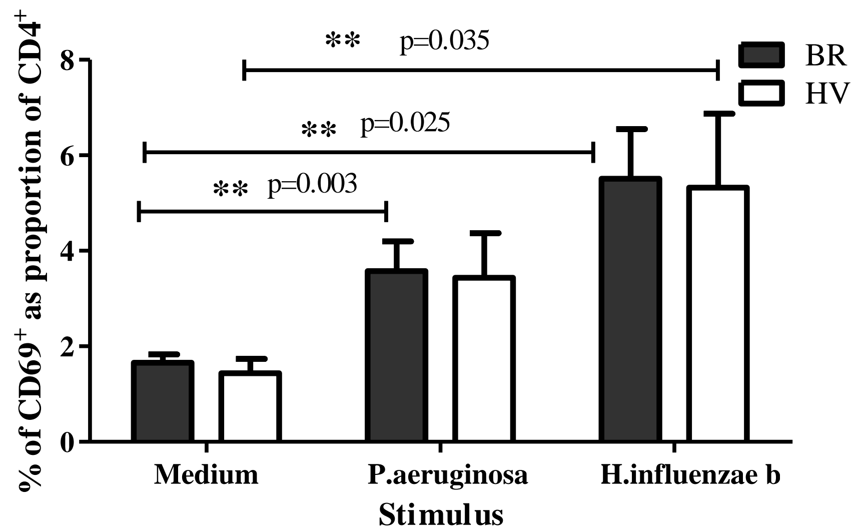


Figure 5.21: Expression of CD69⁺ as a proportion of CD4⁺ following stimulation with *P. aeruginosa* and *H. influenzae* antigens in BR patients and HV.
Data are presented as mean± SEM . ** indicated the significance p value are calculated by Mann-Whitney test. BRn= 10 and HV n=8.

5.2.14 Expression of candidate markers on CD4⁺CD69⁺ lymphocytes

In order to ascertain the phenotype of the responding activated CD4⁺CD69⁺ T cells, the levels of six other candidate markers were assessed. The results in Figure 5.22 show that,

irrespective of the stimuli, activated cells ($CD4^+CD69^+$) expressed similar levels of the other candidate markers when compared to resting ($CD4^+CD69^-$) cells

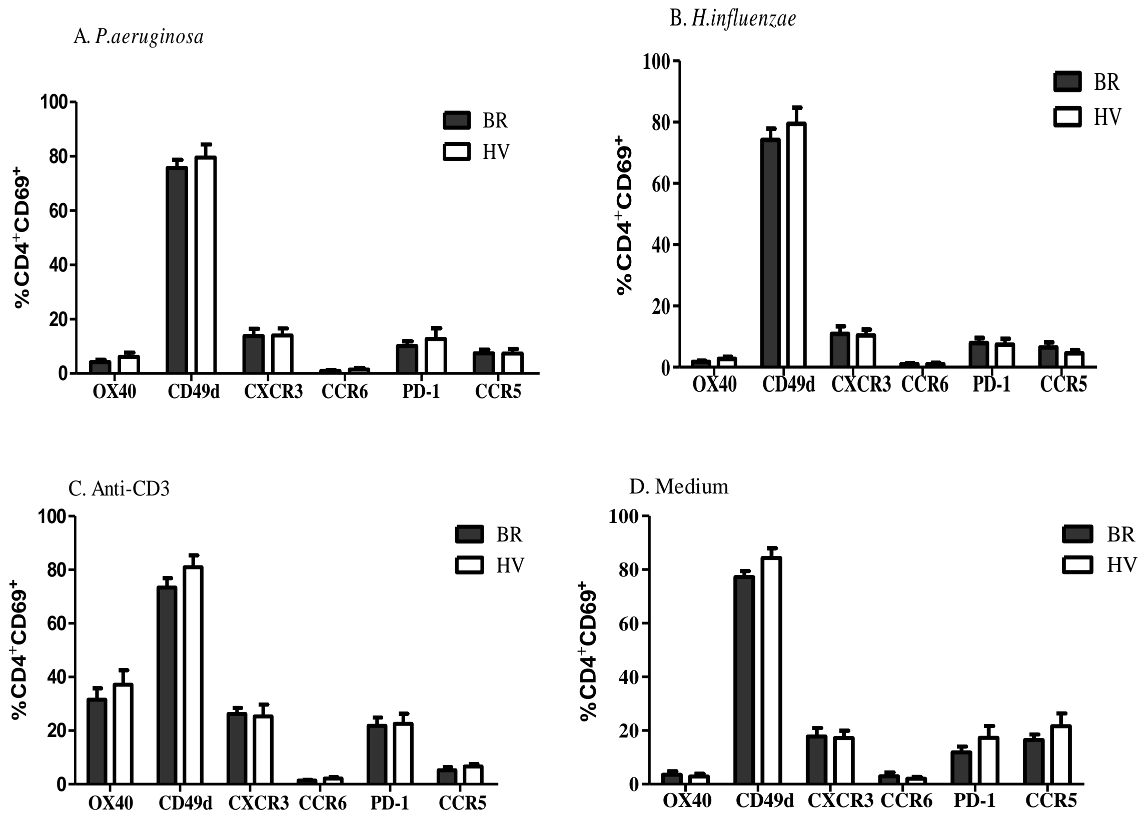


Figure 5.22 (A-D): Expression of candidate markers on $CD4^+CD69^+$ cells in BR patients and HV in response to various stimuli.
(A) PBMCs were stimulated with *P. aeruginosa*; (B) stimulation with *H. influenzae*;(C) stimulation with anti-CD3 and (D) incubation with medium. Data are analysed as mean± SEM. No significant differences were found. BR n= 10 and HV n=8.

5.2.15 Expression of candidate markers on $CD8^+CD69^+$ lymphocytes

By gating on the $CD4^-$ subset of lymphocytes, $CD8^+$ cells could also be analysed. The phenotype of $CD8^+CD69^+$ cells was therefore also assessed in BR patients and healthy controls following stimulation as described in section 2.9.2. The findings in Figure 5.23 show that both activated and resting cells expressed similar levels of the majority phenotype

markers studied. However, OX40 and CCR6 were marginally expressed more highly on $CD8^+$ cells following stimulation with anti-CD3.

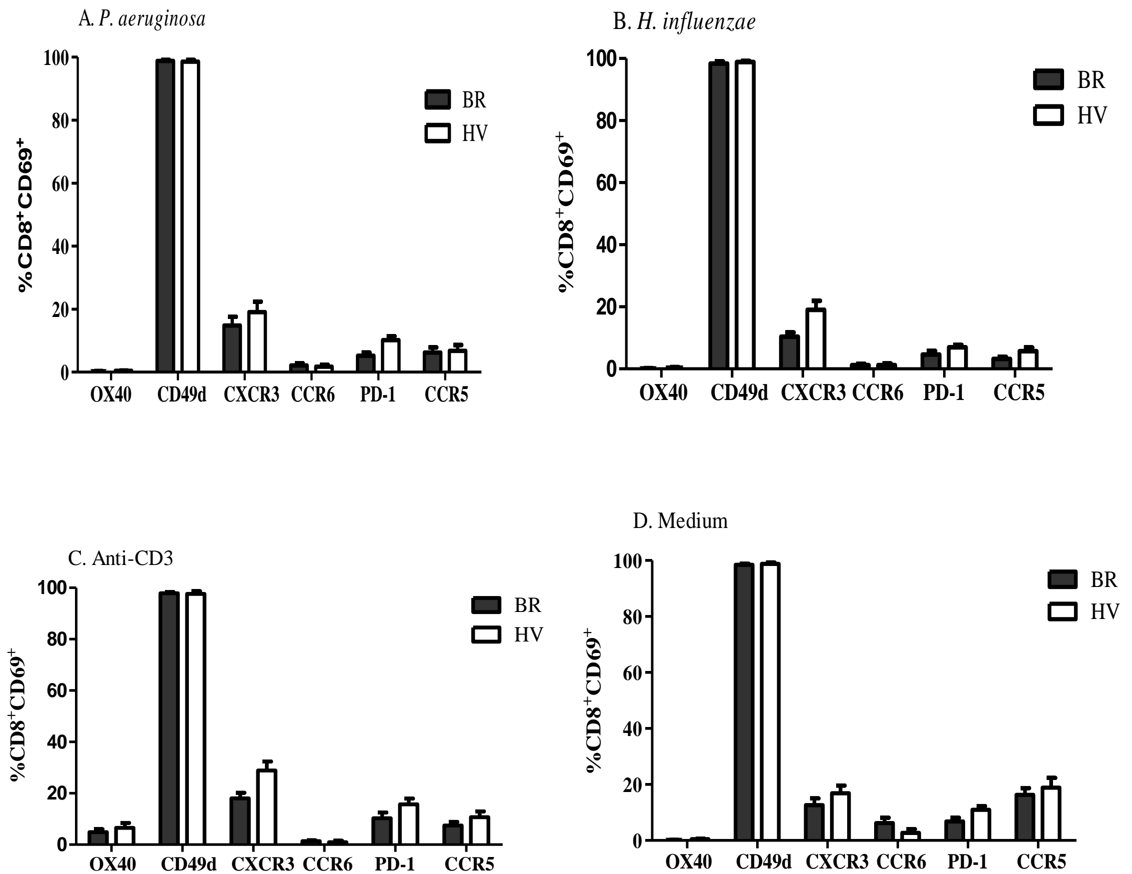


Figure 5.23(A-D): Comparison of different phenotypes on CD8+CD69+ in BR patients and HV in response to different stimuli.

(A) PBMCs were stimulated with *P. aeruginosa*; (B) stimulation with *H. influenzae*;(C) stimulation with anti-CD3 and (D) incubation with medium. Data are analysed as mean. ± SEM No significant differences were found. BR n= 10 and HV n=8.

5.3 Discussion

In this study, it was first confirmed that $CD4^+$ T cells were the predominant T cell responding to the lung antigens. IFNγ production was decreased after $CD4^+$ depletion and it was blocked by anti-MHC class II antibodies, and activated $CD4^+$ T cells producing IFNγ and expressing CD69 were identified by ICS. IFNγ is secreted by $CD4^+$ Th_1 cells, which distinguishes them from other subsets of T cells. Th_1 cells are important in promoting an inflammatory response and eradicating intracellular pathogens (Pollard *et al.*, 2012). Once these findings had been established, experiments were developed to investigate the responses of T cells against lung microbe antigens in patients with lung diseases compared to healthy volunteers.

5.3.1 T cell responses in BR, COPD and asthma patients compared to healthy volunteers

It was found that patients with BR had significantly lower levels of IFNγ compared to healthy volunteers against *P. aeruginosa* (p=0.002), *S. pneumoniae* (p=0.001), *A. fumigatus* (p=0.03), *B. pertussis* (p=0.01) and *M. tuberculosis* (p=0.002). Furthermore, lower levels of IFNγ, but not significant lower, against the other antigens including *H. influenzae*, *M. catarrhalis*, Influenza virus, *B. cepacia* and *M. pneumoniae* were also observed in BR patients. Similarly, COPD patients had significantly reduced T cell responses against *P. aeruginosa* (p=0.014) and S. *pneumoniae* (p=0.031) compared to HV. These patients also had low responses against *H. influenzae*, *M. catarrhalis*, *A. fumigatus*, *B. pertussis*, RSV, *B. cepacia* and *M. pneumoniae.* Moreover, asthmatics had significantly lower levels of IFNγ against *P. aeruginosa* (p=0.040) compared to healthy volunteers. Reduced responses of T cells among asthmatics were also observed against *S. pneumoniae*, *M. catarrhalis*, *A. fumigatus*, *B. pertussis*, and RSV. These findings were in accordance with the previous work that showed decreased responses of T cells to outer membrane protein F (OprF) during *P.*

aeruginosa infections in non-cystic fibrosis bronchiectasis patients despite the high antibody production. The reason for this may be a skewed T cell response to *P. aeruginosa* protein antigen towards Th_2 (Quigley *et al.*, 2015). Furthermore, the reduced response of T cells in BR and COPD patients may be because of high pathogen loads resulting in anergic of T cells specific for the bacteria antigens. It has been shown that, in some chronic infections, high antigen loads generated dysfunction and impaired antigen-specific T cell populations (Han *et al.*, 2010). Furthermore, in patients with COPD, the effector T cells defined as $CD4^+CD127^+$ expressed a high percentage of PD-1, with these cells lacking the energy to proliferate after exposure to bacteria (Singh, 2014). However, reduced IFNγ responses observed among asthmatics patients in our study may be because of the skew of T cell differentiation toward Th_2 cells following antigen presentation, since increased Th_2 cytokines are associated with asthma (Robinson, 2010, Shalaby and Martin, 2010). The lung previously has been shown to became polarized for Th_2 response. It was found that increased Th_2 cytokines (IL-13) occurred in BAL fluid in a murine asthma model following rhinovirus infection (Kim *et al.*, 2013).

In these experiments, bacterial lysate extracts and peptides were utilized to stimulate $CD4^+$ Th_1 cells to produce IFNγ. A previous study has shown robust IFNγ responses by $CD4^+$ T cells and high levels of IL-2 after *Streptococcus agalactiae* infection, suggesting that a type I pro-inflammatory response was generated. The bacterium has a thick capsule polysaccharide (CPS) which is thought to modulate $CD4^+$ T cell activation and to help to induce strong cytokine production. Therefore, the polysaccharide may contribute to the $CD4^+$ Th_1 response to generate a high IFNγ production (Clarke *et al.*, 2016). Interestingly, BR patients produced higher, but not significantly higher, IFNγ in response to Cytomegalovirus (p=0.055), whilst COPD patients showed higher IFNγ response to Cytomegalovirus, *M. tuberculosis* and influenza virus, though not significantly so. This

might be attributable to the responses of both $CD8^+$ cytotoxic T cells and $CD4^+$ T cells during viral persistence (Simpson *et al.*, 2016). This shows that neither disease had reduced global T cell responses. Similarly, a slightly increased response of T cells was observed in asthmatics against *H. influenzae*, *M. tuberculosis* and *M. pneumoniae*. This may be attributable to these patients having more exposure to these pathogens compared to HV.

In contrast, the high magnitude of T cell response observed in healthy volunteers compared to patients with BR, COPD and asthma suggests that these antigens may trigger T cell responses after encountering antigen during natural exposure or infection and that these persist for a few weeks and are then elevated later during secondary immune response (Hus *et al.*, 2015). Previous studies have shown that healthy volunteers have high levels of IFNγ responses against NT*H. influenzae* compared to patients with bronchiectasis. It was shown that the responses of healthy volunteers were made up of Th_1 cells, also expressing CD40L, which promotes antibody production, whereas patients with bronchiectasis exhibited a different immune response comprising Th_2 cells and characterised by the production of IL-4 and IL-10 (King *et al.*, 2003).

In this study, measurements of other cytokines such as IL-2, IL-4, IL-5, IL-13 and IL-17 were also performed, but no significant differences were observed (this Data not shown).

5.3.2 T cell responses versus microbial colonization

In this study, patients who are classified as currently chronically infected have reduced T cell responses. This result contrasts somewhat with the antibody responses shown in sections 4.2.3 and 4.2.4. Chronic microbial infection resulted in reduced T cell responses possibly due to T cell responses being overwhelmed by sustained antigen and becoming anergic progressively , as shown in $CD8^+$ unresponsiveness with chronic viral infection (Mueller and Ahmed, 2009). Continuous bacterial persistence also contributes to dysfunctional T cells

through the down-regulation of newly-primed antigen-specific effector $CD4^+$T cells, and this results in the development of poor memory responses (Han *et al.*, 2010).

5.3.3 T cell responses in exacerbated BR patients and FEV_1% predicted

The study also demonstrated that a significant difference was seen in IFNγ production at > 3 and <3 exacerbation compared to hospitalized status in patients with BR against *M. catarrhalis* but not *P. aeruginosa*. This suggests that IFNγ responses may be elevated and contribute to disease pathology during exacerbations and reduced when the patients were hospitalised. In contrast, no significant correlations were observed between IFNγ production and FEV_1% predicted in BR and COPD patients, and thus T cell responses were not associated with decline lung function in patients with chronic obstructive lung disease.

5.3.4 Relationship between antibody and T cell responses

The relationship between antibody and T cell responses in patients and healthy volunteers was also investigated. The analysis showed a significant negative correlation in COPD patients in response to *M. catarrhalis* (p=0.005). Similarly, a significant negative correlation was seen for healthy volunteers against *M. catarrhalis* (p=0.030) and *H. influenzae* (p=0.017). In this study the response of T cells was assessed by the production of IFNγ in which Th_1 are the main cell sources. Th_1 cytokines predominantly mediate cellular immunity and activate inflammatory responses, while Th_2 cytokines mediate humoral immunity and up-regulate antibody production. Each type of cytokine can cross inhibit the other type of cytokine resulting in mutual exclusion and negative correlation (Kidd, 2003, Maki *et al.*, 2016).

5.3.5 Measurement of cytokines by MSD of T cell-stimulation culture supernatants

Measurements of cytokines by MSD of T cell-stimulation culture supernatants were also performed in this study. Our data showed that apart from IFNγ, the other cytokines were at low levels. Nevertheless, a significant negative correlation was found when comparing IFNγ spot-forming cells and secreted IFNγ against *H. influenzae* (p=0.04). A significant negative correlation was also observed between IFNγ spot-forming cells compared to secreted IL-2 and IL-12 against *H. influenzae* (p=0.003 and p=0.035 respectively). A significant negative correlation was also detected when comparing IFNγ and IL-4 against *P. aeruginosa* (p=0.045). As described in the literature review, Th_1 cells mainly produce IFNγ, IL-2, IL-12 and IL-15, whereas, Th_2 secrete more IL-4, IL-5, IL-10, and IL-13, and these cytokines antagonise each other and block each other's effector functions. (Wilczynski, 2005). Thus, it may be possible here that the high IFNγ level inhibited the production of IL-4 resulting in lower IL-4 response. In contrast, it was surprising that IFNγ negatively correlated with IL-2 since both cytokines are secreted by Th_1 cells. It may be possible that other factors affect the responses of these cytokines, including antigen dose, the nature of the antigens, and the cytokine receptors available on the naive cell (Kidd, 2003). Some limitations of this experiment should be acknowledged, namely the sample sizes were small with only 10 BR patients and 4 healthy controls included.

5.3.6 Expression of $CD4^+CD69^+$ in response to different stimulus in BR patients and healthy volunteers

This study examined the phenotypes of activated lymphocytes in both patients with bronchiectasis and healthy volunteers, as these cells have the potential to play an important role in the pathogenesis of bronchiectasis. The stimulation of PBMC with anti-CD3 was used as a positive control to demonstrate that the cells are healthy and able to respond to a

stimulus. Expression of CD69 was, as expected, significantly increased in both groups following anti-CD3 activation (Figure 5.20). The percentage of $CD4^+$ cells co-expressing $CD69^+$ was significantly higher in patients with BR following activation by *P. aeruginosa* compared to unstimulated (p=0.0036). A significant increase in $CD69^+$ was also observed in both patients and healthy volunteers after *H. influenzae* stimulation (p=0.0025 and p=0.035, respectively) (Figure 5.21). This demonstrates that exposing PBMCs to lung antigens leads to the up-regulation of CD69. $CD69^+$ cells have been suggested to play a major role in the pathogenesis of interstitial lung diseases (Heron *et al.*, 2010), possibly because the rapid expression of $CD69^+$ on T cells promotes activation and differentiation (Sancho *et al.*, 2005). It is therefore likely that such activated cells may also contribute to the pathology of bronchiectasis.

The results also demonstrate that activated T cells ($CD4^+$ and $CD8^+$) in patients and healthy volunteers express similar high levels of CD49d on their surfaces. This possibly indicates the continued stimulation and activation of lymphocytes in response to these stimuli. It has been found that the increased expression of CD49d on $CD4^+$ and $CD8^+$ in patients with asthma indicates the activation of T cells and the priming of these cells to migrate into the site of inflammation in the airway (Bazan-Socha *et al.*, 2012).

5.3.7 Expression of candidate markers on $CD4^+CD69^+$ lymphocytes

By gating on $CD4^+CD69^+$ lymphocytes, the co-expression of several other candidate markers could also be determined. The results show minor expression of OX40, CXCR3, CCR6, CCR5 and PD-1 on $CD4^+CD69^+$ cells from BR patients and healthy controls for both antigens stimulated and resting cells. Stimulation with anti-CD3, however, enhanced the expression of OX40, CXCR3, and PD-1, demonstrating that anti-CD3 provides a powerful stimulus to $CD4^+$ cells and results in them being highly activated. In contrast, the small

percentage of expression of OX40, CXCR3, CCR6, CCR5 and PD-1 on $CD8^+CD69^+$ was observed in both BR patients and healthy controls even after stimulation with anti-CD3. This result agrees with those of a previous study, which found OX40 only slightly up-regulated in $CD8^+$ cells following antigen stimulation (Zhong *et al.*, 2010). This may be because $CD8^+$ T cells were not highly activated here. It has been reported that $CD8^+$ T cells may be rapidly expanded and activated after viral infection and provide long term protection against further viral infection through memory $CD8^+$ T cells (Shaw *et al.*, 2014). Further work using different stimuli such as viral antigens and more participants may be necessary to determine the expression of these molecules on $CD8^+$cells.

6 Conclusion

In conclusion, microbial infections accelerate morbidity and mortality in patients with chronic obstructive pulmonary diseases. Understanding the interaction between the host and the pathogens may provide novel insights into the pathology pathways. In this study, it has been found that bacterial infection results in reduced IFNγ production through impaired Th_1 response or altered the response toward Th_2. The findings showed that IFNγ significantly increased during the course of exacerbations to *M. catarrhalis* but it was not implicated in the worsening of lung functions. Thus, these finding suggest that the role of IFNγ–producing cells may be substantial in protection but are not, or are minimally, implicated in the pathogenesis of the disease.

In this study, 10 BR patients and 8 healthy volunteers were included in the analysis. The study showed that the proportion of $CD4^+$T cells which co-expressed $CD69^+$ was significantly higher following stimulation with anti-CD3, *H. influenzae* and *P. aeruginosa* suggesting that $CD4^+$ Th cells are the main cells activated in response to these stimuli. No significant differences were found for other candidate markers suggesting that further work

may be necessary to achieve a better understanding with more participants and different stimuli (e.g. viral antigens), and candidate markers, being investigated.

6 Chapter Six: Immune responses in patients with cystic fibrosis

Abstract

Aim: Cystic fibrosis (CF) is a chronic progressive lung disease associated with recurrent pulmonary infections and exaggerated inflammatory responses that promote irreversible damage, potentially ending in respiratory failure and death. Antibody responses play an active role in protection against lung infections in CF, whereas the role of T cells in its pathogenesis or protection is still unclear. Therefore, the purpose of this study was to examine specific immune responses against lung-infecting microbes in clinically characterised patients with CF, since immune responses may be contributing to disease pathology as well as protection from infection.

Methods: Peripheral blood samples from CF patients (*P.aeruginosa*-colonised at RVI, or *Burkholderia*-colonised at Manchester) were used to obtain PBMCs and serum. T cell and antibody responses were measured against a series of lung-infecting microbial antigens (bacteria, fungi and viruses) using characterised ELIspot and ELISA assays. Furthermore, the sputum of patients was investigated for culturable microbial colonization. Patients were characterised for lung function (FEV_1 %) and exacerbation frequency.

Result: The microbiology results confirmed that the predominant pathogens in the sputum of the RVI CF patient cohort were *P. aeruginosa,* as well as *Candida* sp, *S. aureus* and *A. fumigatus*. Patients with CF had significantly higher antibody responses compared to healthy controls for *P. aeruginosa*, *S. maltophilia, M. catarrhalis, A. fumigatus* and *H. influenzae.* T cell responses (in the form of IFNγ) were significantly higher in CF patients compared to healthy controls against *B. cenocepacia* (p=0.04). Furthermore, higher T cell responses were observed against *H. influenzae, M. catarrhalis*, *M. pneumoniae* and Cytomegalovirus, but

differences were not statistically significant. However, healthy volunteers had significantly higher T cell responses against *S. pneumoniae* compared to CF patients. Antibody and T cell responses did not correlate in CF except for a negative correlation for *A. fumigatus*. Deterioration of lung functions and impaired cellular immune response, but not antibody response, were significantly associated with exacerbations. The Manchester cohort showed associations of antibody responses with colonisation, and T cells secreting Th_{17} cytokines as well as IFNγ.

Conclusion: The findings show that whilst colonization causes increased antibody levels, these levels are not associated with disease stability and lung function. On the other hand levels of IFNγ-secreting T cells against *P. aeruginosa* do associate positively and may be prognostic for disease, acting directly or through other cytokines and mechanisms.

6.1 Background

Cystic fibrosis (CF) is one of the most common and lethal autosomal recessive disorders of white European populations (Aureli *et al.*, 2016, McGuire, 2015). Most of the morbidity and mortality of CF is caused by a progressive decline in lung function resulting from repeated microbial infection and inflammatory immune response (Milagres *et al.*, 2009, Palaniyar *et al.*, 2015). Typically, *P. aeruginosa*, *S. aureus, B. cepacia complex*, *S. maltophilia* and Non-tuberculous mycobacteria are the most common pathogens detected by conventional microbiology (de Vrankrijker *et al.*, 2010, Emerson *et al.*, 2010). However, molecular techniques have found many microorganisms that were previously unidentified in the CF lung, such as, *Lautropia mirabilis, Fusobacterium gonidiaformans*, and *Bacteroides fragilis,* and thus these species may be novel potential CF-associated microorganisms (Rogers *et al.*, 2003). *P. aeruginosa* is the predominant pathogen associated with chronic infection in CF and is considered to exaggerate the inflammatory response caused by macrophage and

neutrophilic infiltration. These cells migrate to the site of infection and release inflammatory mediators and pro-inflammatory cytokines, causing lung injury and fuelling the vicious cycle of infection (Courtney *et al.*, 2004, Moreau-Marquis *et al.*, 2008). The diagnosis of microbial infections, in particular *P. aeruginosa*, may help in assessing early treatment and preventing disease progression. Therefore, testing for antibodies may provide surrogate markers to define the infection and colonization of CF patients by *P. aeruginosa* and other organisms, especially in young children who are unable to produce sputum (Ratjen *et al.*, 2007). It has been argued that the measurement of antibody against bacteria is an alternative approach to tracking bacterial colonization in cystic fibrosis. Previous studies have shown that a higher antibody titre is correlated with the acquisition of *P. aeruginosa* (Kappler *et al.*, 2006). Increased inflammatory responses associated with bacterial infection have been described in patients with CF (Aldallal *et al.*, 2002). Distinct patterns of pro-inflammatory cytokines have been reported in patents with CF, including of IL-1β, IL-6, IL-8, and TNF-α in the serum and in BAL fluid (Casaulta *et al.*, 2003, Conese *et al.*, 2003). One study has shown that IL-10 plays an effective role in the inhibition of IFNγ and IL-2 synthesis by blocking the co-stimulatory signals for T cell activation, and it may regulate inflammatory responses in CF (Casaulta *et al.*, 2003).

The purpose of the present study is to investigate immune responses in relation to colonizing microorganisms in CF patients. Moreover, the relationship between specific T cell and antibody responses, and whether or not immune responses may contribute to clinical parameters such as pulmonary function and exacerbation in CF patients were investigated.

6.2 Methods

6.2.1 Patient recruitment and protocols

Adult cystic fibrosis patients (n=30) attending the outpatient CF clinic at the Royal Victoria Infirmary, Newcastle upon Tyne were recruited in this study. They were known to harbour *P.aeruginosa*. Twenty-eight healthy volunteers were recruited from the staff of the outpatient clinic at the Freeman Hospital Newcastle upon Tyne. Blood and sputum samples were collected and processed as described in section 2.2.2. Assays were performed as per the patient samples in chapters 3 and 5. Demographic data for the RVI CF patients are shown in Table 6.1. Furthermore, 14 CF patients attending the Manchester Adult Cystic Fibrosis Centre, the University Hospital of South Manchester, were included in this study. Both groups of CF patients were negative for nontuberculous mycobacteria (NTM) The clinical data were collected from each patient (limited data) Table 6.1. Blood specimens were collected from each individual and processed as described in section 2.2.2. Sera and PBMCs were tested for antibody and T cell response against *B. cenocepacia, B. multivorans* and *P. aeruginosa* by indirect ELISA as described in section 2.7 whereas ELI-spot was carried out as described in 2.8.2.

6.3 Results

6.3.1 Demographics data of RVI cystic fibrosis patients

The clinical characteristics and demographic data are shown in Table 6.1. Patients with CF had a high mean value of symptoms. Patients and healthy volunteers underwent a routine respiratory test, as shown; a mean FEV_1 % predicted of less than 50% indicated impaired lung function, while healthy volunteers showed mean FEV_1 values higher than 100% of the predicted value.

Table 6.1. Clinical characteristics of RVI and MAN cystic fibrosis patients

	CF(RVI) N=30	CF(MAN)	HV N=27
Sex (no.) Male/female	19/11	4 / 10	Not av.
Age (y)	29 ±1.72	Not av	54±3.0
Symptoms	75 ±8.17	Not av	Not app.
Exacerbations (per year)	3 ± 0.43	Not av	Not app.
FEV_1 (% predicted)	47 ±4.52	48±5.2	113±2.83
FVC (% predicted)	70 ± 4.29	72±4.7	118±2.7
FEV_1/VC ratio	59 ±2.65	Not av	83±1.75

Abbreviations: FEV_1=forced expiratory volume in one second. FVC=forced vital capacity. The data is presented as mean ± SEM. Not av indicates that data were not available whereas Not app means that category not applicable.

6.3.2 Microbial infections in cystic fibrosis patients

Sputum or swab microbial cultures were carried out at the Freeman Hospital at Microbiology Department. Microbial pathogens were isolated from all patients enrolled in the study, with polymicrobial communities comprising of both bacterial and fungal species being present. The main pathogens isolated from sputum are presented in Table 6.2. The most abundant colonization was seen for *P. aeruginosa* followed by *Candida* sp., and the least was recorded for *E. coli*. These CF patients were selected specifically to possess clinical *P. aeruginosa* infections.

Table 6.2. The abundance of microorganisms from the sputum of RVI CF patients

Organism	Cystic Fibrosis %
P. aeruginosa	100
***Candida* sp.**	86
S. aureus	70
A. fumigatus	60
H. influenzae	56
S. maltophilia	26
S. pneumoniae	10
E.coli	6

The Manchester patients were selected specifically to possess chronic infection by *Burkholderia* species. The following Table 6.3 demonstrates the microbiological isolation of *Burkholderia*. sp and *P. aeruginosa* in the 14 Manchester patients with CF. These patients were classified into no colonization, chronic colonization or intermittent colonization. The microbiology results in Table 6. 3 show that 5 out of 14 patients were chronically colonized by *B. cenocepacia* and 9 patients by *B. multivorans.* Furthermore*,* 4 patients had chronic colonization with *P. aeruginosa,* and 3 had intermittent infection.

Table 6.3. Microbial culture analyses for *B. cenocepacia*, *B. multivorans* and *P. aeruginosa* in Manchester CF patients

CF subject number	*B. cenocepacia*	*B. multivorans*	*P. aeruginosa*
MAN2	Chronic	No	No
MAN9	No	Chronic	Chronic
MAN21	No	Chronic	No
MAN31	No	Chronic	Intermittent
MAN41	No	Chronic	No
MAN52	No	Chronic	Intermittent
MAN65	No	Chronic	Intermittent
MAN74	Chronic	No	Chronic
MAN75	Chronic	No	Chronic
MAN88	No	Chronic	No
MAN111	No	Chronic	No
MAN120	No	Chronic	No
MAN121	Chronic	No	Chronic
MAN122	Chronic	No	No

6.3.3 Comparison of antibody titres in RVI CF patients and healthy volunteers

IgG titre for CF patients and healthy controls against the lung-infecting organisms are shown in Figure 6.1. The results show that significantly higher IgG titres were observed in CF patients against *P. aeruginosa* (p<0.001), *S. maltophilia* (p<0.01), *M. catarrhalis (*p= 0.02) and *A.fumigatus* (p<0.001). CF patients also showed higher anti-NT*H. influenzae* titres but without statistical significance. However, the magnitude of IgG against *H. influenzae* polysaccharide was significantly higher in healthy controls *(*p<0.001*)* when compared to CF patients. IgG titre against *S. pneumoniae* lysate was slightly higher in healthy controls but no significant difference was found.

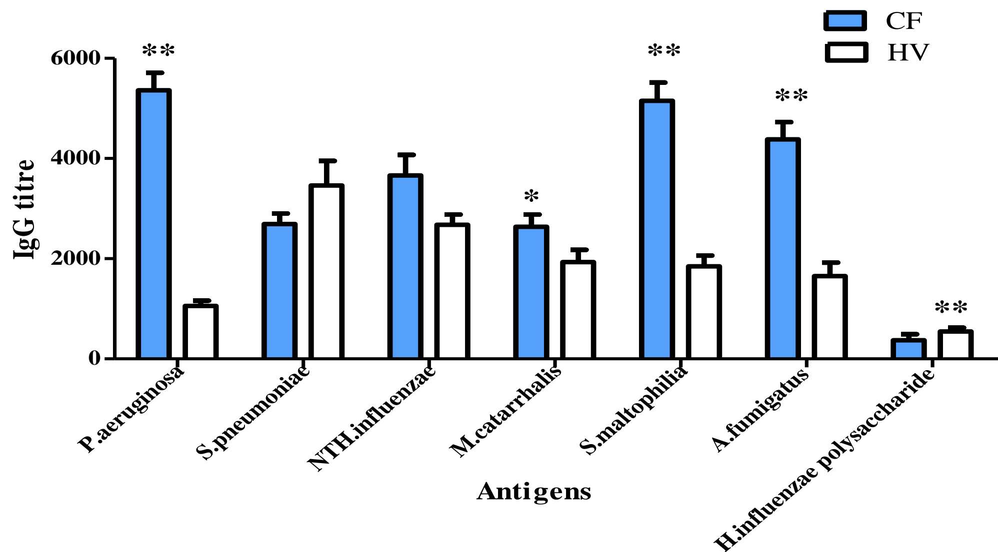


Figure 6.1: IgG titre against the key antigens examined in the study in CF patients and HV. Higher antibody titres were detected in most CF patients compared to the healthy volunteers. ** indicates p-value highly significant p≤0.001. * indicates p-value ≤ 0.05. The data are shown as the mean± SEM. CF n=30 and HV n= 27.

6.3.4 T cell responses in RVI CF patients

Antigen-specific responses of T cells against lung antigens and positive controls were also measured in CF patients compared to healthy volunteers. In CF patients the level of IFNγ was significantly higher against *B. cenocepacia* (p=0.046) compared to healthy volunteers. In contrast, healthy volunteers showed significantly higher response of IFNγ against *S. pneumoniae* (p= 0.011). There was no significant difference between CF patients and healthy subjects in IFNγ specific for *P. aeruginosa*, *A. fumigatus, M. tuberculosis*, Cytomegalovirus, *B. pertussis* and RSV. A higher production of IFNγ was observed in CF patients for *H. influenzae* and *M. catarrhalis*, but this did not yield statistical significance (p= 0.065 and p=0.098, respectively). No differences were observed between responses to positive controls.

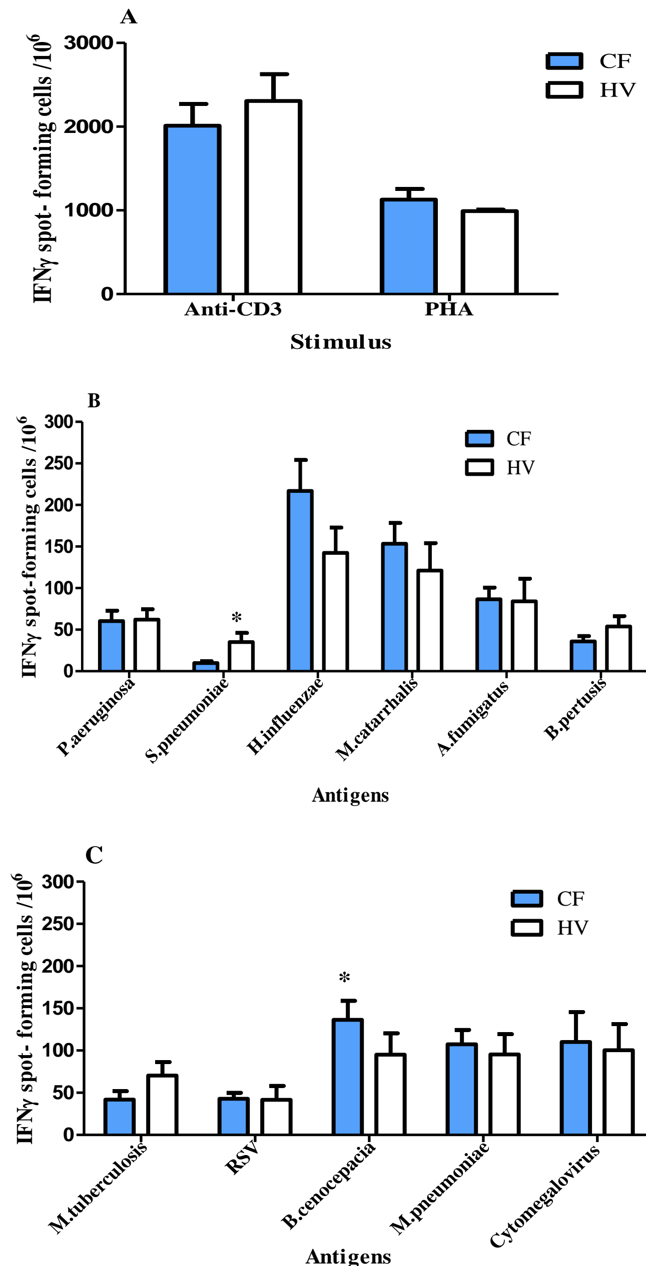


Figure 6.2(A-C): Antigen-specific response of T cells to different lung-infecting organisms. ELIspot was used to measure IFNγ against the lung antigens in CF patients and HV. * indicates p-value ≤ 0.05: (A) positive controls; (B) and (C) lung microbe antigens. The data are shown as mean± SEM. CF n=30 and HV n=27.

6.3.5 Relationship between T cell and antibody responses in CF patients

The relationship between antibody and T cell responses against the main key organisms was assessed for CF patients. A negative correlation was found between T cell and antibody response for *A. fumigatus* (p=0.086; Fig.6.3E), but statistical significance was not reached.

No correlation was observed for the other antigens including *P. aeruginosa, S. pneumoniae*, *H. influenzae* and *M. catarrhalis* in CF patients. The analysis of antibody and T cell responses for healthy volunteers are shown in chapter 5.

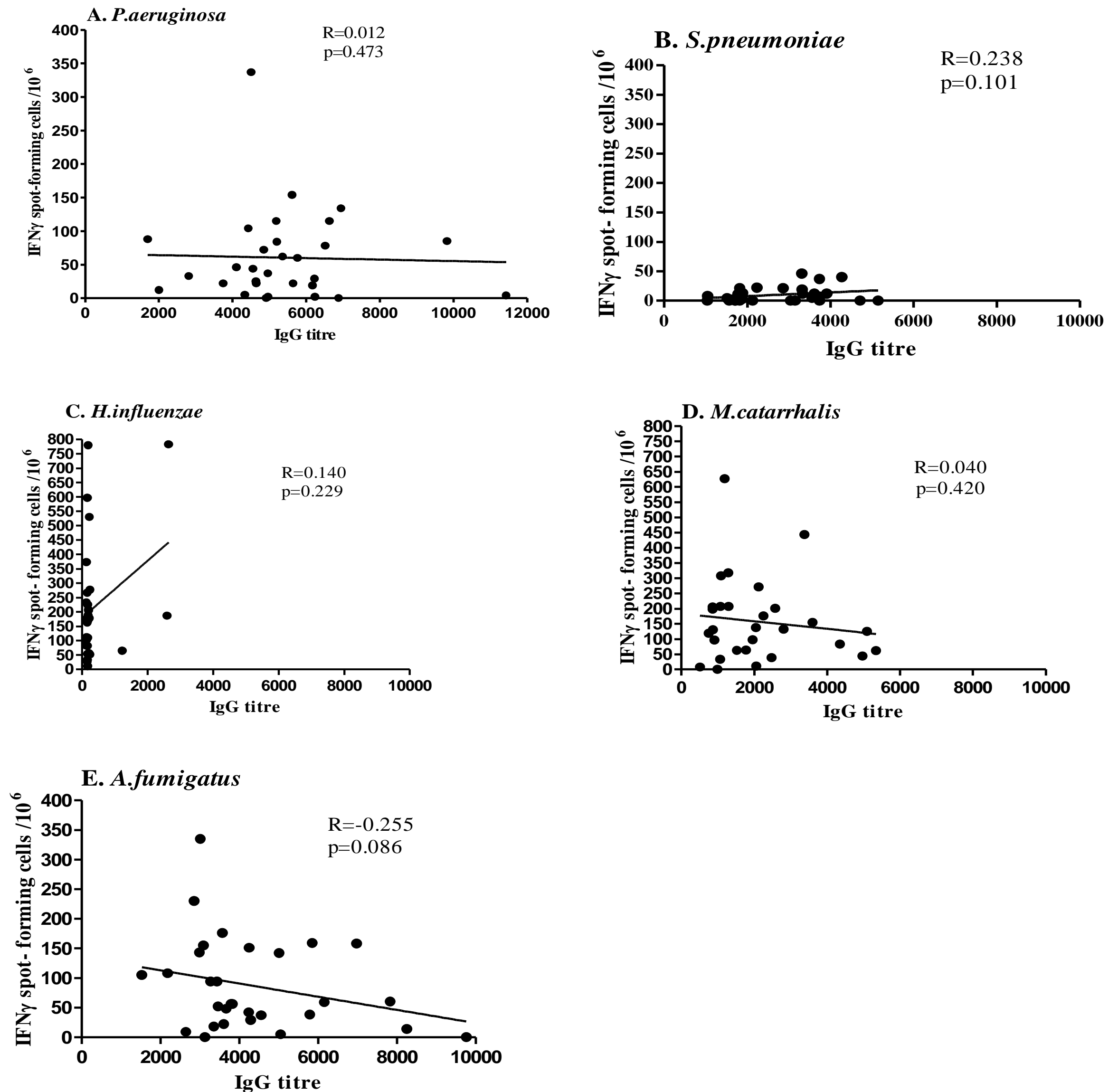


Figure 6.3(A-E): Relationship between antibody titre and T cell responses against lung antigens in CF patients.

(A) *P. aeruginosa*; (B) *S. pneumoniae*; (C) *H. influenzae*; (D) *M. catarrhalis* and (E) *A. fumigatus*; and. Data are shown as antibody titre and spot-forming cells/106 in each patient. Spearman analysis was used for the correlation and Mann-Whitney test for p-value.

6.3.6 Comparison of lung function with the clinical status (exacerbation and Stable patients)

To determine if exacerbations contribute to decline in lung function, the clinical status of patients with exacerbating status and those who were stable were compared for FEV_1 % predicted, as shown in Figure 6.4. A significant reduction in FEV_1 % predicted was found in the exacerbating group compared to stable patients (p=0.037).

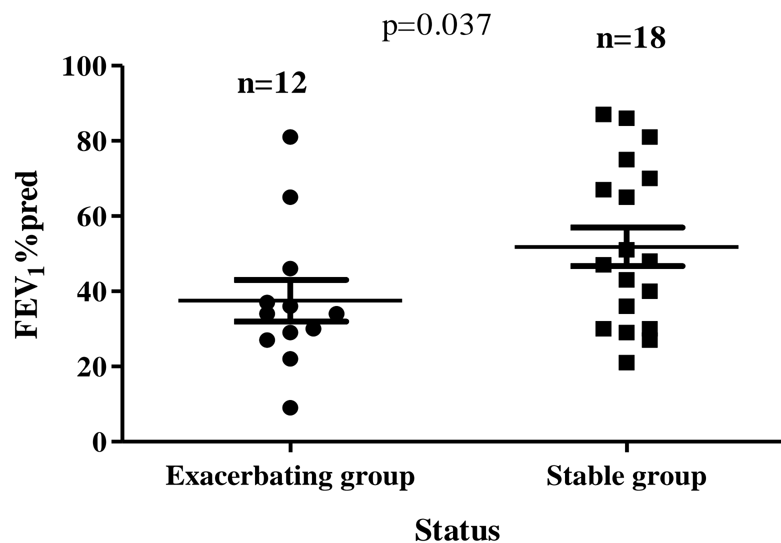


Figure 6.4: FEV_1 % predicted of stable and exacerbated patients.

FEV_1% was significantly reduced in the exacerbating group. Data are presented as mean± SEM. The numbers were in each group are indicated**.**

6.3.7 Antibody response compared with clinical status (exacerbating and stable) in CF patients

IgG titre against *P. aeruginosa* and *H. influenzae* was investigated in exacerbated compared to stable patients to determine any association of IgG antibody titre in both clinical status and its association with pulmonary exacerbation. Figure 6.5 shows that the stable group had significantly higher antibody titre compared to those presenting with exacerbations against *H. influenzae* (p=0.003), whereas there was no significant difference for *P. aeruginosa* titre.

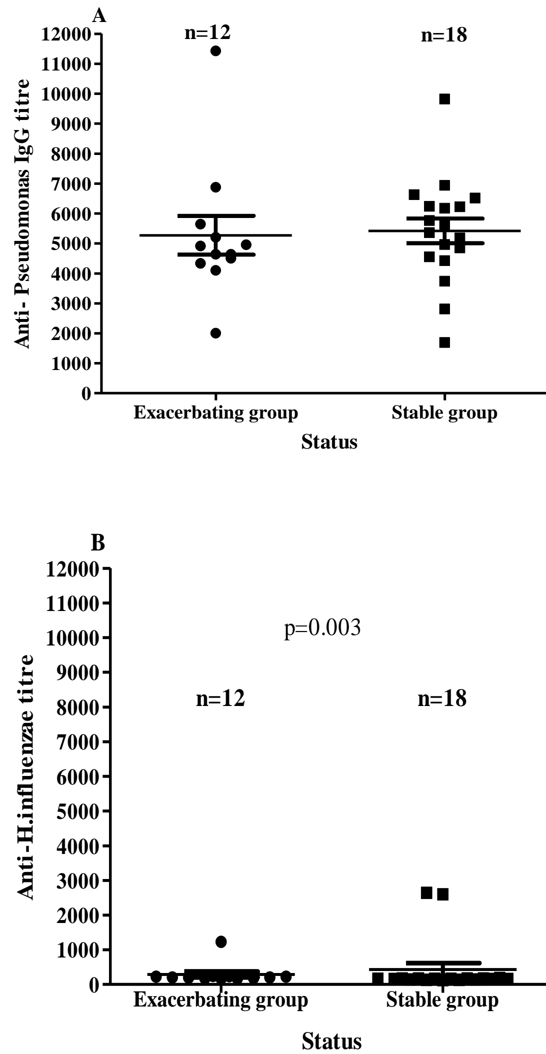


Figure 6.5(A-B): IgG titres compared in exacerbating and stable patients.

(A) antibody titres against *P. aeruginosa*;(B) IgG titre against *H. influenzae*. Data are shown as mean ± SEM. Significant differences between both groups were seen in response to *H. influenzae*.

6.3.8 Correlation between antibody, T cell responses and lung function in CF patients

The relationship between antibody and T cell responses against *P. aeruginosa* and *H. influenza,* in relation to lung function were assessed in CF patients to evaluate if the immune responses may predict subsequent lung function. No correlation was observed between IgG titre and FEV_1 predicted (Figure 6.6). However, a significant positive correlation was seen between T cell responses and FEV_1 % predicted in response to *P. aeruginosa* ($p=0.005$) (Figure 6.7).

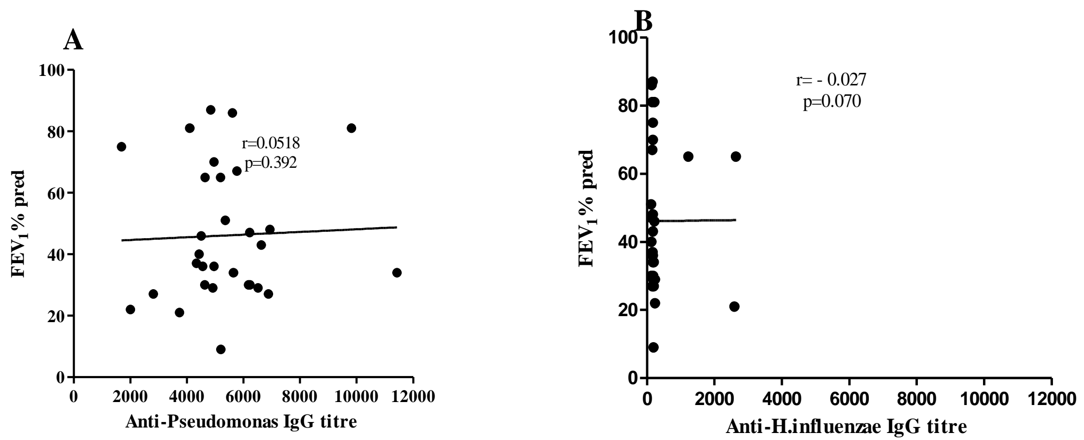


Figure 6.6 (A-B): Relationship between antibody titre and lung function in patients with CF. (A)*P. aeruginosa*;(B) *H. influenzae*. No significant correlation was found (Spearman correlation was used).

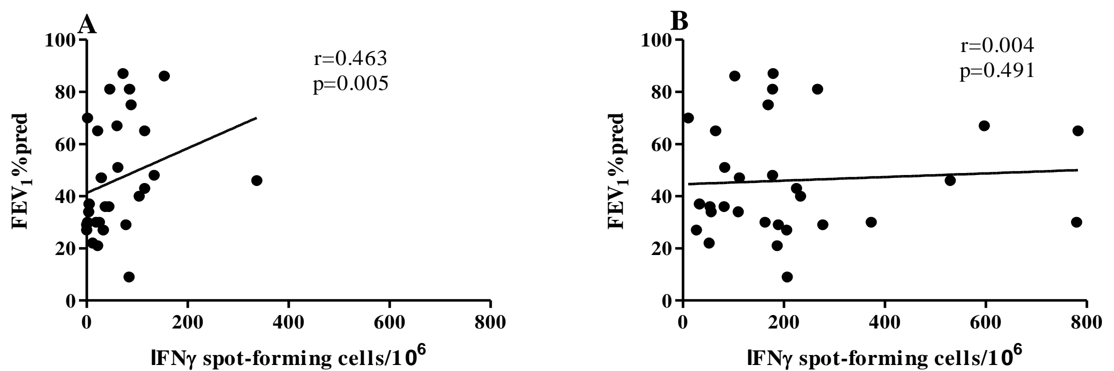


Figure 6.7(A-B): Relationship between T cell responses and lung function in patients with CF. (A) *P. aeruginosa*; (B) *H. influenzae*. A significant positive correlation was found in response to *P. aeruginosa* (Spearman correlation was used).

6.3.9 T cell responses compared with clinical status (exacerbating and stable) in CF patients.

Figure 6.8 shows T cell responses in the form of IFNγ against *P. aeruginosa* and *H. influenzae* in both status groups to see whether or not T cell responses may contribute to exacerbation in CF. The results show significantly reduced T cell responses against *P. aeruginosa* in patients with exacerbation (p=0. 026).

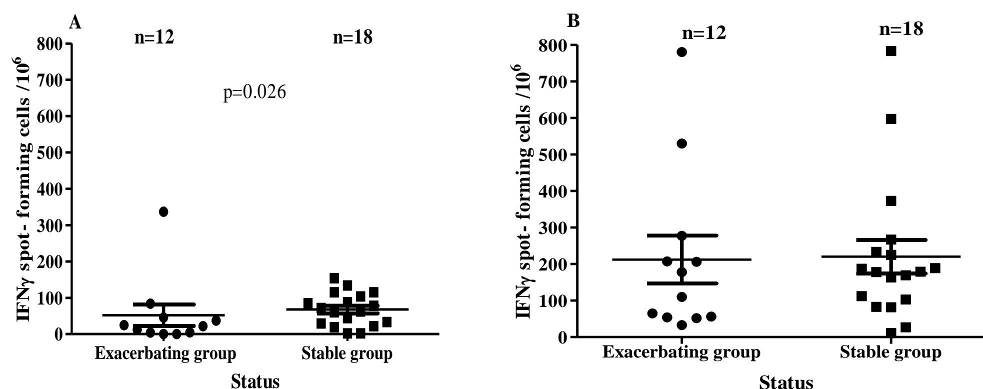


Figure 6.8 (A-B):IFNγ produced by T cells in exacerbating and stable groups.
(A) *P. aeruginosa*; (B) *H. influenzae* measured in patients with exacerbation and those who were stable. Data are shown as mean ±SEM. A significance differences in IFNγ were seen between both groups against *P. aeruginosa*.

6.3.10 Immune responses in Manchester CF (MAN) patients colonized by *Burkholderia* sp and *P. aeruginosa*

The serum IgG to *B. multivorans*, *B. cenocepacia* and *P. aeruginosa* was measured in 14 adult CF (MAN) patients colonized with these pathogenic bacteria as described in Table 6.3. The results in Figure 6.9 show that all patients who were chronically colonized by *B. multivorans* had higher specific antibody titre to this antigen, except for patient MAN111 who had slightly higher anti-*B. cenocepacia*. Similarly, increased antibody titre was also found in CF patients colonized by *B. cenocepacia* except for MAN74. Furthermore, patients who had *Pseudomonas* colonization also tended to have higher anti-Pseudomonas IgG titre, apart from MAN9. Although patients MAN52 and MAN9 were colonized with *B. multivorans,* and *P. aeruginosa,* the responses were only marginally higher versus both antigens. T cell responses were measured by ELIspot in three MAN patients for which PBMCs were available for IFNγ, IL-2, IL-5, IL-17 and IL-22 against lung antigens as described in Table 2.5.

The results in Figure 6.10 show that patient MAN122 had higher IFNγ against most of the antigens as well as IL-17 and IL-22 versus *Candida*. sp. In contrast, patient MAN 88 had higher IL-2, IL-17 and IL-22 against *Candida.* sp. As well as IL-5 against *Candida*.sp and *B.cepacia*. MAN 9 had higher IL-17 and IL-22 against *Candida*. sp.

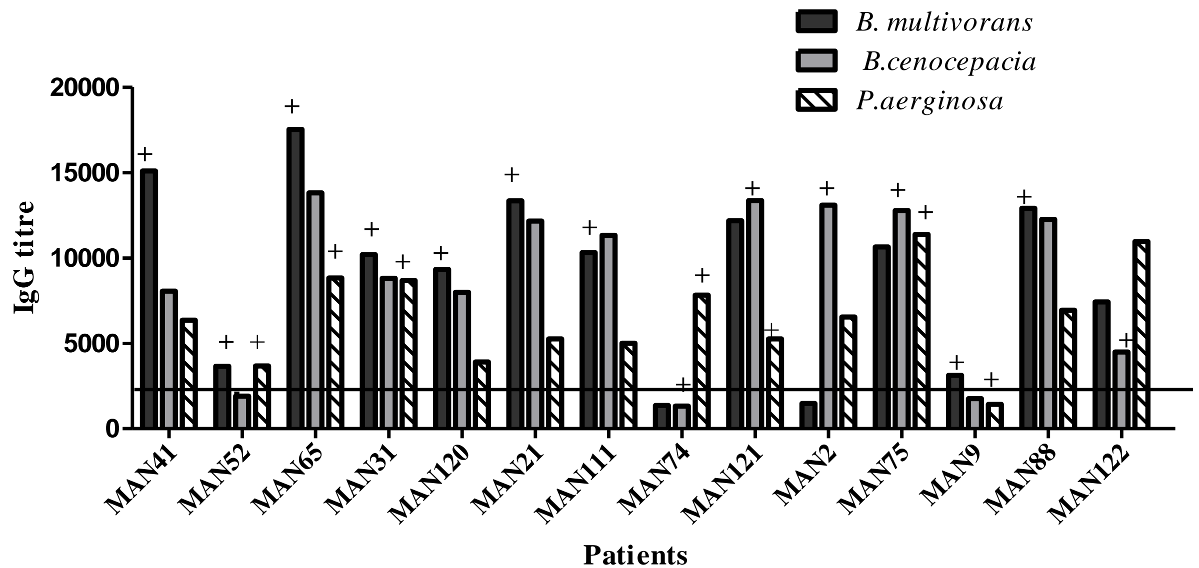


Figure 6.9: Antibody response against *B. multivorans*, *B. cenocepacia* and *P. aeruginosa* in CF Manchester patients.

Higher IgG titres were measured against these antigens in all patients colonized with these bacteria. Data are shown as end point titre for each patient. + indicates bacterial colonization. The straight horizontal line indicates mean anti-Pseudomonas IgG for healthy volunteers as a reference point.

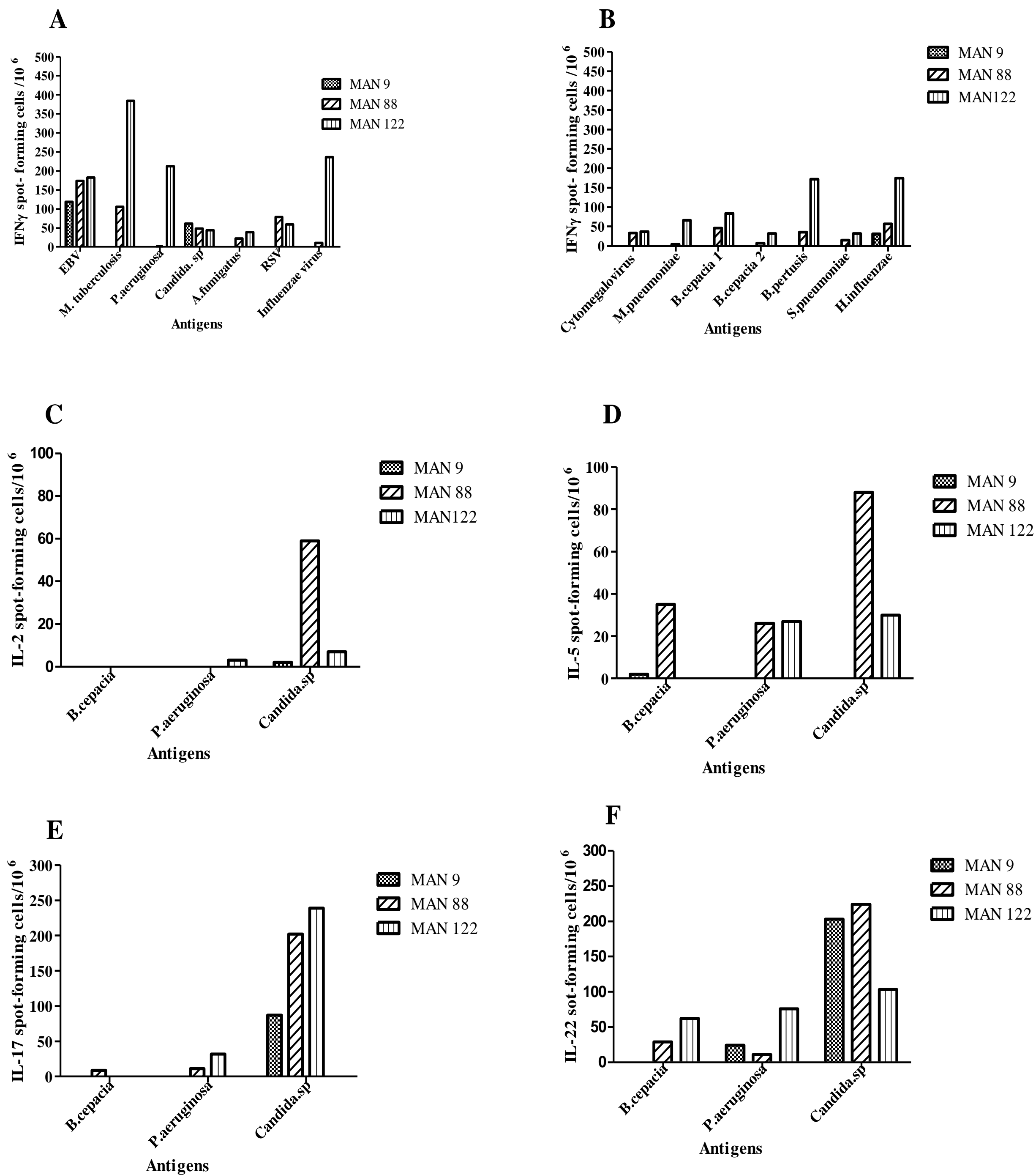


Figure 6.10 (A-F): T cell responses in three MAN samples against a panel of antigens in the form of IFNγ, IL-2, IL-5, IL-17 and IL-22.

Data are represented as spot-forming cell/10^6 for each patient.

6.4 Discussion

The culture-dependent approach revealed the typical microbial species colonizing the lungs of our CF patients, including bacteria and fungi. Table 6.2 confirms that all RVI patients were culture-positive for *P. aeruginosa;* indeed, it is the dominant pathogen isolated from CF airways. Previous studies found that 80-90% of CF patients were colonized with *P.aeruginosa* when associated with hospitalisation (Leid *et al.*, 2005, Moreau-Marquis *et al.*, 2008). The culture results in Tables 6.2 and 6.3 show that patients colonized by *P. aeruginosa* were also colonized by other bacterial species, suggesting that this bacterium can change the environment from aerobic to anaerobic and thus allowing more species to reside in the CF lung (Yoon *et al.*, 2002). Besides, infection with the mucoid phenotype of *Pseudomonas* has been shown to impair the innate host defence and facilitate colonization by other bacteria such as *B. cenocepacia* in CF patients (Chattoraj *et al.*, 2010).

6.4.1 Immune responses in CF patients

Antibody levels may provide an early indicator of microbial infection of the lung in cystic fibrosis, and they may also have a role in protection. Highly significant IgG titres against *P. aeruginosa*, *A. fumigatus*, *S. maltophilia* and *M. catarrhalis* were found in CF patients compared to healthy controls. This reflects the greater degree of exposure to, and colonization with, these organisms, as observed in the microbiology results showing the high number of patients colonized with these organisms (i.e. 100% and 60% of patients colonized with Pseudomonas and Aspergillus, respectively). Indeed, increased antibody levels are strongly associated with the recurrent microbial infection of *P.aeruginosa* and *S. maltophilia* as shown in previous studies (Milagres *et al.*, 2009, Teunis *et al.*, 2012, Wettlaufer *et al.*, 2016). The results showed that IgG titre also associated with *A. fumigatus* and *M. catarrhalis* in patients with CF. The role of IgG titre in the protection against microbial infection in

patients with BR and COPD has been discussed in chapters 3 and 4. However, the response against *H. influenzae* polysaccharide was significantly higher in healthy volunteers compared to CF patients and it may be that the patients failed to give normal IgG responses against polysaccharide antigens. It has been shown by Browning *et al.* (2014) that children with CF have impaired antibody response against unconjugated polysaccharide vaccine. Thus, it is possible that patients produce less IgG compared to healthy volunteers against this antigen. Similarly, the response against *S. pneumoniae* lysate was lower in patients with CF compared to healthy volunteers, indicating infrequent *S. pneumoniae* colonization found in patients with CF and the failure of these patients to produce antibody responses. Healthy volunteers may have higher degrees of antigen exposure as discussed in chapter 3, or may respond better and have higher memory responses than CF patients.

T cell responses against lung-infecting microbes in the form of IFNγ were also investigated in patients with CF compared to healthy volunteers. These findings demonstrate that CF patients had significantly higher IFNγ production compared to healthy volunteers only against *B. cenocepacia* ($p=0.046$). Furthermore, the responses were higher in CF patients compared to healthy controls for *H. influenzae* and *M. catarrhalis*, but neither difference was statistically significant, yielding p-values of 0.065 for *H. influenzae* and 0.098 for *M. catarrhalis.* The increased IFNγ production in patients with CF indicates that the response against these pathogens is a Th_1 response. A significantly lower T cell response against *S. pneumoniae* in CF patients was also observed ($p= 0.011$). Lower T cell responses were also shown by patients with BR, COPD and asthma in the previous chapter .

It has been suggested that Th_1 is characterised by the secretion of IFNγ and decreased antibody response, while Th_2 is characterised by production of IL-4, IL-5, IL-10 and pronounced antibody response through the activation of B cells. Therefore, reduced

production of IFNγ by $CD4^+$ T cells in response to these antigens is an impaired Th_1 function (Behar *et al.*, 2014). Indeed, a previous study conducted by Moss *et al.* (2000) showed that patients with CF produce lower levels of IFNγ and other Th_1 cytokines, whereas higher IL-10, a signature of Th_2 and immune regulation (Lehar and Bevan, 2004). This may be explained by the fact that the up-regulation of IL-10 leads to the inhibition of IFNγ production and decreased co-stimulatory molecules on macrophages, resulting in impedance of antigen presentation and impairing effective immune response against *P. aeruginosa*, *A. fumigatus* and other pathogens (Moss *et al.*, 2000). Further investigation of the role of antibodies, and specific Th_2 and IL-10 responses in CF is required.

Concerning the relationship between antibody and T cell responses in CF patients, no correlation was found between the magnitude of antibody and T cell responses, except for the negative correlation for *A. fumigatus*. Our findings were similar to those of a previous study conducted to examine antibody and T cell responses associated with experimental malaria infections (Walker *et al.*, 2015). As the authors described, the generation of antibody response requires either T cell help through cytokine production to undergo class switching from IgM to IgG, or the activation of B cells through CD40 ligand–expressing T cells, with cytokines being less effective.

It is important to note that in our study the healthy controls (with a mean age of 54 years) were older than the CF patients (mean age 29 years). Therefore, reduced responses in healthy volunteers against certain antigens may be associated with ageing. As has been reported previously, decline in the micro RNA (miRNA) network that is essential for T cell differentiations and consequently $CD4^+$ T cell function, are observed with ageing (Kushwah *et al.*, 2013).

6.4.2 Exacerbation and lung function test

Exacerbation is the main risk factor for progressive obstructive lung disease in CF, which over time is associated with a deterioration in lung function. FEV_1% predicted has been proven to be a marker of disease severity (Amadori *et al.*, 2009, Schluchter *et al.*, 2002). Therefore, FEV_1 in stable patients was compared to those having exacerbation in order to ensure that FEV_1 decline is the main indicator of CF deterioration. A significant reduction in FEV_1 was found in patients having exacerbation compared to those who were stable ($p=0.037$). This is in agreement with a previous study showing impaired lung functions in association with exacerbations in CF patients (Nelson, 2011), and validates the categorization by exacerbation status.

6.4.3 Immune response and exacerbations

In evaluating the role of antibody levels and the risk of pulmonary exacerbation, an analysis was carried out on the patient cohort. The results show that exacerbated patients showed significantly lower levels of IgG titre against *H. influenzae*, whereas no significant differences were found for *P. aeruginosa*. As stated above, higher antibody titres are associated with greater exposure to *P. aeruginosa,* which may be protective. In our study, stable and exacerbated patients were exposed to similar level of *P. aeruginosa* as ascertained by antibody level and microbial colonization. Previous researches has demonstrated no significant differences between bacterial load and exacerbations (Dickson *et al.*, 2014, Nelson, 2011, Tunney *et al.*, 2013). Therefore, as the colonization or exposure levels are similar, we would not expect to see a higher level of antibody in patients with exacerbations.

However, a significantly increased level of T cell responses against *Pseudomonas* was found in patients who were stable. Supporting this, lung function FEV1 % correlated with these T cell levels. The reason for this may be that, during acute exacerbation, T_{reg} cells suppress

pro-inflammatory cytokines such as IFNγ through production of IL-10 and TGF-β which are essential in preventing lung damage (Jin *et al.*, 2014, Tang and Bluestone, 2008).

6.4.4 Antibodies and T cells against *B. cenocepacia*, *B. multivorans* and *P. aeruginosa* in MAN patients

Increased antibody levels were broadly observed in MAN patients chronically colonized with *B. cenocepacia, B. multivorans and P.aeruginosa,* indicating that high microorganism exposure resulted in higher antibody response. Exceptions were that patient MAN74 was chronically colonized by *B. cencepacia* yet had low antibody titre. MAN122 was chronically colonized by *B. cenocepacia* and yet had lower antibody titre against *B.cenocepacia* than *B.multivorans*. It may be that antibodies exhibit cross-reactive activity against *B. cenocepacia*, and *B. multivorans* species (Makidon *et al.*, 2010). The lipid A endotoxic of lipopolysaccharide endotoxins is the main component of *B. multivorans* which is responsible for triggering immune responses, and it is implicated in the pathogenesis of CF (Ierano *et al.*, 2010). In this study, the cell lysate was used as an antigen to stimulate antibody responses , and it is not clear in our result whether the elevated antibody reacted against the outer membrane or the LPS portion.

As demonstrated in chapter four that increased antibody is associated with *P. aeruginosa* colonization, as has also been demonstrated in previous studies (Milagres *et al.*, 2009, Teunis *et al.*, 2012).

The analysis of T cell responses was limited to 3 patients. Despite being heterogeneous in nature, as a group the responses against colonizing bacteria showed secretion of IFNγ, IL-5, IL-17 and IL-22. Although not as high as the anti-candida responses, these results suggest a role of these cytokines in protection against infection and conversely in disease immunopathology.

6.5 Conclusion

Overall, these results show that patients with CF are colonized by various microbial species, with *P. aeruginosa* being the predominant pathogen. These pathogens stimulate immune responses, eliciting high IgG production. By contrast, the chronic microbial infection does not lead to a similarly-boosted T cell response. Moreover, in this study it was demonstrated that patients with exacerbation had significantly reduced lung function, which is a prognostic indicator for disease severity. The IFNγ response of T cells against *Pseudomonas* was also higher in stable patients and correlated positively with lung function, suggesting that these cells (or ones they are associated with) may be implicated to prevent pulmonary destruction and thus can be prognostic for pulmonary exacerbation.

7 Chapter Seven: General Discussion

Diseases of the airway, including bronchiectasis (BR), chronic obstructive pulmonary disease (COPD) and asthma, are characterised by persistent airway inflammation and involve both innate and adaptive arms of the immune response as a result of microbial infection(Dickson *et al.*, 2014). These factors facilitate disease progression and contribute to reduced life expectancy. The adaptive response is involved in the activation of leukocytes and production of inflammatory mediators and the stimulation of other cell types (Panettieri, 2002). These lymphocytes have two potential functions in respect to pulmonary lung diseases: to improve immunity against infection in association with inflammatory mechanisms, and to contribute to disease pathophysiology (Singh, 2014). A balance needs to be struck between the two processes. The challenge of understanding the immunopathogenesis of disease requires knowledge of the role of these cells, specific antibodies and cytokine profiles that may contribute to immunity. This research project was designed to investigate specific immune responses involving both T cells and antibodies in response to microorganisms that are associated with the status and progression of pulmonary conditions including BR, COPD, asthma and CF. A panel of lung antigens including *H. influenzae*, *P. aeruginosa, S. pneumoniae*, *M. catarrhalis* and *A. fumigatus* (see Table 2.1) was selected to test the antibody responses in different clinical groups compared to healthy volunteers. These microorganisms were found to be the most abundant microbes isolated from the sputum of patients with BR, COPD and CF. These disease groups display polymicrobial infection created by combinations of different microorganisms including, viruses, bacteria, fungi, which can facilitate other micro-organisms as co-infecting pathogens (Brogden *et al.*, 2005). In the respiratory tract, infection with viruses can promote secondary infection by bacteria by inducing immunosuppressive effects that lead to severe

complications (Peltola and McCullers, 2004). In contrast, *P. aeruginosa* can lyse other bacteria such as *S. aureus* and use it in certain environments where iron levels are low, contributing to persistent chronic *P. aeruginosa* infection in the CF lung (Mashburn *et al.*, 2005). The clinical groups studied here demonstrate high IgG titre against most of the antigens, being significant antigens. This suggests that microbial infections trigger the adaptive immune system and subsequently the production of high levels of immune responses. The high exposure to pathogenic microbes results in the boosting of the immune response, sustaining high levels of immunoglobulins in circulation (Wettlaufer *et al.*, 2016).

It has been found that increased IgG titres are associated with microbial infections in the lung. Antibodies can neutralize microbes by direct binding to their surface protein, which is important for attachment onto the host cells. Furthermore, antibodies stimulate the complement cascade and antibody-dependent cell-mediated cytotoxicity via the ligation of Fc receptors. The antibody most highly correlated with protection to be identified in the serum was the IgG type (Chiu and Openshaw, 2015). Therefore, it is clear that elevated IgG antibody following microbial infection is critical for protection. In contrast, asthmatic subjects showed lower level of IgG titre compared to other patients groups. This is possibly due to less frequent bacterial infection in patients with asthma. Furthermore, the switching of antibody isotype from IgM to IgE may be implicated in allergic diseases such as asthma (Holgate, 2012). Most previous research has tested the development of antibody responses to *P. aeruginosa* infection in CF patients (Doring and Hoiby, 1983, Winnie and Cowan, 1991). However, in our study, groups of patients with pulmonary conditions, including BR, COPD, asthma and CF, were tested for the relationships between immune responses, infection incidence and clinical status.

The deterioration of lung function was associated with chronic current microbial colonization in both BR and COPD patients for a proportion of the microorganisms.

Colonization of bacteria and the augmentation of inflammatory responses contribute to the progression of damage in the lung architecture, and over years results in the impairment of lung function. It has been found that lung tissue damage increases with age of the disease in patients with CF (Helbich *et al.*, 1999).

While a great deal of research in CF patients has found that exacerbation contributes to lung function decline, the definitive conclusion to determine the cause of exacerbation in BR and COPD is still uncertain (Amadori *et al.*, 2009, Sanders *et al.*, 2011). Several studies using culture approaches have found that there is no correlation between bacterial load and the induction of exacerbation (Dickson *et al.*, 2014, Tunney *et al.*, 2011, Wedzicha and Seemungal, 2007). Furthermore, in this study, reduced FEV_1% predicted was associated with exacerbations in patients with different scores as defined by BSI and compared to the FEV_1% of healthy volunteers. Similarly, impaired lung functions was also shown in exacerbated CF patients. Therefore, the decline in lung function may be a determinant of exacerbations in patients with BR and CF. Further analysis was performed to investigate the role of antibodies against *P. aeruginosa*, *M. catarrhalis* and *S. maltophilia* in patients with different degrees of exacerbation and FEV_1% predicted. These bacteria were found to be pathogens associated with reduced lung functions in BR and COPD. Significant differences in antibody titre were observed between these categories of exacerbations, suggesting that bacterial colonisation may trigger antibody and inflammatory responses leading to the induction of exacerbation (Perera *et al.*, 2007). However, no significant correlations between lung function and IgG titres for patients with BR, COPD, and CF were found. In contrast, exacerbated patients with CF have significant reductions in IgG against *H. influenzae*, which suggests different role for this bacterium in CF.

T lymphocytes are also involved in protection against infections through the secretion of specific cytokine molecules. Intracellular bacteria and viruses broadly stimulate Th_1,

whereas multicellular pathogens promote Th_2 cytokines (Lehar and Bevan, 2004). During infections, dendritic cells recognise the microbial pathogens via TLR and then migrate to the lymphoid organ where they interact with T cells and induce T cell responses. This may contribute to the inability to clear the infections (Lehar and Bevan, 2004). In this study, the clinical groups were tested for T cells responses, and in particular Th_1 through the production of IFNγ. The cells were initially stimulated with a selection of lysates and peptides to ensure that they were specifically activated. The findings show that patients with pulmonary diseases (BR, COPD and asthma) exhibit lower IFNγ production compared to healthy volunteers against most of the antigens. In these circumstances, higher frequencies of microbial infection show a trend for reduced T cell responses due to dysfunctional T cells (Tiringer *et al.*, 2013). Moreover, as the antibody levels were significantly higher against most of the antigens and were correlated with microbial colonisation, the responses of T cells were contradictory. This may indicate that the response of Th_2 response was skewed toward Th_2 rather than Th_1. The high degree of continuing antigenic stimulation correlates with more rapid T cell exhaustion, whereas the lower antigen exposures resulted in intermediate impairment (Behar *et al.*, 2014). It was concluded that the lack correlation between antibody and T cell responses in patients with lung disease is likely to be due to the different patterns of cells involved.

The findings showed that the response of T cells was significantly reduced in BR against *M. catarrhalis* and *P. aeruginosa* in hospitalised and exacerbated patients, respectively. Meanwhile, a significant reduction against *P. aeruginosa* was also found in exacerbated CF patients. Although there is no correlation between antibody levels, T cell response and FEV_1% predicted, a positive correlation was seen in relation to anti-Pseudomonas T cell response and FEV_1% in CF patients. The results also showed that patients with CF have the highest antibody whereas; comparable T cell responses in CF and HV against *P. aeruginosa*

(Figure 7.1). All of these findings suggest the important role of antibodies and T cells as prognostic markers for microbial infection and the progression of clinical outcomes such as reduced pulmonary functions and progression exacerbation.

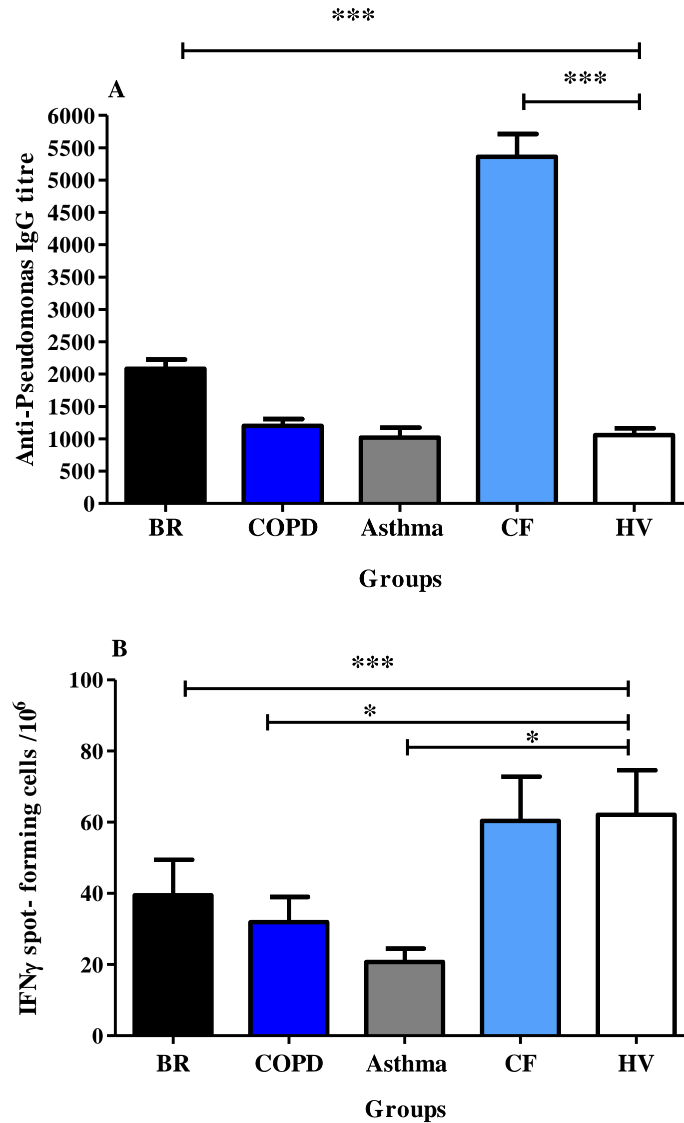


Figure 7.1(A-B): Antibody and T cell responses against *P.aeruginosa* in all clinical groups and healthy volunteers.

A, antibody; B, T cells responses against *P.aeruginosa*. Patients with CF showed the highest antibody response in all groups but T cell responses also were higher compared to patients but comparable to HV.

Conclusion and Future Work

In conclusion, the lungs of the BR, COPD and CF cohorts are colonized by polymicrobial communities comprising bacterial and fungal species. These microorganisms promote the perpetuation of immunological responses and drive a vicious cycle of re-infection and further inflammation. The study shows that patients with current microbial colonization are also associated with impaired $FEV_1\%$, and the lung function of most BR and COPD patients is in the GOLD stages II or III.

Measurement of antibody levels in patients with pulmonary diseases can help to identify those with microbial colonization, especially in individuals unable to produce sputum specimens and those at risk of pulmonary exacerbation who could benefit from earlier antibiotic treatment. Nevertheless, high microbial stimulation results in T cell exhaustion and is likely to lead to the impaired ability of cells to control the infection. T cells responses were not associated with pulmonary exacerbation in BR and COPD patients but they were in CF. Among the pathological mechanisms of established BR, COPD and CF, there are likely to be a large number of factors that interact to cause a specific level of disease. These clearly include the initial aetiology (genetics, triggers), microbial exposure and colonization, innate immunity and adaptive immunity. Any one parameter relating strongly with disease would seem to be difficult to identify. These findings offer merely a snapshot in time of the diseases, but indicate a role for immunological investigations as earlier diagnostic tools to predict microbial colonization using antibodies, and to determine the pulmonary exacerbations and function in certain groups. Further investigations are hence warranted.

Future Work

In this study, anti-microbial immune responses were tested on a single occasion in relation to microbial colonization and clinical status in different groups of pulmonary lung disease. Large datasets were generated from which hypothesis-driven analysis was carried out. Furthermore, the genomic analysis of sputum samples was also carried out, the results of which were not available for inclusion in the analysis in this thesis. Once available, complex multi-factor statistical analysis with multiple corrections would be warranted. Furthermore, the measurement of Th_{17}- and Th_2-cell responses were conducted only in three patients with CF due to the limitations of PBMCs, and therefore further investigation of these cytokines are necessary in a larger cohort to assess their role of the response to microbial infections in different clinical categories.

References

Abbas, A., Burstein, H. and Bogen, S. 1993. Determinants of helper T cell-dependent antibody production. *Semin Immunol.,* 5**,** 441-447.

Abreu, M. T. and Arditi, M. 2004. Innate immunity and toll-like receptors: clinical implications of basic science research. *J of Pediatrics,* 144**,** 421-429.

Acharya, K. R. and Ackerman, S. J. 2014. Eosinophil granule proteins: form and function. *J Biol Chem,* 289**,** 17406-15.

Agnello, D., Denimal, D., Lavaux, A., Blondeau-Germe, L., Lu, B., Gerard, N. P., Gerard, C. and Pothier, P. 2013. Intrarectal immunization and IgA antibody-secreting cell homing to the small intestine. *J Immunol,* 190**,** 4836-47.

Al-Ghamdi, H. S. and Anil, S. 2007. Serum antibody levels in smoker and non-smoker saudi subjects with chronic periodontitis. *J Periodontol,* 78**,** 1043-50.

Alberts, B., Johnson, A., Lewis, J., Raff, M. and Roberts, K. 2002 Molecular Biology of the Cell.4th edition. *B cells and Antibodies* New York: Garland Science.

Albiger, B., Dahlberg, S., Henriques-Normark, B. and Normark, S. 2007. Role of the innate immune system in host defence against bacterial infections: focus on the Toll-like receptors. *J Intern Med,* 261**,** 511-28.

Aldallal, N., McNaughton, E. E., Manzel, L. J., Richards, A. M., Zabner, J., Ferkol, T. W. and Look, D. C. 2002. Inflammatory response in airway epithelial cells isolated from patients with cystic fibrosis. *Am J Respir Crit Care Med,* 166**,** 1248-56.

Amadori, A., Antonelli, A., Balteri, I., Schreiber, A., Bugiani, M. and De Rose, V. 2009. Recurrent exacerbations affect FEV(1) decline in adult patients with cystic fibrosis. *Respir Med,* 103**,** 407-13.

Amin, P. B., Diebel, L. N. and Liberati, D. M. 2010. Secretory immunoglobulin A abrogates inflammatory responses and improves mortality after pseudomonas pneumonia. *J Trauma,* 68**,** 827-33.

Amulic, B., Cazalet, C., Hayes, G. L., Metzler, K. D. and Zychlinsky, A. 2012. Neutrophil function: from mechanisms to disease. *Annu Rev Immunol,* 30**,** 459-89.

Anderson, P., Sundstedt, A., Li, L., O'Neill, E. J., Li, S., Wraith, D. C. and Wang, P. 2003. Differential activation of signal transducer and activator of transcription (STAT)3 and STAT5 and induction of suppressors of cytokine signalling in T(h)1 and T(h)2 cells. *Int Immunol,* 15**,** 1309-17.

Anelli, T. and van Anken, E. 2013. Missing links in antibody assembly control. *Int J Cell Biol,* 2013**,** 1-9.

Angrill, J., Agusti, C., de Celis, R., Rano, A., Gonzalez, J., Sole, T., Xaubet, A., Rodriguez-Roisin, R. and Torres, A. 2002. Bacterial colonisation in patients with bronchiectasis: microbiological pattern and risk factors. *Thorax,* 57**,** 15-9.

Aoshiba, K. and Nagai, A. 2004. Differences in airway remodeling between asthma and chronic obstructive pulmonary disease. *Clinic Rev Allerg Immunol,* 27**,** 35-43.

Aureli, M., Schiumarini, D., Loberto, N., Bassi, R., Tamanini, A., Mancini, G., Tironi, M., Munari, S., Cabrini, G., Dechecchi, M. C. and Sonnino, S. 2016. Unravelling the role of sphingolipids in cystic fibrosis lung disease. *Chem Phys Lipids,* 200**,** 94-103.

Avery, D. T., Bryant, V. L., Ma, C. S., de Waal Malefyt, R. and Tangye, S. G. 2008. IL-21-induced isotype switching to IgG and IgA by human naive B cells is differentially regulated by IL-4. *J Immunol,* 181**,** 1767-1779.

Baldwin, A., Mahenthiralingam, E., Thickett, K. M., Honeybourne, D., Maiden, M. C., Govan, J. R., Speert, D. P., Lipuma, J. J., Vandamme, P. and Dowson, C. G. 2005. Multilocus sequence typing scheme that provides both species and strain differentiation for the Burkholderia cepacia complex. *J Clin Microbiol,* 43**,** 4665-73.

Bals, R. and Hiemstra, P. S. 2004. Innate immunity in the lung: how epithelial cells fight against respiratory pathogens. *Eur Respir j,* 23**,** 327-333.

Bandyopadhyay, D., Bose, S., Majumder, S. and Bandyopadhyay, S. 2004. Young's Syndrome An Uncommon Cause of Bronchiectasis. *Indian Academy of Clinical Medicine.,* 5,.

Barnes, P. J. 2008. The cytokine network in asthma and chronic obstructive pulmonary disease. *J Clinic Invest,* 118**,** 3546–3556.

Barnes, P. J., Shapiro, S. D. and Pauwels, R. A. 2003. Chronic obstructive pulmonary disease: molecular and cellular mechanisms. *Eur Respir J,* 22**,** 672-688.

Barrios, R. J., Kheradmand, F., Batts, L. and Corry, D. B. 2006. Asthma: Pathology and pathophysiology. *Arch Pathol Lab Med,* 130**,** 447-51.

Bayes, H. K., Bicknell, S., MacGregor, G. and Evans, T. J. 2014. T helper cell subsets specific for Pseudomonas aeruginosa in healthy individuals and patients with cystic ibrosis. *PLoS One,* 9.

Bayes, H. K., Ritchie, N. D., Ward, C., Corris, P. A., Brodlie, M. and Evans, T. J. 2016. IL-22 exacerbates weight loss in a murine model of chronic pulmonary Pseudomonas aeruginosa infection. *J Cyst Fibros*.

Bazan-Socha, S., Zuk, J., Jakiela, B., Pulka, G., Pelka, K. and Musial, J. 2012. Increased expression of alpha(2) (CD49b), alpha(4) (CD49d) and beta(1) (CD29) integrin subunits on peripheral blood T lymphocytes in clinically stable mild-to-moderate persistent asthma. *Pol Arch Med Wewn,* 122**,** 585-90.

Beasley, V., Joshi, P. V., Singanayagam, A., Molyneaux, P. L., Johnston, S. L. and Mallia, P. 2012. Lung microbiology and exacerbations in COPD. *Int J Chron Obstruct Pulmon Dis,* 7**,** 555-69.

Beck, J. M., Young, V. B. and Huffnagle, G. B. 2012. The microbiome of the lung. *Transl Res,* 160**,** 258-66.

Behar, S. M., Carpenter, S. M., Booty, M. G., Barber, D. L. and Jayaraman, P. 2014. Orchestration of pulmonary T cell immunity during Mycobacterium tuberculosis infection: Immunity interruptus. *Semin Immunol,* 26**,** 559-577.

Bergin, D. A., Hurley, K., Mehta, A., Cox, S., Ryan, D., O'Neill, S. J., Reeves, E. P. and McElvaney<N.G 2013. Airway inflammatory markers in individuals with cystic fibrosis and non-cystic fibrosis bronchiectasis. *J Inflamm Res,* 6**,** 1–11.

Bergmann, I. E., Neto, D. D. L., Neitzert, E., Malirat, V., Campos, R. D. M., Pulga, M., Muratovik, R., Quintino, D., Morgado, J. C. and Oliveira, M. 2006. Development of an inhibition ELISA test for the detection of non-capsid polyprotein 3ABC in viral suspensions destined for inactivated foot-and-mouth disease vaccines *Dev Biol (Basel),* 126**,** 241 - 250.

Blutt, S. E., Miller, A. D., Salmon, S. L., Metzger, D. W. and Conner, M. E. 2012. IgA is important for clearance and critical for protection from rotavirus infection. *Mucosal Immunol,* 5**,** 712-9.

Bomberger, J. M., Ye, S., MacEachran, D. P., Koeppen, K., Barnaby, R. L. and Stanton, B. A. 2011. A Pseudomonas aeruginosa toxin that hijacks the host ubiquitin proteolytic system. *PLoS Pathogens,* 7**,** e1001325.

Bouchara, J. P., Hsieh, H. Y., Croquefer, S., Barton, R., Marchais, V., Pihet, M. and Chang, T. C. 2009. Development of an oligonucleotide array for direct detection of fungi in sputum samples from patients with cystic fibrosis. *J Clin Microbiol,* 47**,** 142-52.

Bourke, S. J. 2002. Respiratory tract infections: A clinical overview. *Molecular Medical Microbiology ;London:Academic press,* 3**,** 1551-1563.

Boyton, R. J. 2008. Bronchiectasis. *Medicine,* 36**,** 315-320.

Boyton, R. J. 2012. Bronchiectasis. *Medicine,* 40**,** 267-272.

Boyton, R. J., Reynolds, C. J., Quigley, K. J. and Altmann, D. M. 2012. Immune mechanisms and the impact of the disrupted lung microbiome in chronic bacterial lung infection and bronchiectasis. *Clin Exp Immunol,* 171**,** 117–123.

Boyton, R. J., Reynolds, C. J., Quigley, K. J. and Altmann, D. M. 2013. Immune mechanisms and the impact of the disrupted lung microbiome in chronic bacterial lung infection and bronchiectasis. *Clin Exp Immunol,* 171**,** 117-23.

Braido, F., Tarantini, F., Ghiglione, V., Melioli, G. and Canonica, G. W. 2007. Bacterial lysate in the prevention of acute exacerbation of COPD and in respiratory recurrent infections. *Int J Chron Obstruct Pulmon Dis.,* 2**,** 335 - 345.

Brandtzaeg, P. and Johansen, F. E. 2005. Mucosal B cells: phenotypic characteristics, transcriptional,regulation, and homing properties. *Immunol Rev. ,* 206**,** 32–63.

Brett, M. M., Ghoneim, A. T. and Littlewood, J. M. 1986. Serum antibodies to Pseudomonas aeruginosa in cystic fibrosis. *Arch Dis Child,* 61**,** 1114-20.

Brett, M. M., Ghoneim, A. T. and Littlewood, J. M. 1987. Serum IgG antibodies in patients with cystic fibrosis with early Pseudomonas aeruginosa infection. *Arch Dis Child,* 62**,** 357-61.

Brett, M. M., Ghoneim, A. T. and Littlewood, J. M. 1988. An ELISA to detect antipseudomonal IgA antibodies in sera of patients with cystic fibrosis. *J Clin Pathol,* 41**,** 1130-4.

Brett, M. M., Ghoneim, A. T., Littlewood, J. M. and Losowsky, M. S. 1986. Development of enzyme linked immunosorbent assay (ELISA) to detect antibodies to Pseudomonas aeruginosa cell surface antigens in sera of patients with cystic fibrosis. *J Clin Pathol,* 39**,** 1124-9.

Brogden, K. A., Guthmiller, J. M. and Taylor, C. E. 2005. Human polymicrobial infections. *Lancet,* 365**,** 253-5.

Browning, M. J., Lim, M. T., Kenia, P., Whittle, M., Doffinger, R., Barcenas-Morales, G., Kumararatne, D., Viskaduraki, M., O'Callaghan, C. and Gaillard, E. A. 2014. Pneumococcal polysaccharide vaccine responses are impaired in a subgroup of children with cystic fibrosis. *J Cyst Fibros,* 13**,** 632-8.

Brusselle, G. G., Joos, G. F. and Bracke, K. R. 2011. New insights into the immunology of chronic obstructive pulmonary disease. *The Lancet,* 378**,** 1015-26.

Busse, W. and Lemanske, R. F. 2001. Advances in immunology - Asthma. *N Engl J Med,* 344**,** 350 - 362.

Caballero, E., Drobnic, M. E., Perez, M. T., Manresa, J. M., Ferrer, A. and Orriols, R. 2001. Anti-Pseudomonas aeruginosa antibody detection in patients with bronchiectasis without cystic fibrosis. *Thorax,* 56**,** 669-74.

Caccamo, N., Guggino, G., Joosten, S. A., Gelsomino, G., Di Carlo, P., Titone, L., Galati, D., Bocchino, M., Matarese, A., Salerno, A., Sanduzzi, A., Franken, W. P., Ottenhoff, T. H. and Dieli, F. 2010. Multifunctional CD4(+) T cells correlate with active Mycobacterium tuberculosis infection. *Eur J Immunol,* 40**,** 2211-20.

Cai, A. Q., Landman, K. A., Hughes, B. D. and Witt, C. M. 2007. T cell development in the thymus: From periodic seeding to constant output. *J Theor Biol,* 249**,** 384-394.

Caramori, G., Casolari, P., Di Gregorio, C., Saetta, M., Baraldo, S., Boschetto, P., Ito, K., Fabbri, L. M., Barnes, P. J., Adcock, I. M., Cavallesco, G., Chung, K. F. and Papi, A. 2009. MUC5AC expression is increased in bronchial submucosal glands of stable COPD patients. *Histopathology,* 55**,** 321-31.

Carola, R., Harley, J. P. and Noback, C. R. 1990. *Human anatomy and physiology,* New York, McGraw-Hill,.

Casaulta, C., Schoni, M. H., Weichel, M., Crameri, R., Jutel, M., Daigle, I., Akdis, M., Blaser, K. and Akdis, C. A. 2003. IL-10 controls Aspergillus fumigatus- and Pseudomonas aeruginosa-specific T-cell response in cystic fibrosis. *Pediatr Res,* 53**,** 313-9.

Castro, M., Schweiger, T., Yin-DeClue, H., Ramkumar, T., Christie, C., Zheng, J., Cohen, R., Schechtman, K., Strunk, R. and Bacharier, L. 2008. Cytokine response after severe RSV bronchiolitis in early life. *J Allergy Clin Immunol,* 122**,** 726-733.

Cavacini, L. A., Kuhrt, D., Duval, M., Mayer, K. and Posner, M. R. 2003. Binding and neutralization activity of human IgG1 and IgG3 from serum of HIV-infected individuals. *AIDS Res Hum Retroviruses,* 19**,** 785-92.

Cazzola, M. and Matera, M. G. 2012. IL-17 in chronic obstructive pulmonary disease. *Expert Rev Respir Med,* 6**,** 135-8.

Chanez, P., Bourdin, A., Vachier, I., Godard, P., Bousquet, J. and Vignola, A. M. 2004. Effects of inhaled corticosteroids on pathology in asthma and chronic obstructive pulmonary disease. *Proc Am Thorac Soc,* 1**,** 184-90.

Chattoraj, S. S., Murthy, R., Ganesan, S., Goldberg, J. B., Zhao, Y., Hershenson, M. B. and Sajjan, U. S. 2010. Pseudomonas aeruginosa alginate promotes Burkholderi cenocepacia persistence in cystic fibrosis transmembrane conductance regulator knockout mice. *Infect Immun,* 78**,** 984-93.

Chaudhry, R., Ghosh, A. and Chandolia, A. 2016. Pathogenesis of Mycoplasma pneumoniae: An update. *Indian J Med Microbiol,* 34**,** 7-16.

Chaudhuri, J. and Jasin, M. 2007. Antibodies Get a Break. *Science,* 315**,** 335-336.

Chawla, K., Vishwanath, S., Manu, M. K. and Lazer, B. 2015. Influence of pseudomonas aeruginosa on exacerbation in patients with bronchiectasis. *J Glob Infect Dis,* 7**,** 18-22.

Chen, J., Trounstine, M., Alt, F. W., Young, F., Kurahara, C., Loring, J. F. and Huszar, D. 1993. Immunoglobulin gene rearrangement in B cell deficient mice generated by targeted deletion of the JH locus. *Int Immunol,* 5**,** 647-56.

Chen, K. and Cerutti, A. 2011. The function and regulation of immunoglobulin D. *Curr Opin Immunol,* 23**,** 345-352.

Cheng, S. L., Wang, H. C., Yu, C. J., Tsao, P. N., Carmeliet, P., Cheng, S. J. and Yang, P. C. 2009. Prevention of elastase-induced emphysema in placenta growth factor knock-out mice. *Respir Res,* 10**,** 115.

Cheroutre, H. and Lambolez, F. 2008. Doubting the TCR coreceptor function of CD8alphaalpha. *Immunity,* 28**,** 149-59.

Chhabra, N. 2014. *Colorimetry- Simplified* [Online]. Dr. Namrata Chhabra. [Accessed 08/03/2016 2016].

Chiu, C. and Openshaw, P. J. 2015. Antiviral B cell and T cell immunity in the lungs. *Nat Immunol,* 16**,** 18-26.

Choi, W. T. and An, J. 2011. Biology and clinical relevance of chemokines and chemokine receptors CXCR4 and CCR5 in human diseases. *Exp Biol Med (Maywood),* 236**,** 637-47.

Cigana, C., Lore, N. I., Riva, C., De Fino, I., Spagnuolo, L., Sipione, B., Rossi, G., Nonis, A., Cabrini, G. and Bragonzi, A. 2016. Tracking the immunopathological response to Pseudomonas aeruginosa during respiratory infections. *Sci Rep,* 6**,** 21465.

Clancy, J. and Blake, D. 2013. Pathophysiology and pharmacological management of asthma from a nature-nurture perspective. *Primary Health Care.*
, 23**,** 34-41.

Clarke, D., Letendre, C., Lecours, M. P., Lemire, P., Galbas, T., Thibodeau, J. and Segura, M. 2016. Group B Streptococcus Induces a Robust IFN-gamma Response by CD4(+) T Cells in an In Vitro and In Vivo Model. *J Immunol Res,* 2016**,** 5290604.

Cole, P. J. 1986. Inflammation: a two-edged sword--the model of bronchiectasis. *Eur J Respir Dis Suppl,* 147**,** 6-15.

Conese, M., Copreni, E., Gioia, S. D., Rinaldis, P. D. and Fumarulo, R. 2003. Neutrophil recruitment and airway epithelial cell involvement in chronic cystic fibrosis lung disease. *J Cyst Fibrosis,* 2**,** 129-135.

Conley, J. M., Gallagher, M. P. and Berg, L. J. 2016. T cells and gne regulation: the switching on and turning up of genes after T cell receptor stimulation in CD8 T cells. *Front Immunol,* 7**,** 76.

Cooper, M. D. and Alder, M. N. 2006. The evolution of adaptive immune systems. *Cell,* 124**,** 815-822.

Cordon, S. M., Elborn, J. S., Rayner, R. J., Hiller, E. J. and Shale, D. J. 1992. IgG antibodies in early Pseudomonas aeruginosa infection in cystic fibrosis. *Arch Dis Child,* 67**,** 737-40.

Cosio, M. G., Saetta, M. and Agusti, A. 2009. Immunologic aspects of chronic obstructive pulmonary disease . Mechanisms of disease. *N Engl J Med,* 360**,** 2445-54.

Courtney, J. M., Ennis, M. and Elborn, J. S. 2004. Cytokines and inflammatory mediators in cystic fibrosis. *J Cyst Fibrosis,* 3**,** 223–231.

Crawford, A., Angelosanto, J. M., Kao, C., Doering, T. A., Odorizzi, P. M., Barnett, B. E. and Wherry, E. J. 2014. Molecular and transcriptional basis of CD4(+) T cell dysfunction during chronic infection. *Immunity,* 40**,** 289-302.

Croft, M., So, T., Duan, W. and Soroosh, P. 2009. The significance of OX40 and OX40L to T-cell biology and immune disease. *Immunol Rev,* 229**,** 173-91.

Cukor, G., Blacklow, N. R., Nowak, N. A., Rich, C. M., Braverman, L. E. and Fischer, R. A. 1983. Comparative analysis of serum antibody responses to Pseudomonas aeruginosa exotoxin A by cystic fibrosis and intensive care unit patients. *J Clin Microbiol,* 18**,** 457-62.

Cullen, L. and McClean, S. 2015. Bacterial adaptation during chronic respiratory infections. *Pathogens,* 4**,** 66-89.

D'hulst, A. I., Maes, T., Bracke, K. R., Demedts, I. K., Tournoy, K. G., Joos, G. F. and Brusselle, G. G. 2005. Cigarette smoke-induced pulmonary emphysema in scid-mice. Is the acquired immune system required? *Respiratory Research,* 6**,** 147.

Danska, J. S., Livingstone, A. M., Paragas, V., Ishihara, T. and Fathman, C. G. 1990. The presumptive CDR3 regions of both T cell receptor alpha and beta chains determine T cell specificity for myoglobin peptides. *J Exp Med,* 172**,** 27-33.

Davies, G., Wells, A. U., Doffman, S., Watanabe, S. and Wilson, R. 2006. The effect of Pseudomonas aeruginosa on pulmonary function in patients with bronchiectasis. *Eur Respir J,* 28**,** 974-9.

de Bree, G. J., Daniels, H., Schilfgaarde, M., Jansen, H. M., Out, T. A., van Lier, R. A. and Jonkers, R. E. 2007. Characterization of CD4+ memory T cell responses directed against common respiratory pathogens in peripheral blood and lung. *J Infect Dis,* 195**,** 1718-25.

De Maria, R., Cifone, M. G., Trotta, R., Rippo, M. R., Festuccia, C., Santoni, A. and Testi, R. 1994. Triggering of human monocyte activation through CD69, a member of the natural killer cell gene complex family of signal transducing receptors. *J Exp Med,* 180**,** 1999-2004.

de Vrankrijker, A. M. M., Wolfs, T. F. W. and van der Ent, C. K. 2010. Challenging and emerging pathogens in cystic fibrosis. *Paediatr Respir Rev.,* 11**,** 246-254.

Decramer, M., Janssens, W. and Miravitlles, M. 2012. Chronic obstructive pulmonary disease. *The Lancet,* 379**,** 1341-1351.

Decramer, M. and Vestbo, J. 2014. Global strategy for the dagnosis, managment, and prevention of chronic obstructive pulmonary disease *Global Initative For Chronic Obstructive lung Disease.*

Demedts, I. K., Bracke, K. R., Van Pottelberge, G., Testelmans, D., Verleden, G. M., Vermassen, F. E., Joos, G. F. and Brusselle, G. G. 2007. Accumulation of dendritic cells and increased CCL20 levels in the airways of patients with chronic obstructive pulmonary disease. *Am J Respir Crit Care Med,* 175**,** 998-1005.

den Haan, J. M. M., Arens, R. and van Zelm, M. C. 2014. The activation of the adaptive immune system: Cross-talk between antigen-presenting cells, T cells and B cells. *Immunol Lett,* 162**,** 103-112.

Dickson, R. P., Erb-Downward, J. R. and Huffnagle, G. B. 2013. The role of the bacterial microbiome in lung disease. *Expert Rev Respir Med,* 7**,** 245-57.

Dickson, R. P., Martinez, F. J. and Huffnagle, G. B. 2014. The role of the microbiome in exacerbations of chronic lung diseases. *Lancet,* 384**,** 691-702.

Dogru, D., Nik-Ain, A., Kiper, N., Gocmen, A., Ozcelik, U., Yalcin, E. and Aslan, A. T. 2005. Bronchiectasis: the consequence of late diagnosis in chronic respiratory symptoms. *J Trop Pediatr,* 51**,** 362-5.

Dolan, D. E. and Gupta, S. 2014. PD-1 pathway inhibitors: changing the landscape of cancer immunotherapy. *Cancer Control,* 21**,** 231-7.

Donne, M., Lechner, A. and Rock, J. 2015. Evidence for lung epithelial stem cell niches. *BMC Dev Biol, ,* 15**,** 32.

Doring, G. and Hoiby, N. 1983. Longitudinal study of immune response to Pseudomonas aeruginosa antigens in cystic fibrosis. *Infect Immun,* 42**,** 197-201.

Du, Q., Jin, J., Liu, X. and Sun, Y. 2016. Bronchiectasis as a Comorbidity of Chronic Obstructive Pulmonary Disease: A Systematic Review and Meta-Analysis. *PLoS One,* 11**,** e0150532.

Dubin, P. J., McAllister, F. and Kolls, J. K. 2007. Is cystic fibrosis a TH17 disease? *Inflamm Res.,* 56**,** 221-7.

Dutta, N. 2013. Diagnosis of COPD. *InnovAiT: The RCGP Journal for Associates in Training,* 6**,** 223-231.

Edholm, E. S., Bengten, E. and Wilson, M. 2011. Insights into the function of IgD. *Dev Comp Immunol,* 35**,** 1309-16.

Edwards, M. R., Bartlett, N. W., Hussell, T., Openshaw, P. and Johnston, S. L. 2012. The microbiology of asthma. *Nat Rev Microbiol,* 10**,** 459-71.

Emerson, J., McNamara, S., Buccat, A., Worrell, K. and Burns, J. 2010. Changes in cystic fibrosis sputum microbiology in the United States between 1995 and 2008. *Pediatr Pulmonol,* 45**,** 363-370.

Fireman, P. 2003. Understanding Asthma Pathophysiology. *Allergy Asthma Proc. ,* 24**,** 79-83.

Fireman, P. Understanding asthma pathophysiology. Allergy Asthma Proc., 2003. OceanSide Publications, Inc, 79-83.

Flajnik, M. F. and Du Pasquier, L. 2004. Evolution of innate and adaptive immunity: can we draw a line? *Trends Immunol,* 25**,** 640-4.

Forman, R., Bramhall, M., Logunova, L., Svensson-Frej, M., Cruickshank, S. M. and Else, K. J. 2016. Eosinophils may play regionally disparate roles in influencing IgA(+) plasma cell numbers during large and small intestinal inflammation. *BMC Immunol,* 17**,** 12.

Fremd, C., Schuetz, F., Sohn, C., Beckhove, P. and Domschke, C. 2013. B cell-regulated immune responses in tumor models and cancer patients. *Oncoimmunology,* 2**,** e25443.

Fuchs, T. A., Abed, U., Goosmann, C., Hurwitz, R., Schulze, I., Wahn, V., Weinrauch, Y., Brinkmann, V. and Zychlinsky, A. 2007. Novel cell death program leads to neutrophil extracellular traps. *J Cell Biol,* 176**,** 231-41.

Fuschillo, S., De Felice, A. and Balzano, G. 2008. Mucosal inflammation in idiopathic bronchiectasis: cellular and molecular mechanisms. *Eur Respir J,* 31**,** 396-406.

Gadgil, A. and Duncan, S. 2008. Role of T-lymphocytes and pro-inflammatory mediators in the pathogenesis of chronic obstructive pulmonary disease. *Int J Chron Obstruct Pulmon Dis,* 3**,** 531–541.

Gaga, M., Bentley, A. M., Humbert, M., Barkans, J., O'Brien, F., Wathen, C. G., Kay, A. B. and Durham, S. R. 1998. Increases in CD4+ T lymphocytes, macrophages, neutrophils and interleukin 8 positive cells in the airways of patients with bronchiectasis. *Thorax,* 53**,** 685-91.

Gangadharan, D. and Cheroutre, H. 2004. The CD8 isoform CD8αα is not a functional homologue of the TCR co-receptor CD8αβ. *Curr Opin Immunol,* 16**,** 264-270.

Ganjalikhani Hakemi, M., Ghaedi, K., Andalib, A., Hosseini, M. and Rezaei, A. 2011. Optimization of human Th17 cell differentiation in vitro: evaluating different polarizing factors. *In Vitro Cell Dev Biol Anim,* 47**,** 581-92.

Garbino, J., Soccal, P. M., Aubert, J.-D., Rochat, T., Meylan, P., Thomas, Y., Tapparel, C., Bridevaux, P.-O. and Kaiser, L. 2009. Respiratory viruses in bronchoalveolar lavage: a hospital-based cohort study in adults. *Thorax,* 64**,** 399-404.

Garcia, G., Taille, C., Laveneziana, P., Bourdin, A., Chanez, P. and Humbert, M. 2013. Anti-interleukin-5 therapy in severe asthma. *Eur Respir Rev,* 22**,** 251 - 257

Garraud, O. and Nutman, T. B. 1996. The role of cytokines in human B-cell differentiation into immunoglobulin-secreting cells. *Bulletin de l'Institut Pasteur,* 94**,** 285-309.

Gessner, E. J., Heiken, H., Tamm, A. and Schmidt, E. R. 1998. *The IgG Fc receptor family*.

Gillissen, A. and Paparoupa, M. 2014. Inflammation and infections in asthma. *Clin Respir J* 9**,** 257–269.

Giscombe, R., Wang, X. B., Kakoulidou, M. and Lefvert, A. K. 2006. Characterization of the expanded T-cell populations in patients with Wegener's granulomatosis. *J Intern Med,* 260**,** 224-30.

Goldklang, M., Golovatch, P., Zelonina, T., Trischler, J., Rabinowitz, D., Lemaitre, V. and D'Armiento, J. 2012. Activation of the TLR4 signaling pathway and abnormal cholesterol efflux lead to emphysema in ApoE-deficient mice. *Am J Physiol Lung Cell Mol Physiol,* 302**,** L1200-8.

Goldsby, R. A., Osborne, B. A., Kuby, J. K. and Thomas, J. 1939. *Kuby Immunology,* New York: USA, W.H. Freeman and Company

Gompertz, S., O'Brien, C., Bayley, D. L., Hill, S. L. and Stockley, R. A. 2001. Changes in bronchial inflammation during acute exacerbations of chronic bronchitis. *Eur Respir J,* 17**,** 1112-9.

Gonzalez, D., van der Burg, M., Garcia-Sanz, R., Fenton, J. A., Langerak, A. W., Gonzalez, M., van Dongen, J. J., San Miguel, J. F. and Morgan, G. J. 2007. Immunoglobulin gene rearrangements and the pathogenesis of multiple myeloma. *Blood,* 110**,** 3112-21.

Gorska, K., Krenke, R., Kosciuch, J., Korczynski, P., Zukowska, M., Domagala-Kulawik, J., Maskey-Warzechowska, M. and Chazan, R. 2009. Relationship between airway inflammation and remodeling in patients with asthma and chronic obstructive pulmonary disease. *Eur J Med Res,* 14 Suppl 4**,** 90-6.

Gramaglia, I., Jember, A., Pippig, S. D., Weinberg, A. D., Killeen, N. and Croft, M. 2000. OX40 induces CCL20 expression in the context of antigen stimulation: an expanding role of co-stimulatory molecules in chemotaxis. *J Immunol,* 165**,** 3043-50.

Grimwood, K. 2011. Airway microbiology and host defences in paediatric non-CF bronchiectasis. *Paediatr Respir Rev,* 12**,** 111-118.

Grumelli, S., Corry, D. B., Song, L.-Z., Song, L., Green, L., Huh, J., Hacken, J., Espada, R., Bag, R., Lewis, D. E., Kheradmand, F. and Barnes, P. J. 2004. An immune basis for lung parenchymal destruction in chronic obstructive pulmonary disease and emphysema. *PLOS Medicine,* 1**,** e8.

Guan, W. J., Gao, Y. H., Xu, G., Lin, Z. Y., Tang, Y., Li, H. M., Lin, Z. M., Jiang, M., Zheng, J. P., Chen, R. C. and Zhong, N. S. 2015. Inflammatory responses, spirometry, and quality of life in subjects with bronchiectasis exacerbations. *Respir Care,* 60**,** 1180-9.

Gwyer Findlay, E. and Hussell, T. 2012. Macrophage-mediated inflammation and disease: a focus on the lung. *Mediators Inflamm,* 2012**,** 1-6.

Haas, K. M., Poe, J. C., Steeber, D. A. and Tedder, T. F. 2005. B-1a and B-1b cells exhibit distinct developmental requirements and have unique functional roles in innate and adaptive immunity to S. pneumoniae. *Immunity,* 23**,** 7-18.

Hamilton, R. G., MacGlashan, D. W., Jr. and Saini, S. S. 2010. IgE antibody-specific activity in human allergic disease. *Immunol Res,* 47**,** 273-84.

Han, M. K., Sethi, S., Toews, G. B., Young, V. B., Wolfgang, M. C., Huffnagle, G. B., Martinez, F. J., Huang, Y. J., LiPuma, J. J., Boushey, H. A., Boucher, R. C., Cookson, W. O., Curtis, J. L., Erb-Downward, J. and Lynch, S. V. 2012 Significance of the microbiome in obstructive lung disease. *Thorax,* 67**,** 456 - 463

Han, S., Norimine, J., Brayton, K. A., Palmer, G. H., Scoles, G. A. and Brown, W. C. 2010. Anaplasma marginale infection with persistent high-load bacteremia induces a dysfunctional memory CD4+ T lymphocyte response but sustained high IgG titers. *Clin Vaccine Immunol,* 17**,** 1881-90.

Hazeldine, V. 2013. Pharmacological management of acute asthma exacerbations in adults. *Nurs Stand.,* 27**,** 43-9.

Helbich, T. H., Heinz-Peer, G., Eichler, I., Wunderbaldinger, P., Gotz, M., Wojnarowski, C., Brasch, R. C. and Herold, C. J. 1999. Cystic fibrosis: CT assessment of lung involvement in children and adults. *Radiology,* 213**,** 537-44.

Henke, M. O., John, G., Germann, M., Lindemann, H. and Rubin, B. K. 2007. MUC5AC and MUC5B mucins increase in cystic fibrosis airway secretions during pulmonary exacerbation. *Am J Respir Crit Care Med,* 175**,** 816-21.

Henrique, M. C. 2007. Burkholderia cepacia complex: Virulence characteristics, importance and relationship with cystic fibrosis. *Indian J Med Sci,* 61**,** 422-9.

Heron, M., Claessen, A. M., Grutters, J. C. and van den Bosch, J. M. 2010. T-cell activation profiles in different granulomatous interstitial lung diseases-a role for CD8+CD28(null) cells?. *Clin Exp Immunol,* 160**,** 256-65.

Hirano, T., Kikuchi, T., Tode, N., Santoso, A., Yamada, M., Mitsuhashi, Y., Komatsu, R., Kawabe, T., Tanimoto, T., Ishii, N., Tanaka, Y., Nishimura, H., Nukiwa, T., Watanabe, A. and Ichinose, M. 2016. OX40 ligand newly expressed on bronchiolar progenitors mediates influenza infection and further exacerbates pneumonia. *EMBO Mol Med,* 8**,** 422-36.

Hogg, J. C. 2004. Pathophysiology of airflow limitation in chronic obstructive pulmonary disease. *Lancet,* 364**,** 709-21.

Hogg, J. C. 2006. State of the art. Bronchiolitis in chronic obstructive pulmonary disease. *Proc Am Thorac Soc,* 3**,** 489-493.

Holgate, S. T. 2012. Innate and adaptive immune responses in asthma. *Nat Med,* 18**,** 673-83.

Hollingsworth, M. A. and Swanson, B. J. 2004. Mucins in cancer: protection and control of the cell surface. *Nat Rev Cancer,* 4**,** 45-60.

Horio, Y., Ichiyasu, H., Kojima, K., Saita, N., Migiyama, Y., Iriki, T., Fujii, K., Niki, T., Hirashima, M. and Kohrogi, H. 2017. Protective effect of Galectin-9 in murine model of lung emphysema: Involvement of neutrophil migration and MMP-9 production. *PLoS One,* 12**,** e0180742.

Hus, I., Gonet-Sebastianka, J., Surdacka, A., Bojarska-Junak, A. and Rolinski, J. 2015. Analysis of peripheral blood immune cells after prophylactic immunization with HPV-16/18 ASO4-adjuvanted vaccine. *Postepy Hig Med Dosw (Online),* 69**,** 543-8.

Ibralic, M. and Beslic, S. 2009. Hrct in diagnosis of bronchiectasis. *Acta Informatica Medica,* 17**,** 213.

Ierano, T., Cescutti, P., Leone, M. R., Luciani, A., Rizzo, R., Raia, V., Lanzetta, R., Parrilli, M., Maiuri, L., Silipo, A. and Molinaro, A. 2010. The lipid A of Burkholderia multivorans C1576 smooth-type lipopolysaccharide and its pro-inflammatory activity in a cystic fibrosis airways model. *Innate Immun,* 16**,** 354-65.

Irish, J. M., Czerwinski, D. K., Nolan, G. P. and Levy, R. 2006. Kinetics of B cell receptor signaling in human B cell subsets mapped by phosphospecific flow cytometry. *J Immunol,* 177**,** 1581-9.

Ito, T., Carson, W. F. t., Cavassani, K. A., Connett, J. M. and Kunkel, S. L. 2011. CCR6 as a mediator of immunity in the lung and gut. *Exp Cell Res,* 317**,** 613-9.

Ivanov, I., Zhou, L. and Littman, D. 2007. Transcriptional regulation of Th17 cell differentiation. *Semin Immunol,* 19**,** 409-417.

Iwasaki, A. and Medzhitov, R. 2004. Toll-like receptor control of the adaptive immune responses. *Nat Immunol,* 5**,** 987-95.

Jackson, R. and Pencharz, P. 2003. Cystic fibrosis. *Best Pract Res Clin Gastroenterol,* 17**,** 213–235.

Jacquota, J., Tabarya, O., Le Rouzica, P. and Clementa, A. 2008. Airway epithelial cell inflammatory signalling in cystic fibrosis. *Int J Biochem Cell Biol,* 40**,** 1703–1715.

Janeway , C. J., Travers, P. and Walport , M. 2001. Immunobiology: The Immune System in Health and Disease. 5th edition. *The distribution and functions of immunoglobulin isotypes.* New York: Garland Science.

Jin, Y., Wan, Y., Chen, G., Chen, L., Zhang, M. Q., Deng, L., Zhang, J. C., Xiong, X. Z. and Xin, J. B. 2014. Treg/IL-17 ratio and Treg differentiation in patients with COPD. *PLoS One,* 9**,** e111044.

Jones, W., P 2009. ST George's Respiratory Questionnaire MANUAL. *In:* LONDON, U. O. (ed.) 2.3 ed.

Jutel, M. and Akdis, C. 2011. T-cell subset regulation in atopy. *Curr Allergy Asthma Rep,* 11**,** 139-45.

Kadowaki, T., Yano, S., Wakabayashi, K., Kobayashi, K., Ishikawa, S., Kimura, M. and Ikeda, T. 2015. An analysis of etiology, causal pathogens, imaging patterns, and treatment of Japanese patients with bronchiectasis. *Respir Investig.,* 53**,** 37-44.

Kaminski, D. and Stavnezer, J. 2004. Antibody class switching: uncoupling S region accessibility from transcription. *Trends Genet.,* 20**,** 337-340.

Kaneko, Y., Nimmerjahn, F. and Ravetch, J. 2006. Anti-inflammatory activity of immunoglobulin G resulting from Fc sialylation. *Science,* 313**,** 670-673.

Kappler, M., Kraxner, A., Reinhardt, D., Ganster, B., Griese, M. and Lang, T. 2006. Diagnostic and prognostic value of serum antibodies against Pseudomonas aeruginosa in cystic fibrosis. *Thorax,* 61**,** 684-8.

Kato, A., Hulse, K. E., Tan, B. K. and Schleimer, R. P. 2013. B-lymphocyte lineage cells and the respiratory system. *J Allergy Clin Immunol,* 131**,** 933-57.

Kaur, D. and Brightling, C. 2012. OX40/OX40 ligand interactions in T-cell regulation and asthma. *Chest,* 141**,** 494-9.

Kepler, T. B. 2013. Reconstructing a B-cell clonal lineage. I. Statistical inference of unobserved ancestors. *F1000Res. ,* 2**,** 103.

Kheradmand, F., Shan, M., Xu, C. and Corry, D. 2012. Autoimmunity in chronic obstructive pulmonary disease: clinical and experimental evidence. *Expert Rev Clin Immunol. ,* 8**,** 285-92.

Kidd, P. 2003. Th1/Th2 balance: the hypothesis, its limitations, and implications for health and disease. *Altern Med Rev,* 8**,** 223-46.

Kim, E., Lee, H., Kim, H. S., Won, S., Lee, E. K., Kim, H. S., Bang, K., Chun, Y. H., Yoon, J. S., Kim, H. H., Kim, J. T. and Lee, J. S. 2013. The effect of rhinovirus on airway inflammation in a murine asthma model. *Korean J Pediatr,* 56**,** 482-9.

Kim, J. J. and Khan, W. I. 2013. Goblet cells and mucins: role in innate defense in enteric infections. *Pathogens,* 2**,** 55-70.

Kim, J. K., Lee, S. H., Lee, B. H., Lee, C. Y., Kim do, J., Min, K. H., Kim, S. K., Yoo, K. H., Jung, K. S. and Hwang, Y. I. 2016. Factors associated with exacerbation in mild- to-moderate COPD patients. *Int J Chron Obstruct Pulmon Dis,* 11**,** 1327-33.

Kim, W. S., Jong-Seok, K., Cha, S. B., Kim, H., Kee, K. W., Kim, S. J., Han, S. J., Choi, S. Y., Sang-Nae, C., Jong-Hwan, P. and Shin, S. J. 2016. Mycobacterium tuberculosis drives Th1-type T cell immunity via TLR2-mediated activation of dendritic cells and displays vaccine potential against the hyper-virulent Beijing K strain. *Oncotarget.*

Kimura, A. and Kishimoto, T. 2011. Th17 cells in inflammation. *Int Immunopharmacol,* 11**,** 319-322.

King, P. 2011. Pathogenesis of bronchiectasis. *Paediatr Respir Rev.,* 12**,** 104-110.

King, P., Holdsworth, S., Freezer, N. and Holmes, P. 2006. Bronchiectasis. *Intern Med J,* 36**,** 729–737.

King, P., Hutchinson, P., Johnson, P., Holmes, P. and et al. 2003. Adaptive immunity to nontypeable Haemophilus influenzae. *Am J Respir Crit Care Med,* 167**,** 587-92.

King, P. T. 2009. The pathophysiology of bronchiectasis. *Int J Chron Obstruct Pulmon Dis,* 4**,** 411-9.

King, P. T., Francis, M., Moshinsky, R., Bardin, P. G., Holmes, P. W., Holdsworth, S. R., Lim, S., Pick, A., Ngui, J., Prodanovic, Z., Downey, W., Choong, C., Kelman, A. and Baranyai, E. 2013. Lung T-cell responses to nontypeable Haemophilus influenzae in patients with chronic obstructive pulmonary disease. *J Allergy Clin Immunol,* 131**,** 1314-1321.e14.

King, P. T., Holdsworth, S. R., Freezer, N. J., Villanueva, E. and Holmes, P. W. 2007. Microbiologic follow-up study in adult bronchiectasis. *Respir Med,* 101**,** 1633-1638.

Kitaguchi, Y., Yasuo, M. and Hanaoka, M. 2016. Comparison of pulmonary function in patients with COPD, asthma-COPD overlap syndrome, and asthma with airflow limitation. *Int J Chron Obstruct Pulmon Dis,* 11**,** 991-7.

Klein, U., Rajewsky, K. and Küppers, R. 1998. Human Immunoglobulin (Ig)M+IgD+ peripheral blood B cells expressing the CD27 cell surface antigen carry somatically mutated variable region genes: CD27 as a general marker for somatically mutated (Memory) B Cells. *J Exp Med,* 188**,** 1679-1689.

Kleinewietfeld, M. and Hafler, D. A. 2013. The plasticity of human Treg and Th17 cells and its role in autoimmunity. *Semin Immunol,* 25**,** 305-312.

Klepac-Ceraj, V., Lemon, K. P., Martin, T. R., Allgaier, M., Kembel, S. W., Knapp, A. A., Lory, S., Brodie, E. L., Lynch, S. V., Bohannan, B. J., Green, J. L., Maurer, B. A. and Kolter, R. 2010. Relationship between cystic fibrosis respiratory tract bacterial communities and age, genotype, antibiotics and Pseudomonas aeruginosa. *Environ Microbiol,* 12**,** 1293-303.

Knowles, M. R. and Boucher, R. C. 2002. Mucus clearance as a primary innate defense mechanism for mammalian airways. *J Clin Invest,* 109**,** 571-7.

Kono, H. and Rock, K. L. 2008. How dying cells alert the immune system to danger. *Nat Rev Immunol,* 8**,** 279-89.

Kreider, T., Anthony, R. M., Urban, J. F., J. and Gause, W. C. 2007. Alternatively activated macrophages in helminth infections. *Curr Opin Immunol,* 19**,** 448-53.

Krzysiek, R., Lefevre, E. A., Bernard, J., Foussat, A., Galanaud, P., Louache, F. and Richard, Y. 2000. Regulation of CCR6 chemokine receptor expression and responsiveness to macrophage inflammatory protein-3alpha/CCL20 in human B cells. *Blood,* 96**,** 2338-45.

Kucharzik, T., Hudson, J. T., 3rd, Waikel, R. L., Martin, W. D. and Williams, I. R. 2002. CCR6 expression distinguishes mouse myeloid and lymphoid dendritic cell subsets: demonstration using a CCR6 EGFP knock-in mouse. *Eur J Immunol,* 32**,** 104-12.

Kudo, M., Ishigatsubo, Y. and Aoki, I. 2013. Pathology of asthma. *Front Microbiol,* 4**,** 1-16.

Kumar, H., Kawai, T. and Akira, S. 2009. Toll-like receptors and innate immunity. *Biochem Biophys Res Commun,* 388**,** 621-625.

Kushwah, R., Gagnon, S. and Sweezey, N. 2013. Intrinsic predisposition of naive cystic fibrosis T cells to differentiate towards a Th17 phenotype. *Respir Res,* 14**,** 138.

Lacy, P. and Moqbel, R. 2000. Eosinophil cytokines. *Chem Immunol. ,* 76**,** 134 - 155.

Lane, P. 2000. Role of OX40 signals in coordinating CD4 T cell selection, migration, and cytokine differentiation in T helper (Th)1 and Th2 cells. *J Exp Med,* 191**,** 201-6.

Lanzavecchia, A. and Sallusto, F. 2007. Toll-like receptors and innate immunity in B-cell activation and antibody responses. *Curr Opin Immunol.,* 19**,** 268-274.

Leahr, S. M. and Bevan, M. J. 2004. Polarizing a T-cell response. *Nature,* 430**,** 150-1.

LeBien, T. W. and Tedder, T. F. 2008. B lymphocytes: how they develop and function. *Blood,* 112**,** 1570-80.

Lehar, S. M. and Bevan, M. J. 2004. Immunology: polarizing a T-cell response. *Nature,* 430**,** 150-1.

Leid, J. G., Willson, C. J., Shirtliff, M. E., Hassett, D. J., Parsek, M. R. and Jeffers, A. K. 2005. The exopolysaccharide alginate protects Pseudomonas aeruginosa biofilm bacteria from IFN-γ-mediated macrophage killing. *J Immunol,* 175**,** 7512-7518.

Lesinski, G. B. and Westerink, M. A. 2001. Novel vaccine strategies to T-independent antigens. *J Microbiol Methods,* 47**,** 135-49.

Leung, M. Y., Liu, C., Koon, J. C. and Fung, K. P. 2006. Polysaccharide biological response modifiers. *Immunol Lett* 105**,** 101-114.

Li, Y., Wang, Y. and Liu, X. 2012. The role of airway epithelial cells in response to Mycobacteria infection. *Clin Dev Immunol***,** 791392.

Li, Y., Yin, Y. and Mariuzza, R. A. 2013. Structural and biophysical insights into the role of CD4 and CD8 in T cell activation. *Front Immunol,* 4**,** 206.

Li, Z., Woo, C. J., Iglesias-Ussel, M. D., Ronai, D. and Scharff, M. D. 2004. The generation of antibody diversity through somatic hypermutation and class switch recombination. *Genes Dev,* 18**,** 1-11.

Liang, Z., Zhang, Q., Thomas, C. M. R., Chana, K. K., Gibeon, D., Barnes, P. J., Chung, K. F., Bhavsar, P. K. and Donnelly, L. E. 2014. Impaired macrophage phagocytosis of bacteria in severe asthma. *Respir Res,* 15**,** 72.

Liao, L. Y., Chen, K. M., Chung, W. S. and Chien, J. Y. 2015. Efficacy of a respiratory rehabilitation exercise training package in hospitalized elderly patients with acute exacerbation of COPD: a randomized control trial. *Int J Chron Obstruct Pulmon Dis,* 10**,** 1703-9.

Liu, S., Zhou, Y., Wang, X., Wang, D., Lu, J., Zheng, J., Zhong, N. and Ran, P. 2007. Biomass fuels are the probable risk factor for chronic obstructive pulmonary disease in rural South China. *Thorax,* 62**,** 889-897.

Lode, H., Allewelt, M., Balk, S., De Roux, A., Mauch, H., Niederman, M. and Schmidt-Ioanas, M. 2007. A prediction model for bacterial etiology in acute exacerbations of COPD. *Infection,* 35**,** 143-9.

Loscertales, M., Nicolaou, F., Jeanne, M., Longoni, M., Gould, D. B., Sun, Y., Maalouf, F. I., Nagy, N. and Donahoe, P. K. 2016. Type IV collagen drives alveolar epithelial-endothelial association and the morphogenetic movements of septation. *BMC Biol,* 14**,** 59.

Lu, Y.-C., Yeh, W.-C. and Ohashi, P. S. 2008. LPS/TLR4 signal transduction pathway. *Cytokine,* 42**,** 145-151.

Lundy, S. K., Lira, S. A., Smit, J. J., Cook, D. N., Berlin, A. A. and Lukacs, N. W. 2005. Attenuation of allergen-induced responses in CCR6-/- mice is dependent upon altered pulmonary T lymphocyte activation. *J Immunol,* 174**,** 2054-60.

Ma, Y., Niu, Y., Tian, G., Wei, J. and Gao, Z. 2015. Pulmonary function abnormalities in adult patients with acute exacerbation of bronchiectasis: A retrospective risk factor analysis. *Chron Respir Dis,* 12**,** 222-9.

MacFarlane, A. W., Jillab, M., Plimack, E. R., Hudes, G. R., Uzzo, R. G., Litwin, S., Dulaimi, E., Al-Saleem, T. and Campbell, K. S. 2014. PD-1 expression on peripheral blood cells increases with stage in renal cell carcinoma patients and is rapidly reduced after surgical tumor resection. *Cancer Immunol Res,* 2**,** 320-31.

MacLennan, I., Toellner, K., Cunningham, A., Serre, K., Sze, D., Zuniga, E., Cook, M. and Vinuesa, C. 2003. Extrafollicular antibody responses. *Immunol Rev,* 194**,** 8–18.

Macpherson, A. J., McCoy, K. D., Johansen, F. E. and Brandtzaeg, P. 2008. The immune geography of IgA induction and function. *Mucosal Immunol,* 1**,** 11-22.

Maino, V. C., Suni, M. A. and Ruitenberg, J. J. 1995. Rapid flow cytometric method for measuring lymphocyte subset activation. *Cytometry,* 20**,** 127-33.

Máiz, L., Cuevas, M., Lamas, A., Sousa, A., Quirce, S. and Suárez, L. 2008. Aspergillus fumigatus and Candida albicans in cystic fibrosis: clinical significance and specific immune response involving serum immunoglobulins G, A, and M. *Arch Bronconeumol. ((English Edition)),* 44**,** 146-151.

Majid, A., Lin, T. T., Best, G., Fishlock, K., Hewamana, S., Pratt, G., Yallop, D., Buggins, A. G. S., Wagner, S. and Kennedy, B. J. 2011. CD49d is an independent prognostic marker that is associated with CXCR4 expression in CLL. *Leuk Res,* 35**,** 750-756.

Mak, T. W. and Saunders, M. E., Ph.D. 2005. *The immune response: basic and clinical principles,* New York, Elsevier/Academic.

Maki, Y., Nishimura, Y., Toyooka, S., Soh, J., Tsukuda, K., Shien, K., Furukawa, M., Muraoka, T., Ueno, T., Tanaka, N., Yamamoto, H., Asano, H., Maeda, M., Kumagai-Takei, N., Lee, S., Matsuzaki, H., Otsuki, T. and Miyoshi, S. 2016. The

proliferative effects of asbestos-exposed peripheral blood mononuclear cells on mesothelial cells. *Oncol Lett,* 11**,** 3308-3316.

Makidon, P. E., Knowlton, J., Groom, J. V., 2nd, Blanco, L. P., LiPuma, J. J., Bielinska, A. U. and Baker, J. R., Jr. 2010. Induction of immune response to the 17 kDa OMPA Burkholderia cenocepacia polypeptide and protection against pulmonary infection in mice after nasal vaccination with an OMP nanoemulsion-based vaccine. *Med Microbiol Immunol,* 199**,** 81-92.

Mannino, D. M. and Buist, A. S. 2007. Global burden of COPD: risk factors, prevalence, and future trends. *Lancet.,* 370**,** 765-73.

Marieb, E. N. and Hoehn, K. 2007. *Human anatomy & physiology* U.S.A, Pearson Education,Inc.

Marin, A., Monso, E., Garcia-Nunez, M., Sauleda, J., Noguera, A., Pons, J., Agusti, A. and Morera, J. 2010. Variability and effects of bronchial colonisation in patients with moderate COPD. *Eur Respir J,* 35**,** 295-302.

Martínez-García, M. Á., Perpiñá-Tordera, M., Román-Sánchez, P., Soler-Cataluña, J. J., Carratalá, A., Yago, M. and José Pastor, M. 2008. Association between bronchiectasis, systemic inflammation, and tumor necrosis factor α. *Arch Bronconeumol. ((English Edition)),* 44**,** 8-14.

Martinez-Garcia, M. A., Soler-Cataluna, J. J., Perpina-Tordera, M., Roman-Sanchez, P. and Soriano, J. 2007. Factors associated with lung function decline in adult patients with stable non-cystic fibrosis bronchiectasis. *Chest,* 132**,** 1565-72.

Martinez, F. O., Sica, A., Mantovani, A. and Locati, M. 2008. Macrophage activation and polarization. *Front Biosci,* 13**,** 453-61.

Martz, E., Canner, D., Decatur, W., Berchansky, A. and Harel, M. 2013. Antibody. [Accessed 11/01/2016].

Mashburn, L. M., Jett, A. M., Akins, D. R. and Whiteley, M. 2005. Staphylococcus aureus serves as an iron source for Pseudomonas aeruginosa during in vivo coculture. *J Bacteriol,* 187**,** 554-66.

Matkovic, Z. and Miravitlles, M. 2013. Chronic bronchial infection in COPD. Is there an infective phenotype?. *Respir Med. ,* 107**,** 10-22.

Matsunaga, K., Klein, T. W., Friedman, H. and Yamamoto, Y. 2001. Involvement of nicotinic acetylcholine receptors in Suppression of antimicrobial activity and cytokine responses of alveolar macrophages to Legionella pneumophila infection by nicotine. *J Immunol,* 167**,** 6518-6524.

Mayer, G. 2015 Microbiology and Immunology -online. *In:* HUNT, R. (ed.) *Immunology - Structure and Function.* University of South Carolina.

McGuire, J. K. 2015. Regulatory T cells in cystic fibrosis lung disease. More answers, more questions. *Am J Respir Crit Care Med,* 191**,** 866-8.

McShane, P. J., Naureckas, E. T., Tino, G. and Strek, M. E. 2013. Non-cystic fibrosis bronchiectasis. *Am J Respir Crit Care Med,* 188**,** 647-56.

Metaxas, E. I., Balis, E., Papaparaskevas, J., Spanakis, N., Tatsis, G. and Tsakris, A. 2015. Bronchiectasis exacerbations: The role of atypical bacteria, respiratory syncytial virus and pulmonary function tests. *Can Respir J,* 22**,** 163-6.

Micol, R., Kayal, S., Mahlaoui, N., Beaute, J., Brosselin, P., Dudoit, Y., Obenga, G., Barlogis, V., Aladjidi, N. and Kebaili, K. 2012. Protective effect of IgM against colonization of the respiratory tract by nontypeable Haemophilus influenzae in patients with hypogammaglobulinemia. *J Allergy Clin Immunol,* 129**,** 770-7.

Milagres, L. G., Castro, T. L., Garcia, D., Cruz, A. C., Higa, L., Folescu, T. and Marques, E. A. 2009. Antibody response to Pseudomonas aeruginosa in children with cystic fibrosis. *Pediatr Pulmonol,* 44**,** 392-401.

Milam, A. V. and Allen, P. M. 2015. Functional heterogeneity in CD4(+) T Cell responses against a bacterial pathogen. *Front Immunol,* 6**,** 621.

Millares, L., Ferrari, R., Gallego, M., Garcia-Nunez, M., Perez-Brocal, V., Espasa, M., Pomares, X., Monton, C., Moya, A. and Monso, E. 2014. Bronchial microbiome of severe COPD patients colonised by Pseudomonas aeruginosa. *Eur J Clin Microbiol Infect Dis,* 33**,** 1101-11.

Mohammad, J. S. and Dhanashree, B. 2012. Streptococcus pseudopneumoniae: an emerging respiratory tract pathogen. *Indian J Med Res.,* 136**,** 877-80.

Molfetta, R., Quatrini, L., Gasparrini, F., Zitti, B., Santoni, A. and Paolini, R. 2014. Regulation of fc receptor endocytic trafficking by ubiquitination. *Front Immunol,* 5**,** 449.

Mond, J. J., Vos, Q., Lees, A. and Snapper, C. M. 1995. T cell independent antigens. *Curr Opin Immunol,* 7**,** 349-354.

Moreau-Marquis, S., Stanton, B. A. and O'Toole, G. A. 2008. Pseudomonas aeruginosa biofilm formation in the cystic fibrosis airway. *Pulm Pharmacol Ther. ,* 21**,** 595-599.

Moss, R. B., Hsu, Y. P. and Olds, L. 2000. Cytokine dysregulation in activated cystic fibrosis (CF) peripheral lymphocytes. *Clin Exp Immunol,* 120**,** 518-25.

Mosser, D. M. and Edwards, J. P. 2008. Exploring the full spectrum of macrophage activation. *Nat Rev Immunol,* 8**,** 958-69.

Mueller, S. N. and Ahmed, R. 2009. High antigen levels are the cause of T cell exhaustion during chronic viral infection. *Proc Natl Acad Sci U S A,* 106**,** 8623-8.

Muramatsu, M., Kinoshita, K., Fagarasan, S., Yamada, S., Shinkai, Y. and Honjo, T. 2000. Class switch recombination and hypermutation require activation-induced cytidine deaminase (AID), a potential RNA editing enzyme. *Cell,* 102**,** 553-563.

Murphy, K. 2011. *Janeway's Immunobiology,* New York and Abingodon UK, Graland Science,Taylor&Francis Group, LLC

Murphy, T. F. 2003. Immunity to nontypeable Haemophilus influenzae: elucidating protective responses. *Am J Respir Crit Care Med,* 167**,** 486-7.

Murray, M. P., Turnbull, K., Macquarrie, S. and Hill, A. T. 2009. Assessing response to treatment of exacerbations of bronchiectasis in adults. *Eur Respir J,* 33**,** 312-8.

Naito, T., Tanaka, H., Naoe, Y. and Taniuchi, I. 2011. Transcriptional control of T-cell development. *Int Immunol. ,* 23**,** 661-668.

Nelson, A. 2011. *An investigation of the polymicrobial nature of lower respiratory tract infections in cystic fibrosis patients.* PhD, Northumbria University at Newcastle up on Tyne

Neves, P. C., Guerra, M., Ponce, P., Miranda, J. and Vouga, L. 2011. State-of-the-art - Pulmonary Non-cystic fibrosis bronchiectasis. *Interact Cardiovasc Thorac Surg.,* 13**,** 619-625.

Nguyen, A. T., Jones, J. W., Camara, M., Williams, P., Kane, M. A. and Oglesby-Sherrouse, A. G. 2016. Cystic fibrosis isolates of Pseudomonas aeruginosa retain iron-regulated antimicrobial activity against Staphylococcus aureus through the action of multiple alkylquinolones. *Front Microbiol,* 7**,** 1171.

Nicol, L., Wilkie, H., Gossner, A., Watkins, C., Dalziel, R. and Hopkins, J. 2016. Variations in T cell transcription factor gene structure and expression associated with the two disease forms of sheep paratuberculosis. *Vet Res,* 47**,** 83.

Niemann, C. U. and Wiestner, A. 2013. B-cell receptor signaling as a driver of lymphoma development and evolution. *Semin Cancer Biol,* 23**,** 410-21.

Nikota, J. K. and Stämpfli, M. R. 2012. Cigarette smoke-induced inflammation and respiratory host defense: Insights from animal models. *Pulm Pharmacol Ther. ,* 25**,** 257-262.

O'Donnell, R., Breen, D., Wilson, S. and Djukanovic, R. 2006. Inflammatory cells in the airways in COPD. *Thorax,* 61**,** 448-454.

O'Sullivan, B. P. and Freedman, S. D. 2009. Cystic fibrosis. *Lancet,* 373**,** 1891–904.

Obukhanych, T. V. and Nussenzweig, M. C. 2006. T-independent type II immune responses generate memory B cells. *J Exp Med,* 203**,** 305-10.

Ostridge, K., Williams, N., Kim, V., Harden, S., Bourne, S., Coombs, N. A., Elkington, P. T., Estepar, R. S., Washko, G., Staples, K. J. and Wilkinson, T. M. 2016. Distinct emphysema subtypes defined by quantitative CT analysis are associated with specific pulmonary matrix metalloproteinases. *Respir Res,* 17**,** 92.

Otsuka, A. and Kabashima, K. 2015. Contribution of basophils to cutaneous immune reactions and Th2-mediated allergic responses. *Front Immunol,* 6**,** 393.

Palaniyar, N., Mall, M. A., Taube, C., Worgall, S. and Grasemann, H. 2015. New developments in cystic fibrosis airway inflammation. *Mediators Inflamm,* 2015**,** 769425.

Panettieri, R. A., Jr. 2002. Airway smooth muscle: an immunomodulatory cell. *J Allergy Clin Immunol,* 110**,** S269-74.

Papazian, D., Hansen, S. and Wurtzen, P. A. 2015. Airway responses towards allergens - from the airway epithelium to T cells. *Clin Exp Allergy,* 45**,** 1268-87.

Parham, P. 2009. *The immune system,* New York ,US, Garland Science,Taylor &Francis group,LLC.

Parker, D. and Prince, A. 2011. Innate immunity in the respiratory epithelium. *Am J Respir Cell Mol Biol* 45**,** 189-201.

Pasteur, M. C., Helliwell, S. M., Houghton, S. J., Webb, S. C., Foweraker, J. E., Coulden, R. A., Flower, C. D., Bilton, D. and Keogan, M. T. 2000. An investigation into causative factors in patients with bronchiectasis. *Am J Respir Crit Care Med,* 162**,** 1277-84.

Pedersen, S. S., Espersen, F., Hoiby, N. and Jensen, T. 1990. Immunoglobulin A and immunoglobulin G antibody responses to alginates from Pseudomonas aeruginosa in patients with cystic fibrosis. *J Clin Microbiol,* 28**,** 747-55.

Pei, J., Ding, X., Fan, Y., Rice-Ficht, A. and Ficht, T. A. 2012. Toll-like receptors are critical for clearance of Brucella and play different roles in development of adaptive immunity following aerosol challenge in mice. *Front Cell Infect Microbiol,* 2**,** 115.

Peltola, V. T. and McCullers, J. A. 2004. Respiratory viruses predisposing to bacterial infections: role of neuraminidase. *Pediatr Infect Dis J,* 23**,** S87-97.

Perera, W. R., Hurst, J. R., Wilkinson, T. M., Sapsford, R. J., Mullerova, H., Donaldson, G. C. and Wedzicha, J. A. 2007. Inflammatory changes, recovery and recurrence at COPD exacerbation. *Eur Respir J,* 29**,** 527-34.

Petrović-Đjergović, D. M., Rakin, A. K., Kuštrimović, N. Z., Ristovski, J. S., Dimitrijevic, L. A. and Mileva, M. V. 2007. Somatostatin modulates T cells development in adult rat thymus. *Regulatory Peptides,* 142**,** 101-110.

Pettersen, C. A. and Adler, K. B. 2002. Airways inflammation and COPD: Epithelial-neutrophil interactions. *Chest,* 121**,** 142S-150S.

Pieper, K., Grimbacher, B. and Eibel, H. 2013. B-cell biology and development. *J Allergy Clin Immunol,* 131**,** 959-71.

Pollard, K. M., Hultman, P., Toomey, C. B., Cauvi, D. M., Hoffman, H. M., Hamel, J. C. and Kono, D. H. 2012. Definition of IFN-gamma-related pathways critical for chemically-induced systemic autoimmunity. *J Autoimmun,* 39**,** 323-31.

Pragman, A. A., Kim, H. B., Reilly, C. S., Wendt, C. and Isaacson, R. E. 2012. The lung microbiome in moderate and severe chronic obstructive pulmonary disease. *PloS one,* 7**,** 1.

Pressler, T., Pedersen, S. S., Espersen, F., Hoiby, N. and Koch, C. 1990. IgG subclass antibodies to Pseudomonas aeruginosa in sera from patients with chronic P. aeruginosa infection investigated by ELISA. *Clin Exp Immunol,* 81**,** 428-34.

Prussin, C. and Metcalfe, D. D. 2006. 5. IgE, mast cells, basophils, and eosinophils. *J Allergy Clin Immunol,* 117**,** S450-6.

Quie, P. G. 1986. Lung defense against infection. *J Pediatr,* 108**,** 813-6.

Quigley, K. J., Reynolds, C. J., Goudet, A., Raynsford, E. J., Sergeant, R., Quigley, A., Worgall, S., Bilton, D., Wilson, R., Loebinger, M. R., Maillere, B., Altmann, D. M. and Boyton, R. J. 2015. Chronic Infection by Mucoid Pseudomonas aeruginosa Associated with Dysregulation in T-Cell Immunity to Outer Membrane Porin F. *Am J Respir Crit Care Med,* 191**,** 1250-64.

Racusin, D. A., Fox, K. A. and Ramin, S. M. 2013. Severe acute asthma. *Semin Perinatol* 37**,** 234-245.

Rademacher, J. and Welte, T. 2011. Bronchiectasis—diagnosis and treatment. *Deutsches Ärzteblatt International,* 108**,** 809–815.

Rajavelu, P., Chen, G., Xu, Y., Kitzmiller, J. A., Korfhagen, T. R. and Whitsett, J. A. 2015. Airway epithelial SPDEF integrates goblet cell differentiation and pulmonary Th2 inflammation. *J Clin Invest,* 125**,** 2021-31.

Ramakrishna, L., de Vries, V. C. and Curotto de Lafaille, M. A. 2012. Cross-roads in the lung: immune cells and tissue interactions as determinants of allergic asthma. *Immunol Res,* 53**,** 213-28.

Rasam, S. A., Apte, K. K. and Salvi, S. S. 2015. Infection control in the pulmonary function test laboratory. *Lung India,* 32**,** 359-66.

Rastawicki, W., Smietanska, K., Rokosz, N. and Jagielski, M. 2012. [Effect of multiple freeze-thaw cycles on detection of IgA, IgG and IgM antibodies to selected bacterial antigens]. *Med Dosw Mikrobiol,* 64**,** 79-85.

Ratjen, F. 2006. Diagnosing and managing infection in CF. *Paediatr Respir Rev,* 7 Suppl 1**,** S151-3.

Ratjen, F., Walter, H., Haug, M., Meisner, C., Grasemann, H. and Döring, G. 2007. Diagnostic value of serum antibodies in early Pseudomonas aeruginosa infection in cystic fibrosis patients. *Pediatr Pulmonol,* 42**,** 249-255.

Redding, G. J. 2011. Update on treatment of childhood bronchiectasis unrelated to cystic-fibrosis. *Paediatr Respir Rev.,* 12**,** 119-123.

Reed, T. and Neil, S. 2013. Diagnosis and treatment of asthma in children. *Practice Nursing,* 24**,** 222-229.

Reibman, J., Hsu, Y., Chen, L. C., Bleck, B. and Gordon, T. 2003. Airway epithelial cells release MIP-3alpha/CCL20 in response to cytokines and ambient particulate matter. *Am J Respir Cell Mol Biol,* 28**,** 648-54.

Robinson, D. S. 2010. The role of the T cell in asthma. *J Allergy Clin Immunol,* 126**,** 1081-1091.

Roche Innovatis AG CASY. 2002. *CASY®Cell Counter + Analyser System* [Online]. Roche Innovatis AG. [Accessed 08/12/2016.

Rogers, G. B., Hart, C. A., Mason, J. R., Hughes, M., Walshaw, M. J. and Bruce, K. D. 2003. Bacterial diversity in cases of lung infection in cystic fibrosis patients: 16S ribosomal DNA (rDNA) length heterogeneity PCR and 16S rDNA terminal restriction fragment length polymorphism profiling. *J Clin Microbiol,* 41**,** 3548-58.

Romagnani, S. 2006. Regulation of the T cell response. *Clin Exp Allergy,* 36**,** 1357-1366.

Rose, D. M., Han, J. and Ginsberg, M. H. 2002. α4 integrins and the immune response. *Immunol Rev.,* 186**,** 118-124.

Ross, M. H. and Pawlina, W. 2011. *Histology: a text and atlas: with correlated cell and molecular biology-6thed,* Philadelphia, Lippincott Williams & Wilkins Health.

Ruby, C. E., Montler, R., Zheng, R., Shu, S. and Weinberg, A. D. 2008. IL-12 is required for anti-OX40-mediated CD4 T cell survival. *J Immunol,* 180**,** 2140-8.

Rybakin, V., Clamme, J.-p., Ampudia, J., Yachi, P. P. and Gascoigne, N. R. J. 2011. CD8[alpha][alpha] and -[alpha][beta] isotypes are equally recruited to the immunological synapse through their ability to bind to MHC class I. *EMBO Reports,* 12**,** 1251-6.

Sabanci University Nanotechnology Research and Application. 2013. *Roche Casy Cell Counter and Analyzer* [Online]. Tuzla-İstanbul: Sabanci University Available: http://sunum.sabanciuniv.edu/en [Accessed 08/12/2016 2016].

Sadikot, R. T., Blackwell, T. S., Christman, J. W. and Prince, A. S. 2005. Pathogen-host interactions in Pseudomonas aeruginosa Pneumonia. *Am J Respir Crit Care Med,* 171**,** 1209-23.

Salmon, J. E. and Pricop, L. 2001. Human receptors for immunoglobulin G: key elements in the pathogenesis of rheumatic disease. *Arthritis Rheum,* 44**,** 739-50.

Sancho, D., Gómez, M. and Sánchez-Madrid, F. 2005. CD69 is an immunoregulatory molecule induced following activation. *Trends Immunol,* 26**,** 136-140.

Sanders, D. B., Bittner, R. C., Rosenfeld, M., Redding, G. J. and Goss, C. H. 2011. Pulmonary exacerbations are associated with subsequent FEV1 decline in both adults and children with cystic fibrosis. *Pediatr Pulmonol,* 46**,** 393-400.

Schluchter, M. D., Konstan, M. W. and Davis, P. B. 2002. Jointly modelling the relationship between survival and pulmonary function in cystic fibrosis patients. *Stat Med,* 21**,** 1271-87.

Schneider, E., Thieblemont, N., De Moraes, M. L. and Dy, M. 2010. Basophils: new players in the cytokine network. *Eur Cytokine Netw,* 21**,** 142-53.

Schroeder, H. W. and Cavacini, L. 2010. Structure and function of immunoglobulins. *J Allergy Clin Immunol,* 125**,** S41-52.

Scullion, J. and Holmes, S. 2013. Diagnosis and management of patients with bronchiectasis. *Nurs Stand (through 2013),* 27**,** 49-55.

Seeley, R. R., Stephens, T. D. and Tate, P. 2008. *Anatomy and Physiology* New York, McGraw-Hill Companies,Inc.

Seemungal, T., Harper-Owen, R., Bhowmik, A., Moric, I., Sanderson, G., Message, S., MacCallum, P., Meade, T. W., Jeffries, D. J., Johnston, S. L. and Wedzicha, J. A.

2001. Respiratory viruses, symptoms, and inflammatory markers in acute exacerbations and stable chronic obstructive pulmonary disease. *Am J Respir Crit Care Med.,* 164**,** 1618-1623.

Sethi, S. 2010. Infection as a comorbidity of COPD. *Eur Respir J,* 35**,** 1209-1215.

Sethi, S. and Murphy, T. F. 2001. Bacterial infection in chronic obstructive pulmonary disease in 2000: a state-of-the-art review. *Clin Microbiol Rev,* 14**,** 336-63.

Shalaby, K. H. and Martin, J. G. 2010. Overview of asthma; the place of the T cell. *Curr Opin Pharmacol. ,* 10**,** 218-225.

Shaw, P. J., Weidinger, C., Vaeth, M., Luethy, K., Kaech, S. M. and Feske, S. 2014. CD4(+) and CD8(+) T cell-dependent antiviral immunity requires STIM1 and STIM2. *J Clin Invest,* 124**,** 4549-63.

Shoemark, A., Ozerovitch, L. and Wilson, R. 2007. Aetiology in adult patients with bronchiectasis. *Respir Med,* 101**,** 1163-1170.

Shum, D. K., Chan, S. C. and Ip, M. S. 2000. Neutrophil-mediated degradation of lung proteoglycans: stimulation by tumor necrosis factor-alpha in sputum of patients with bronchiectasis. *Am J Respir Crit Care Med,* 162**,** 1925-31.

Siddiqui, S., Mistry, V., Doe, C., Stinson, S., Foster, M. and Brightling, C. 2010. Airway wall expression of OX40/OX40L and interleukin-4 in asthma. *Chest,* 137**,** 797-804.

Simpson, R. J., Bigley, A. B., Spielmann, G., LaVoy, E. C., Kunz, H. and Bollard, C. M. 2016. Human cytomegalovirus infection and the immune response to exercise. *Exerc Immunol Rev,* 22**,** 8-27.

Singh, D. 2014. Reduced antibacterial immunity in chronic obstructive pulmonary disease: a key role for lymphocytes? *Am J Respir Crit Care Med,* 190**,** 3-4.

Sohal, S. S., Ward, C., Danial, W., Wood-Baker, R. and Walters, E. H. 2013. Recent advances in understanding inflammation and remodeling in the airways in chronic obstructive pulmonary disease. *Expert Rev Respir Med,* 7**,** 275-88.

Sollberger, G., Amulic, B. and Zychlinsky, A. 2016. Neutrophil Extracellular Trap Formation Is Independent of De Novo Gene Expression. *PLoS One,* 11**,** e0157454.

Song, Z., Wu, H., Ciofu, O., Kong, K. F., Hoiby, N., Rygaard, J., Kharazmi, A. and Mathee, K. 2003. Pseudomonas aeruginosa alginate is refractory to Th1 immune response and impedes host immune clearance in a mouse model of acute lung infection. *J Med Microbiol,* 52**,** 731-40.

Stavnezer, J. 1996. Immunoglobulin class switching. *Curr Opin Immunol,* 8**,** 199-205.

Stavnezer, J. 2011. Complex regulation and function of activation-induced cytidine deaminase. *Trends Immunol.*

Stead, A., Douglas, J. G., Broadfoot, C. J., Kaminski, E. R. and Herriot, R. 2002. Humoral immunity and bronchiectasis. *Clin Exp Immunol,* 130**,** 325-30.

Stentzel, S., Teufelberger, A., Nordengrun, M., Kolata, J., Schmidt, F., van Crombruggen, K., Michalik, S., Kumpfmuller, J., Tischer, S., Schweder, T. and Hecker, M. 2016. Staphylococcal serine protease-like proteins are pacemakers of allergic airway reactions to Staphylococcus aureus. *J Allergy Clin Immunol.*

Strieter, R. M., Belperio, J. A. and Keane, M. P. 2002. Cytokines in innate host defense in the lung. *J Clinic Invest,* 109**,** 699 - 705

Sunderkotter, C., Nikolic, T., Dillon, M. J., Van Rooijen, N., Stehling, M., Drevets, D. A. and Leenen, P. J. 2004. Subpopulations of mouse blood monocytes differ in maturation stage and inflammatory response. *J Immunol,* 172**,** 4410-7.

Szczepanska, M., Sedek, L., Makulska, I., Szprynger, K., Mazur, B., Bulsa, J., Zwolinska, D., Karpe, J., Ziora, K. and Szczepanski, T. 2015. Expression of chemokine receptors on peripheral blood T cells in children with chronic kidney disease. *Mediators Inflamm,* 2015**,** 536894.

Tan, H.-L., Regamey, N., Brown, S., Bush, A., Lloyd, C. M. and Davies, J. C. 2011. The Th17 pathway in cystic fibrosis lung disease. *Am J Respir Crit Care Med,* 184**,** 252-8.

Tan, W. C., Xiang, X. Y., Qiu, D. W., Ng, T. P., Lam, S. F. and Hegele, R. G. 2003. Epidemiology of respiratory viruses in patients hospitalized with near-fatal asthma, acute exacerbation's of asthma, or chronic obstructive pulmonary disease. *American Journal of Medicine,* 115**,** 272-277.

Tang, Q. and Bluestone, J. A. 2008. The Foxp3+ regulatory T cell: a jack of all trades, master of regulation. *Nat Immunol,* 9**,** 239-44.

Teunis, P. F., van Eijkeren, J. C., Ang, C. W., van Duynhoven, Y. T., Simonsen, J. B., Strid, M. A. and van Pelt, W. 2012. Biomarker dynamics: estimating infection rates from serological data. *Stat Med,* 31**,** 2240-8.

Teunis, P. F. M., Van Der Heijden, O. G., De Melker, H. E., Schellekens, J. F. P., Versteegh, F. G. A. and Kretzschmar, M. E. E. 2002. Kinetics of the IgG antibody response to pertussis toxin after infection with B. pertussis. *Epidemiol Infect. ,* 129**,** 479.

Tewari, M. K., Sinnathamby, G., Rajagopal, D. and Eisenlohr, L. C. 2005. A cytosolic pathway for MHC class II-restricted antigen processing that is proteasome and TAP dependent. *Nat Immunol* [Online], 6. Available: http://www.ncbi.nlm.nih.gov/pubmed/15711549 [Accessed Mar].

Tiegs, S. L., Russell, D. M. and Nemazee, D. 1993. Receptor editing in self-reactive bone marrow B cells. *J Exp Med.,* 177**,** 1009-1020.

Tiringer, K., Treis, A., Fucik, P., Gona, M., Gruber, S., Renner, S., Dehlink, E., Nachbaur, E., Horak, F. and Jaksch, P. 2013. A Th17- and Th2-skewed cytokine profile in cystic fibrosis lungs represents a potential risk factor for Pseudomonas aeruginosa infection. *Am J Respir Crit Care Med,* 187**,** 621-9.

Toor, A. A., Toor, A. A., Rahmani, M. and Manjili, M. H. 2016. On the organization of human T-cell receptor loci: log-periodic distribution of T-cell receptor gene segments. *J R Soc Interface,* 13**,** 20150911.

Triantafilou, K., Triantafilou, M. and Dedrick, R. L. 2001. A CD14-independent LPS receptor cluster. *Nat Immunol. ,* 2**,** 338-45.

Tubo, N. J. and Jenkins, M. K. 2014. CD4+ T Cells: guardians of the phagosome. *Clin Microbiol Rev,* 27**,** 200-13.

Tunney, M. M., Einarsson, G. G., Wei, L., Drain, M., Klem, E. R., Cardwell, C., Ennis, M., Boucher, R. C., Wolfgang, M. C. and Elborn, J. S. 2013. Lung mcrobiota and bacterial abundance in patients with bronchiectasis when clinically stable and during exacerbation. *Am J Respir Crit Care Med.,* 187**,** 1118-1126.

Tunney, M. M., Klem, E. R., Fodor, A. A., Gilpin, D. F., Moriarty, T. F., McGrath, S. J., Muhlebach, M. S., Boucher, R. C., Cardwell, C., Doering, G., Elborn, J. S. and Wolfgang, M. C. 2011. Use of culture and molecular analysis to determine the effect of antibiotic treatment on microbial community diversity and abundance during exacerbation in patients with cystic fibrosis. *Thorax,* 66**,** 579-84.

Twigg, H. L., 3rd 2005. Humoral immune defense (antibodies): recent advances. *Proc Am Thorac Soc,* 2**,** 417-21.

Tzortzaki, E. G., Proklou, A. and Siafakas, N. M. 2011. Asthma in the Elderly: Can We Distinguish It from COPD? *J Allergy (Cairo),* 2011**,** 843543.

Vargas-Rojas, M. I., Ramírez-Venegas, A., Limón-Camacho, L., Ochoa, L., Hernández-Zenteno, R. and Sansores, R. H. 2011. Increase of Th17 cells in peripheral blood of patients with chronic obstructive pulmonary disease. *Respir Med,* 105**,** 1648-54.

Vermeire, K., Schols, D. and Bell, T. W. 2006. Inhibitors of HIV infection via the cellular CD4 receptor. *Curr Med Chem. ,* 13**,** 731-43.

Vestbo, J., Hurd, S. S., Agusti, A. G., Jones, P. W., Vogelmeier, C., Anzueto, A., Barnes, P. J., Fabbri, L. M., Martinez, F. J. and Nishimura, M. 2013. Global strategy for the diagnosis, management, and prevention of chronic obstructive pulmonary disease: GOLD executive summary. *Am J Respir Crit Care Med,* 187**,** 347-65.

Vidarsson, G., Dekkers, G. and Rispens, T. 2014. IgG subclasses and allotypes: from structure to effector functions. *Front Immunol,* 5**,** 520.

Voss, M., Wonnenberg, B., Honecker, A., Kamyschnikow, A., Herr, C., Bischoff, M., Tschernig, T., Bals, R. and Beisswenger, C. 2015. Cigarette smoke-promoted acquisition of bacterial pathogens in the upper respiratory tract leads to enhanced inflammation in mice. *Respir Res,* 16**,** 41.

Walker, K. M., Okitsu, S., Porter, D. W., Duncan, C., Amacker, M., Pluschke, G., Cavanagh, D. R., Hill, A. V. S. and Todryk, S. M. 2015. Antibody and T-cell responses associated with experimental human malaria infection or vaccination show limited relationships. *Immunology,* 145**,** 71-81.

Wang, L. D. and Clark, M. R. 2003. B-cell antigen-receptor signalling in lymphocyte development. *Immunology,* 110**,** 411-420.

Wang, Y. H. and Liu, Y. J. 2007. OX40-OX40L interactions: a promising therapeutic target for allergic diseases?. *J Clin Invest,* 117**,** 3655-7.

Wedzicha, J. A. and Seemungal, T. A. 2007. COPD exacerbations: defining their cause and prevention. *Lancet,* 370**,** 786-796.

Wells, T. J., Whitters, D., Sevastsyanovich, Y. R., Heath, J. N., Pravin, J., Goodall, M., Browning, D. F., O'Shea, M. K., Cranston, A., De Soyza, A., Cunningham, A. F., MacLennan, C. A., Henderson, I. R. and Stockley, R. A. 2014. Increased severity of respiratory infections associated with elevated anti-LPS IgG2 which inhibits serum bactericidal killing. *J Exp Med,* 211**,** 1893-904.

Wettlaufer, J., Klingel, M., Yau, Y., Stanojevic, S., Tullis, E., Ratjen, F. and Waters, V. 2016. Longitudinal study of Stenotrophomonas maltophilia antibody levels and outcomes in cystic fibrosis patients. *J Cyst Fibros*.

Whitters, D. and Stockley, R. 2012. Immunity and bacterial colonisation in bronchiectasis. *Thorax,* 67**,** 1006-13.

Whitters, D. and Stockley, R. A. 2013. Bronchiectasis in older patients with chronic obstructive pulmonary disease : prevalence, diagnosis and therapeutic management. *Drugs Aging,* 30**,** 215-25.

Wilczynski, J. R. 2005. Th1/Th2 cytokines balance--yin and yang of reproductive immunology. *Eur J Obstet Gynecol Reprod Biol,* 122**,** 136-43.

Winnie, G. B. and Cowan, R. G. 1991. Respiratory tract colonization with Pseudomonas aeruginosa in cystic fibrosis: correlations between anti-Pseudomonas aeruginosa antibody levels and pulmonary function. *Pediatr Pulmonol,* 10**,** 92-100.

Winter, A. G., Samuel, K., Hsia, K.-T. and Melton, D. W. 2003. The repair and recombination enzyme ERCC1 is not required for immunoglobulin class switching. *DNA Repair,* 2**,** 561-569.

Wood, A. M. and Stockley, R. A. 2006. The genetics of chronic obstructive pulmonary disease. *Respir Res,* 7**,** 130.

Woof, J. M. and Burton, D. R. 2004. Human antibody-Fc receptor interactions illuminated by crystal structures. *Nat Rev Immunol,* 4**,** 89-99.

Wozniak, B., Wozniak, A., Konca, J., Gorecki, D., Mila-Kierzenkowska, C., Szpinda, M., Sutkowy, P. and Wesolowski, R. 2015. Activity of alpha 1-antitrypsin and some lysosomal enzymes in the blood serum of patients with chronic obstructive pulmonary disease after smoking cessation. *Biomed Res Int,* 2015**,** 176582.

Yamauchi, K., Kasuya, Y., Kuroda, F., Tanaka, K., Tsuyusaki, J., Ishizaki, S., Matsunaga, H., Iwamura, C., Nakayama, T. and Tatsumi, K. 2011. Attenuation of lung inflammation and fibrosis in CD69-deficient mice after intratracheal bleomycin. *Respir Res,* 12**,** 131.

Yao, H. and Rahman, I. 2011. Current concepts on oxidative/carbonyl stress, inflammation and epigenetics in pathogenesis of chronic obstructive pulmonary disease. *Toxicol Appl Pharmacol. ,* 254**,** 72-85.

Yeshanew, A. G., Gizaw, A. K. and Tebeje, B. M. 2015. Effect of highly active antiretroviral therapy on T-cell sub-population profile in humanimmunodeficiency virus-1 infected patients. *Int Immunol,* 3**,** 57-71.

Yin, Y., Wang, X. X. and Mariuzza, R. A. 2012. Crystal structure of a complete ternary complex of T-cell receptor, peptide–MHC, and CD4. *Proc Natl Acad Sci U S A,* 109**,** 5405-5410.

Yonker, L. M., Cigana, C., Hurley, B. P. and Bragonzi, A. 2015. Host-pathogen interplay in the respiratory environment of cystic fibrosis. *J Cyst Fibros,* 14**,** 431-9.

Yoon, S. S., Hennigan, R. F., Hilliard, G. M., Ochsner, U. A., Parvatiyar, K., Kamani, M. C., Allen, H. L., DeKievit, T. R., Gardner, P. R. and Schwab, U. 2002. Pseudomonas aeruginosa anaerobic respiration in biofilms: relationships to cystic fibrosis pathogenesis. *Dev Cell*

3**,** 593-603.

Yoshida, T. and Tuder, R. M. 2007. Pathobiology of cigarette smoke-induced chronic obstructive Pulmonary Disease. *Physiol Rev,* 87**,** 1047-1082.

Yu, S., Kim, H. Y., Chang, Y. J., DeKruyff, R. H. and Umetsu, D. T. 2014. Innate lymphoid cells and asthma. *J Allergy Clin Immunol,* 133**,** 943-50; quiz 51.

Zhang, P., Summer, W. R., Bagby, G. J. and Nelson, S. 2000. Innate immunity and pulmonary host defense. *Immunol Rev,* 173**,** 39-51.

Zhang, X. and Mosser, D. M. 2008. Macrophage activation by endogenous danger signals. *J Pathol,* 214**,** 161-78.

Zhao, H., Jung, J. A., Briles, D. E., Kita, H., Tsigrelis, C. and Juhn, Y. J. 2013. Asthma and antibodies to pneumococcal virulence proteins. *Infection,* 41**,** 927-34.

Zheng, L., Shum, I. H., Tipoe, G. L., Leung, R., Lam, W. K., Ooi, G. C. and Tsang, K. W. 2001. Macrophages, neutrophils and tumour necrosis factor- α expression in bronchiectatic airways in vivo. *Respir Med,* 95**,** 792-798.

Zhong, W., Zhang, Z., Hinrichs, D., Wu, X., Hall, M., Xia, Z. and Rosenbaum, J. T. 2010. OX40 induces CCL20 expression in the context of antigen stimulation: an expanding role of co-stimulatory molecules in chemotaxis. *Cytokine,* 50**,** 253-9.

Appendices

Appendix 1: Ethical approval

Health Research Authority

NRES Committee North East - County Durham & Tees Valley

Room 002
TEDCO Business Centre
Viking Industrial Park
Rolling Mill Road
Jarrow
Tyne & Wear
NE32 3DT

Telephone: 0191 4283545 Facsimile: 0191 4283432

26 July 2012

Dr Anthony DeSoyza

Senior Lecturer, Honorary Consultant in Respiratory medicine

Newcastle Upon Tyne Hospitals NHS Foundation Trust
Sir William Leech Centre for Lung Research

Freeman Hospital

Newcastle Upon Tyne

NE7 7DN

Dear Dr DeSoyza

Study title: **Inflammation, aging and lung disease study**

REC reference: 12/NE/0248

Thank you for your e-mail correspondence of 23rd July 2012 responding to the Committee's request for further information on the above research and submitting revised documentation. The further information has been considered on behalf of the Committee by the Chair.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised, subject to the conditions specified below.

Ethical review of research sites

NHS sites

The favourable opinion applies to all NHS sites taking part in the study, subject to management permission being obtained from the NHS/HSC R&D office prior to the start of the study (see "Conditions of the favourable opinion" below).

Non-NHS sites

Conditions of the favourable opinion

The favourable opinion is subject to the following conditions being met prior to the start of the study.

Management permission or approval must be obtained from each host organisation prior to the start of the study at the site concerned.

Management permission ("R&D approval") should be sought from all NHS organisations involved in the study in accordance with NHS research governance arrangements.

Guidance on applying for NHS permission for research is available in the Integrated Research Application System or at http://www.rdforum.nhs.uk.

Where a NHS organisation's role in the study is limited to identifying and referring potential participants to research sites ("participant identification centre"), guidance should be sought from the R&D office on the information it requires to give permission for this activity.

For non-NHS sites, site management permission should be obtained in accordance with the procedures of the relevant host organisation.

Sponsors are not required to notify the Committee of approvals from host organisations

It is the responsibility of the sponsor to ensure that all the conditions are complied with before the start of the study or its initiation at a particular site (as applicable).

Approved documents

The final list of documents reviewed and approved by the Committee is as follows:

Document	*Version*	*Date*
Covering Letter		12 June 2012
GP/Consultant Information Sheets	GP Letter Version 1.0	24 January 2012
Investigator CV	Dr DeSoyza	04 April 2012
Investigator CV	Jodie Birch	11 June 2012
Other: Normal Volunteers Consent Form	1.0	28 January 2012
Participant Consent Form	1.0	28 January 2012
Participant Information Sheet: Patient Information Leaflet	Version 1.1	23 July 2012
Participant Information Sheet: Volunteer information Leaflet	Version 1.1	23 July 2012

Protocol	1.0	14 February 2012
REC application		
Response to Request for Further Information		

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

After ethical review

Reporting requirements

The attached document *"After ethical review – guidance for researchers"* gives detailed guidance on reporting requirements for studies with a favourable opinion, including:

Notifying substantial amendments

- Adding new sites and investigators
- Notification of serious breaches of the protocol
- Progress and safety reports
- Notifying the end of the study

The NRES website also provides guidance on these topics, which is updated in the light of changes in reporting requirements or procedures.

Feedback

You are invited to give your view of the service that you have received from the National Research Ethics Service and the application procedure. If you wish to make your views known please use the feedback form available on the website.

Further information is available at National Research Ethics Service website > After Review

12/NE/0248	**Please quote this number on all correspondence**

With the Committee's best wishes for the success of this project

Yours sincerely

pp

Dr A MacSween Chair

Email: hayley.jeffries@nhs.net

Enclosures: "After ethical review – guidance for researchers" *[SL-AR2]*

Copy to: Jill Peacock, Newcastle upon Tyne Hospitals NHS Foundation Trust

Appendix 2: Microbiological Reagents

Media for culturing bacteria lysate

Brain heart infusion broth	1.85 g
Deionised water	50 ml

Bradford Reagent

Bradford Dye Reagent (blue)

BSA (Bovine serum albumin)

96 well ELISA Plate

Appendix 3: SDS-PAGE for protein analysis

SDS-PAGE running buffer X10

Running buffer was made up to 10X concentration and (diluted to 1X before use).

Tris-HCl	30.3 g
Glaycine	144.0g
SDS	10.0 g

12% (W/V) SDS Acrylamide resolving gel components

40% (w/v) solution (37.5:1 acrylamide:biscrylamide)	3.0 ml
Solution B*	2.5ml
Deionised water	4.50 ml
10% (w/v) ammonium persulphate (ASP)	50 µl
TEMED	10 µl

***Solution B**

2M Tri-HCL, PH 8.8	75ml	per 100 ml
10%(w/v) SDS	4ml	per 100 ml

4% (W/V) Acrylamide stacking gel components

40% Acrylamide	250 ml
10% ammonium persulphate (APS)	30µl
Deionised water	1.2 ml
Solution C*	500 µl
TEMED	10 µl

*** Solution C**

1M Tris-HCL, PH 6.8	50 ml per 100 ml
10 %(w/v) SDS	4 ml per 100 ml

SDS-PAGE sample buffer

60mM Tris-HCL pH 6.8	0.6ml per 10 ml
10 %(w/v) SDS	2.0 ml per 10 ml

50% (v/v) glycerol	5.0ml per 10 ml
14.4 mM β- Mercaptoethanol	0.5 ml per10 ml
0.1% (w/v) Bromophenol Blue	1.0 ml per 10 ml
Deionised water	0.9ml per 10ml

Coomassie blue gel stain solution

Coomassie Brilliant Blue R250	1.0g per L
Glacial acetic acid	100.0 ml per L
Methanol	450.0 ml per L

Coomassie gel destain solution

Glacial acetic acid	100.0 ml per L
Methanol	100.0ml per L

Appendix 4: Pierce® LAL Chromogenic Endotoxin Quantitation Kit

Endotoxin-free-water 1 vial, 30mL

Limulus Amebocyte Lysate (LAL), lyophilized, 2vial, 1.4mL

E. coli Endotoxin standard (011: B4), lyophilized, 1 vial,15-40 endotoxin unit (EU)/mL upon reconstitution 28EU/ml

Chromogenic substrate, lyophilized, 1 vial, 6.5mL/vial upon reconstitution (7mg of lyophilized substrate was reconstituted in 9 ml of in endotoxin-free water)

Acetic acid

Appendix 5: Reagents for ELISA

Phosphate Buffer Saline PH =7.0 (PBS)

Phosphate Buffer was made up to 10X concentration (and diluted to 1X before use)

NaCl 81.7 g

Na_2PO_4 11.2 g

NaH_2PO_4.H2O 2.2g

Deionised water up to 1L

PBS/0.05 Tween 20 (washing buffer in-between steps)

Phosphate Buffer Saline (1XPBS) 1L

Tween 20 0.5 ml

PBS Tween (PBST) + skimmed milk (blocking buffer)

Skimmed milk powder 50 g

PBS- Tween 20 up to 50ml

PBS Tween (PBST) + skimmed milk for diluent solution

Blocking buffer 5ml

PBST 45 ml

Diluent solution for keeping sera long time

Diluent solution 50 ml

Azide 50 µl

Citrate phosphate Buffer (for substrate solution)

Citric acid 5.1g

Na_2HPO_4 7.31g

Deionised water up to 1L

Substrate solution

Citrate buffer 25ml

Orthophenylenediamine (OPD) 20 mg tablet

H_2O_2	20 µl

1.2.7. Stopping reaction

Sulphuric acid	2M

All chemicals were from Sigma-Aldrich, Gillingham, Kent

Appendix 6: Reagents for ELIspot

Bicarbonate Buffer, 0.05 M pH 9.6 for coating

Carbonate –bicarbonate buffer 1 capsule

Deionised water 100 ml

Phosphate Buffer Saline (PBSx1) for washing (see 3.1.2.1)

Substrate solution

The solution made for 1 plate

AP Conjugate substrate kit 50 µl of each (A&B)

AP development buffer 200 µl

Deionised water 4700 µl

Medium for washing the cells (R0)

RPMI-1640 500ml

Medium for culturing the cells (R10)

RPMI-1640 500ml

Supplemented with penicillin (100U/ml), streptomycin (100mg/ml), L-glutamine (2mM) (Invitrogen, UK) and 10% heat-inactivated FBS (Biosera, UK) 5ml

Appendix 7: Flow cytometry reagents

Anti-Mouse Ig, κ/Negative Control Compensation Particles Set 6.0 ml (1 ea)

Brilliant Stain Buffer (BD Horizon™) 50 µl

Permeabilization Buffer 10X (100ml)

Fixation Buffer (IC Fixation Buffer 125ml)

Brefeldin A (Golgi plug, BD Bioscience

Appendix8: Meso Scale Discovery for cytokines ELISA

Cytokines panel1 (human) multi-spot 96 well (10 spot plates)

Pro-inflammatory panel 1(human) multi-spot 96well (10 spot plates

MSD Blocker B 1g

MSD Diluent1 50ml

Diluent 3 5ml

MSD Read Buffer (4X) with surfactant 50ml

Detector antibody pro-inflammatory panel 1(human) kit (calibrator blend):

Anti-human IL-2 antibody

Anti-human IL-4 antibody

Anti-human IL-10 antibody

Anti-human IL-13 antibody

Anti-human IFNγ antibody

Anti-human IL-12P70 antibody

Cytokines panel 1 human kit (calibrator blend)

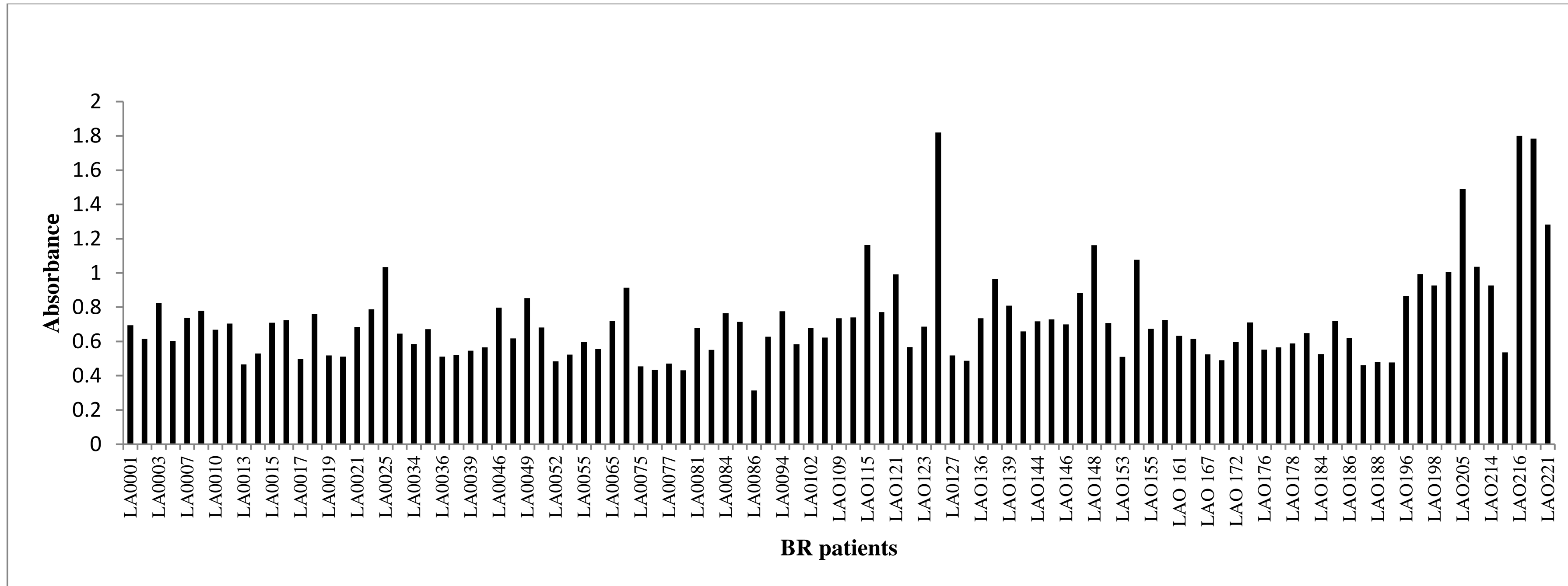
Anti-human IL-5 antibody

Anti-human IL-17A antibody

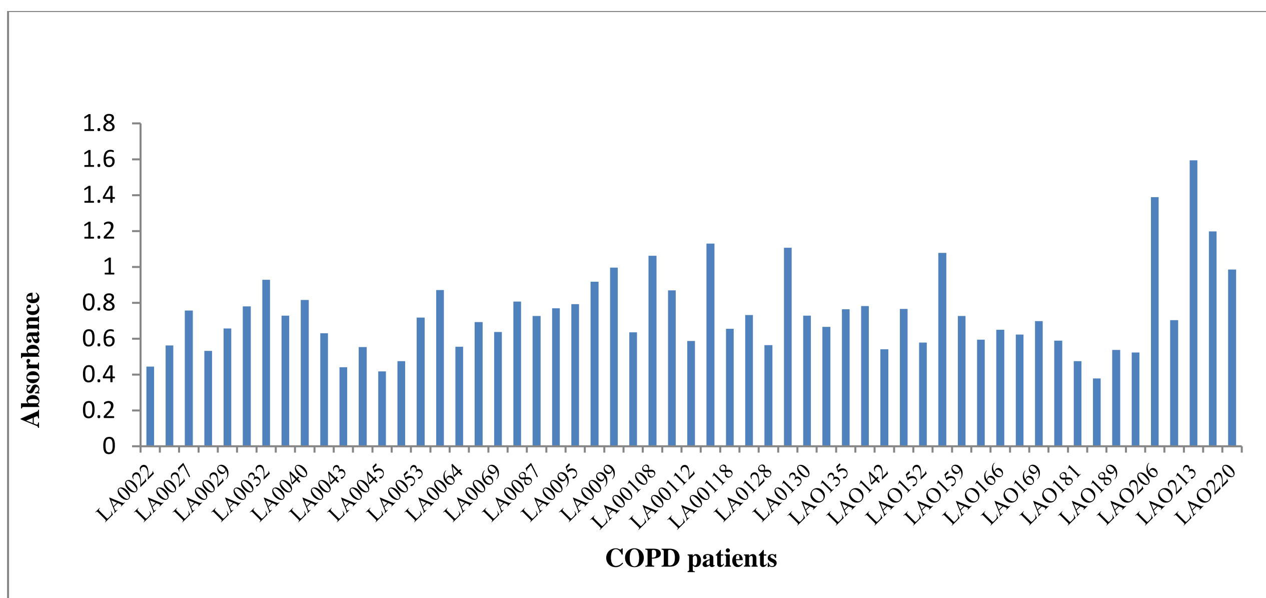
Capture antibody

IFN-γ: Mouse Monoclonal
IL-1β: Mouse Monoclonal
IL-2: Mouse Monoclonal
IL-4: Mouse Monoclonal
IL-5: Mouse Monoclonal
IL-8: Mouse Monoclonal
IL-10: Rat Monoclonal
IL-12p70: Mouse Monoclonal
IL-13: Rat Monoclonal
TNF-α: Mouse Monoclonal

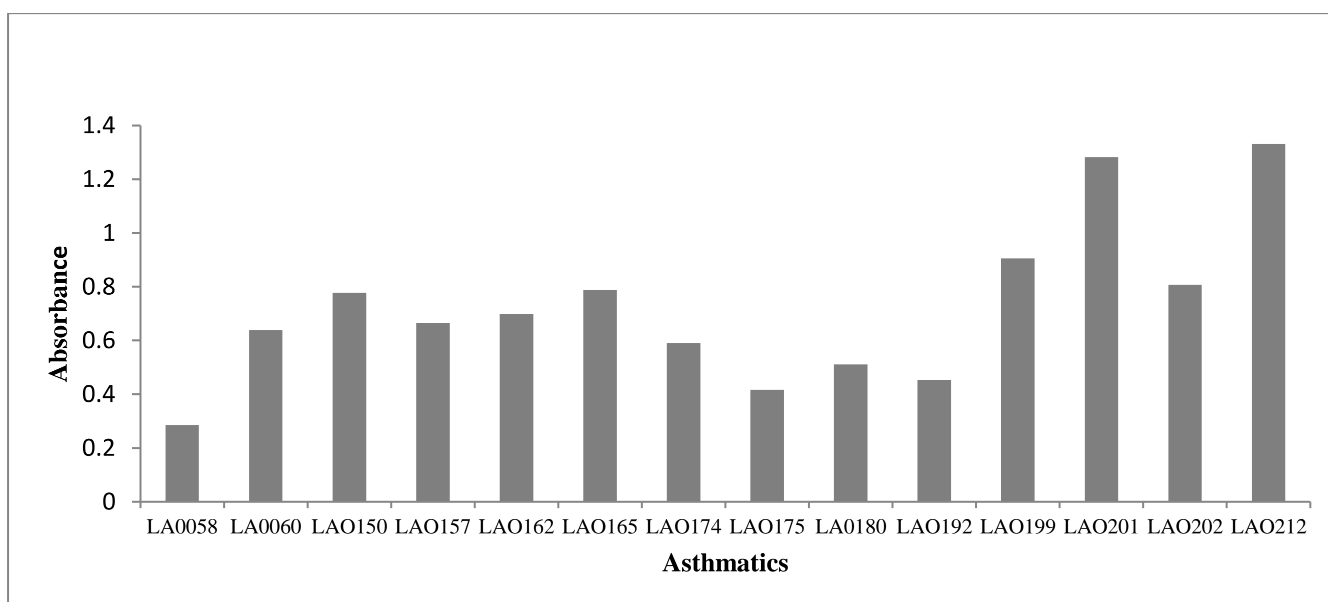
Appendix 9: Antibody screening for BR, COPD, asthma and healthy volunteers



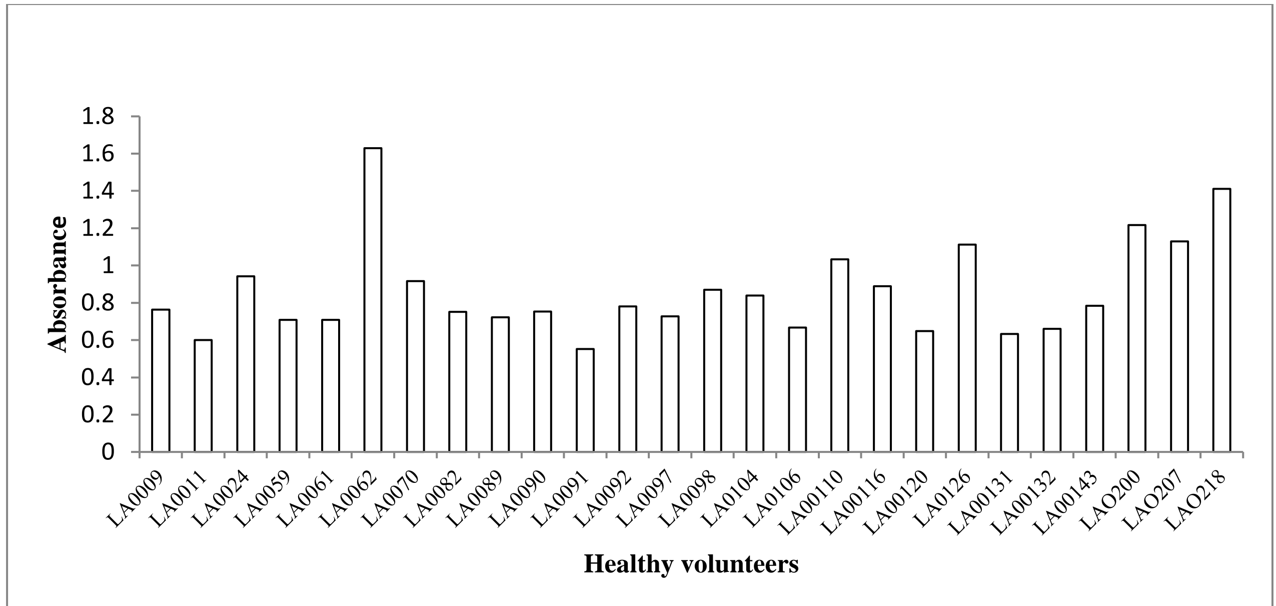
Screening profile of BR sera against *S. pneumoniae* at 1in 25 to establish the first dilution for antibody titration. Absorbance values for each BR patient are given.



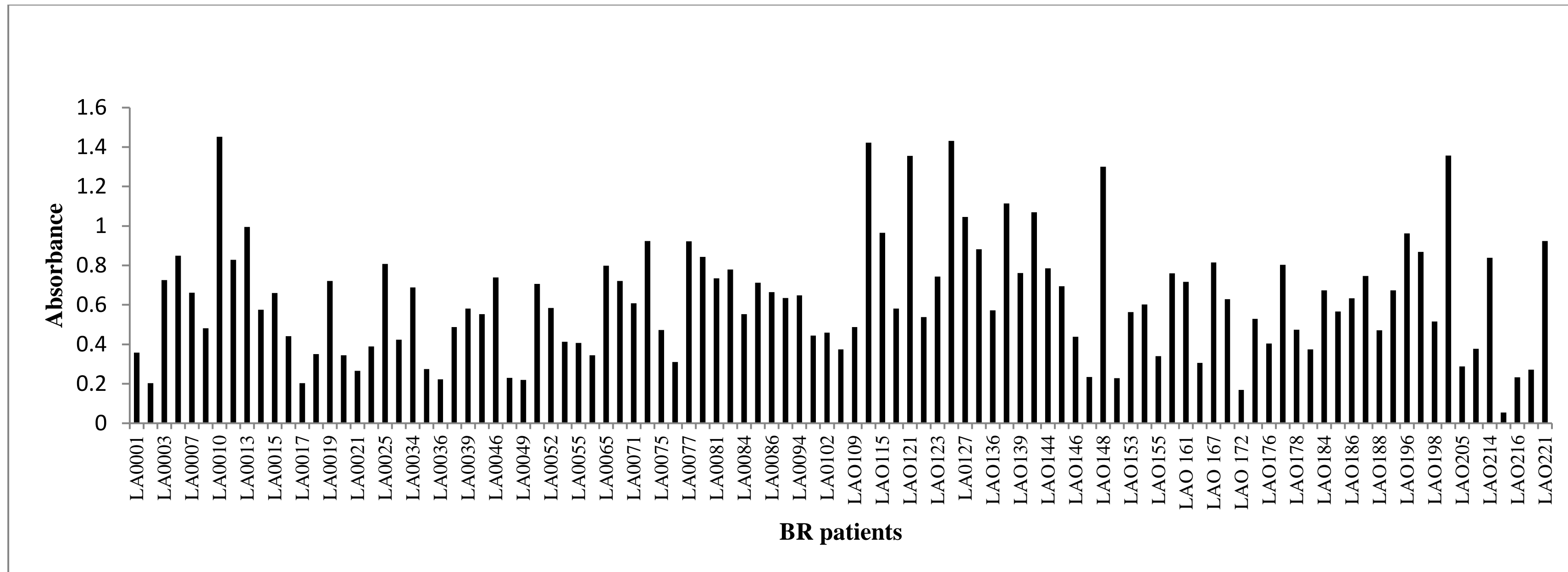
Screening of COPD sera against *S.pneumoniae* at 1:25 to establish the first dilution for antibody titration.



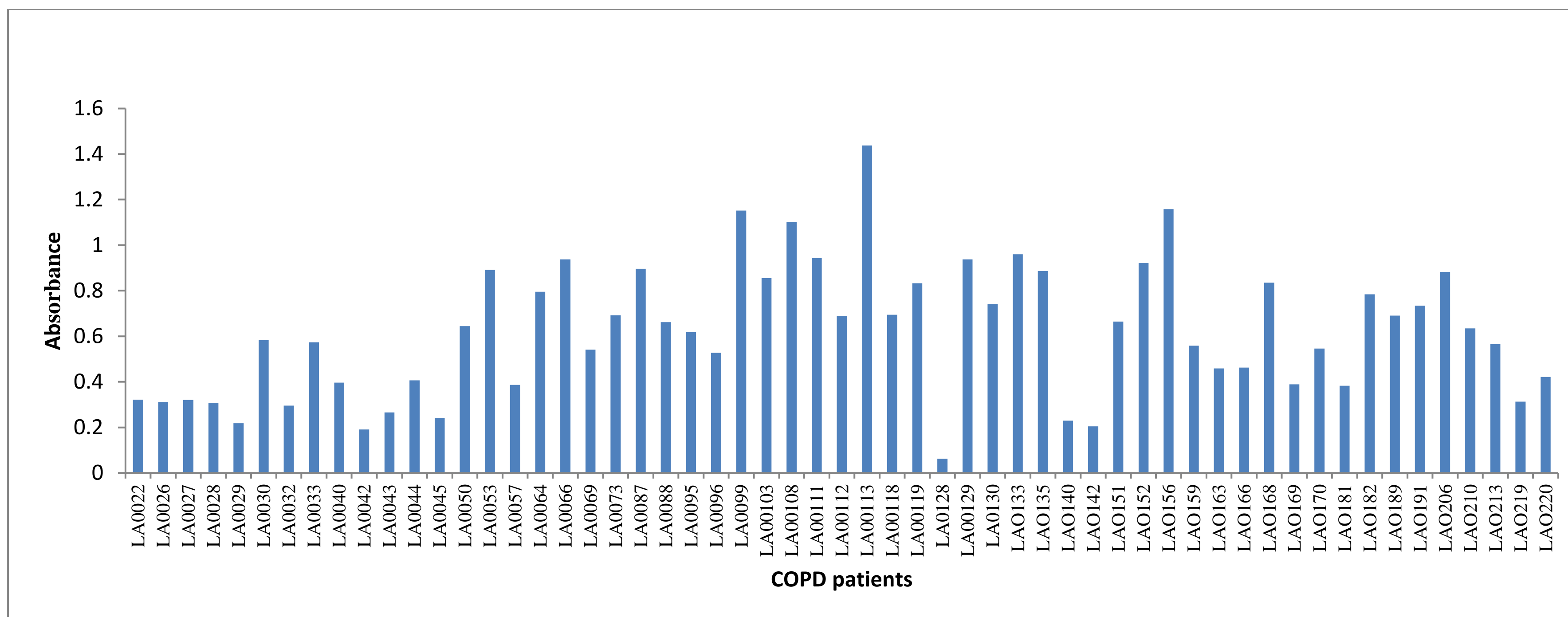
Screening of asthmatics sera against *S.pneumoniae* at 1:25 to establish the first dilution for antibody titration.



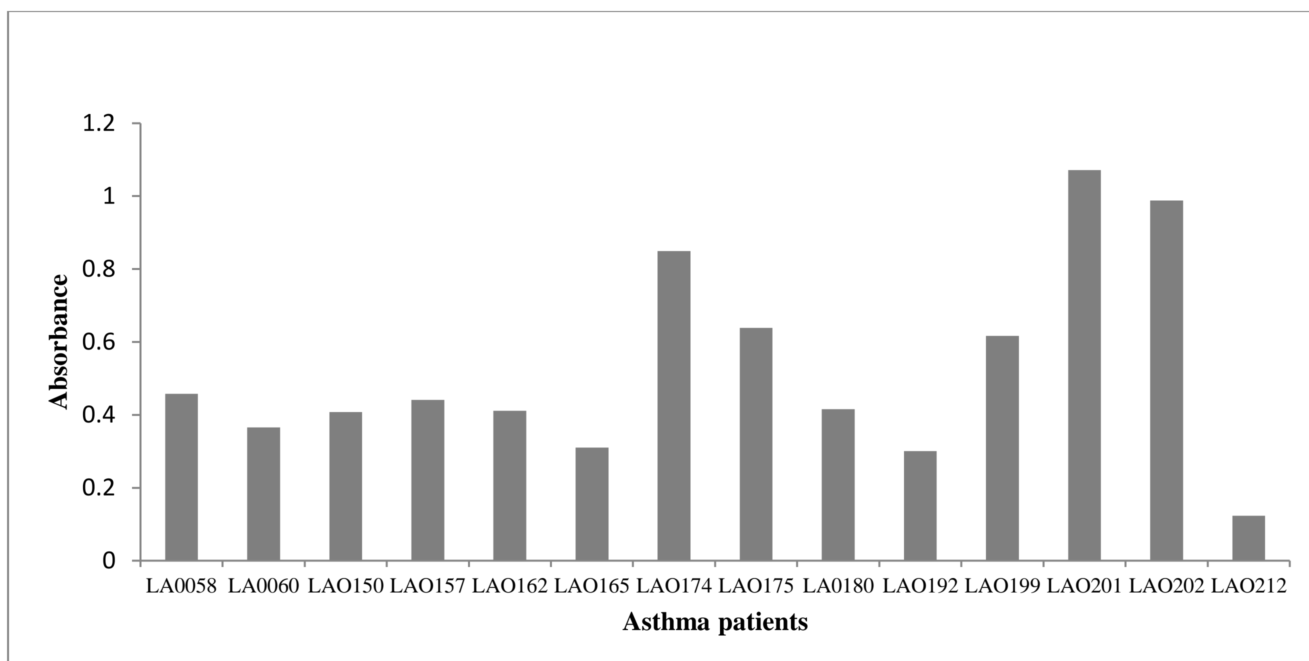
Screening of healthy volunteers sera against *S.pneumoniae* at 1:25 to establish the first dilution for antibody titration.



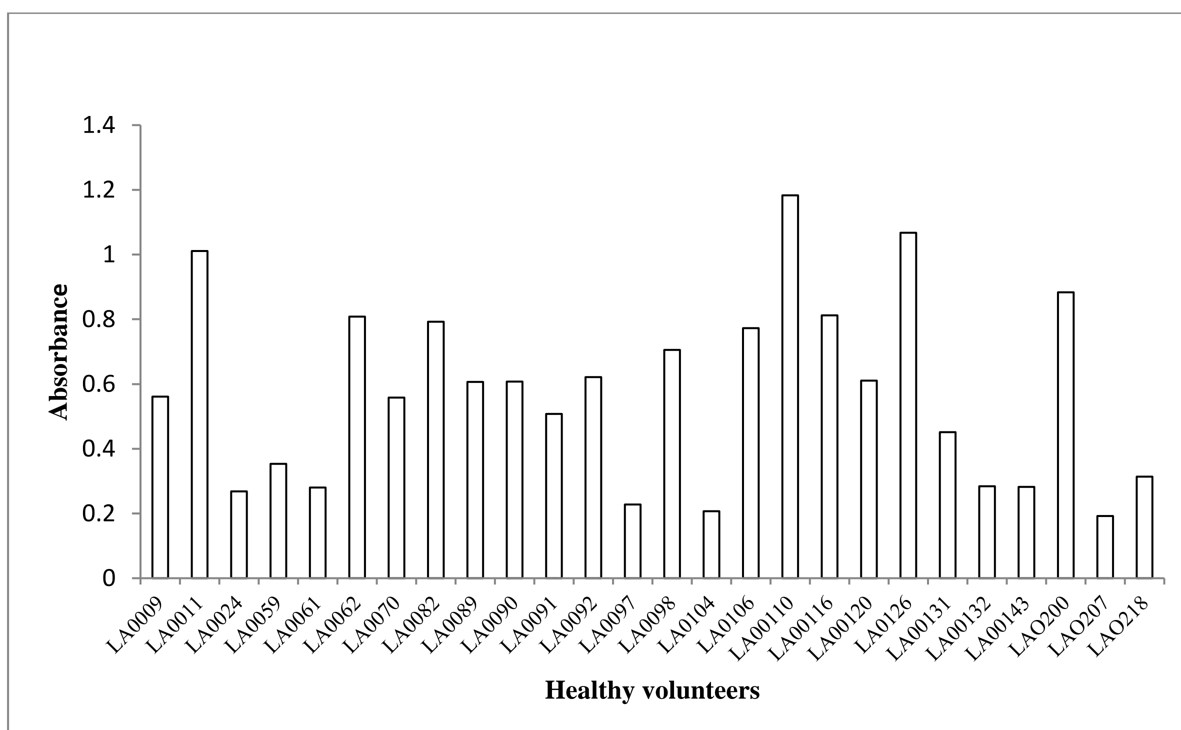
Screening of BR sera against *A. fumigatus* at 1in25 to establish the first dilution for antibody titration.



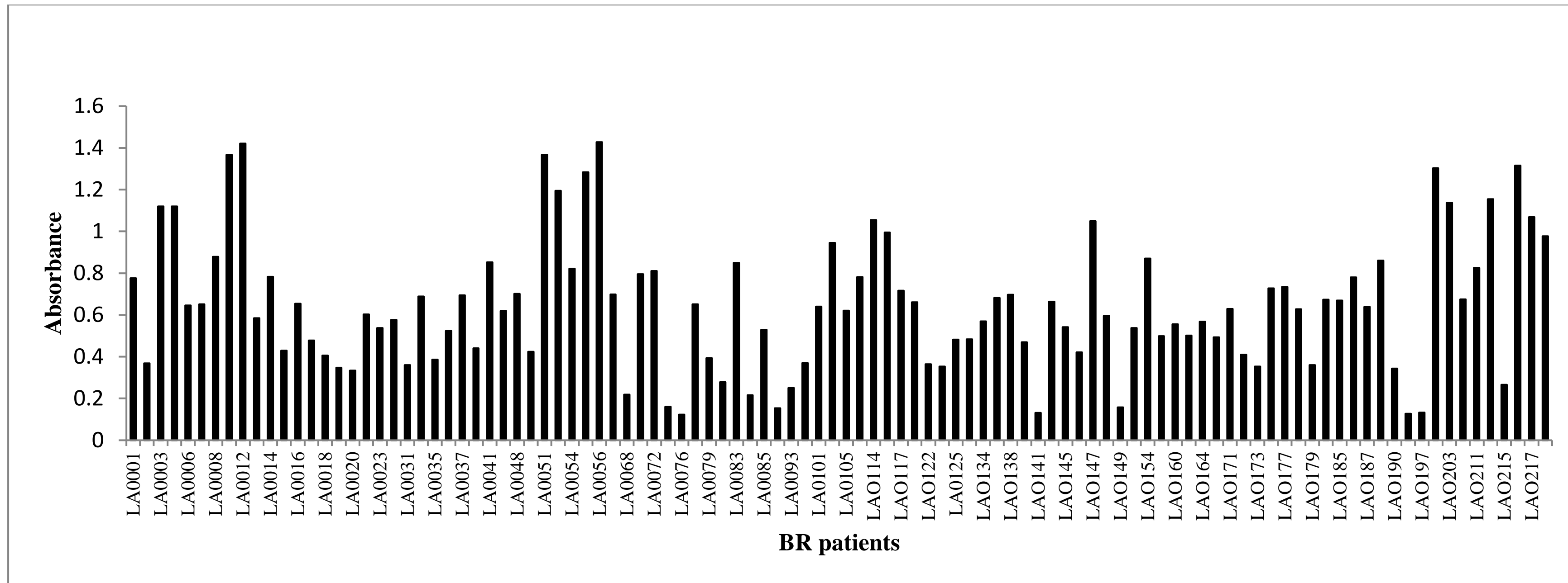
Screening of COPD sera against *A.fumigatus* at 1in 25 to establish the first dilution for antibody titration.



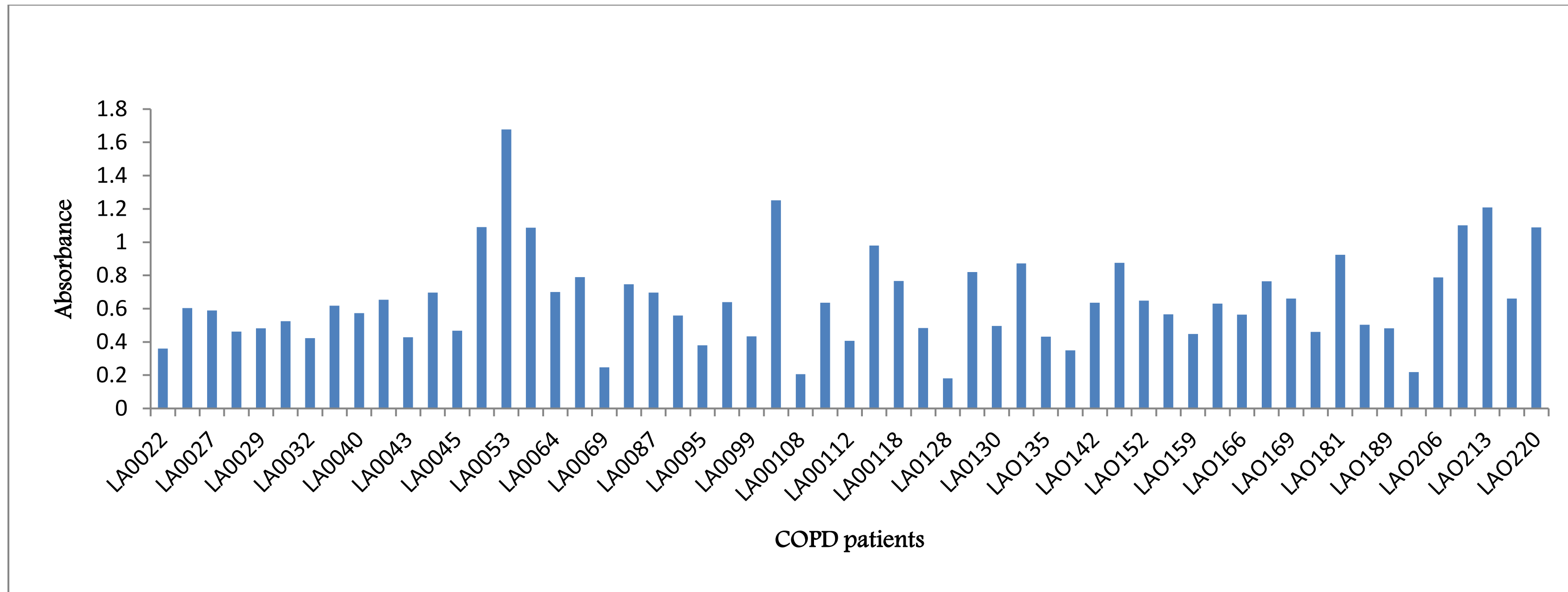
Screening of asthmatics sera against *A.fumigatus at* 1in25 to establish the first dilution for antibody titration.



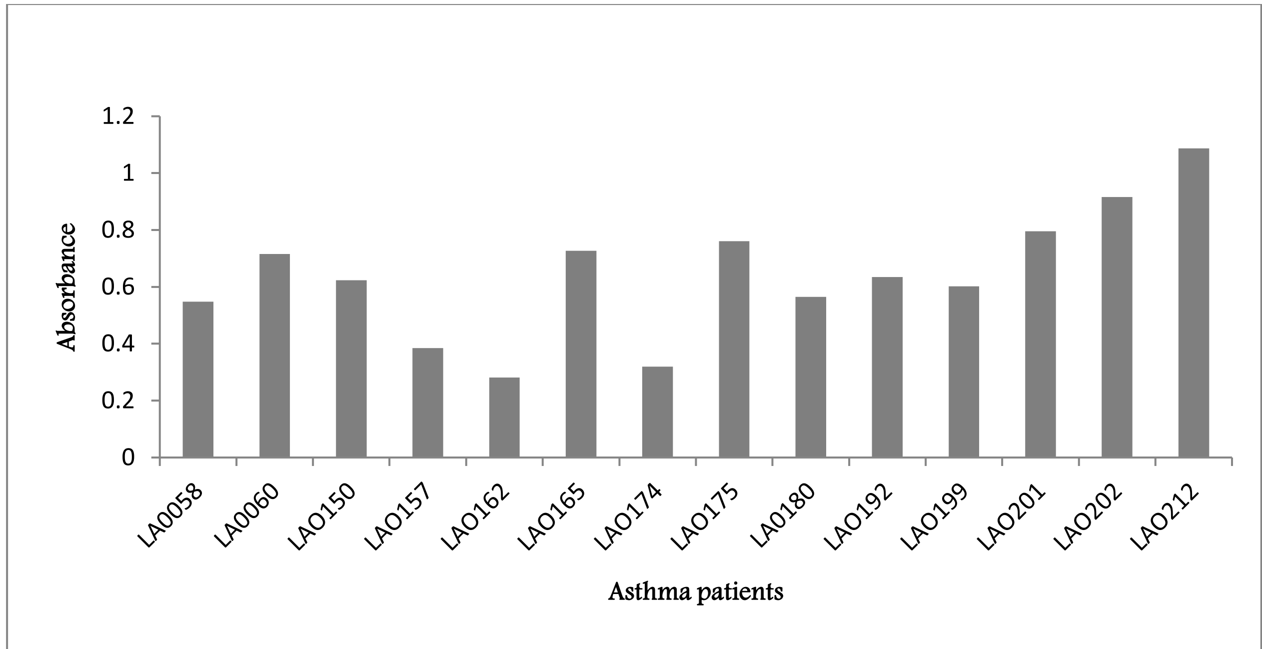
Screening of healthy sera against *A.fumigatus at* 1:25 to establish the first dilution for antibody titration.



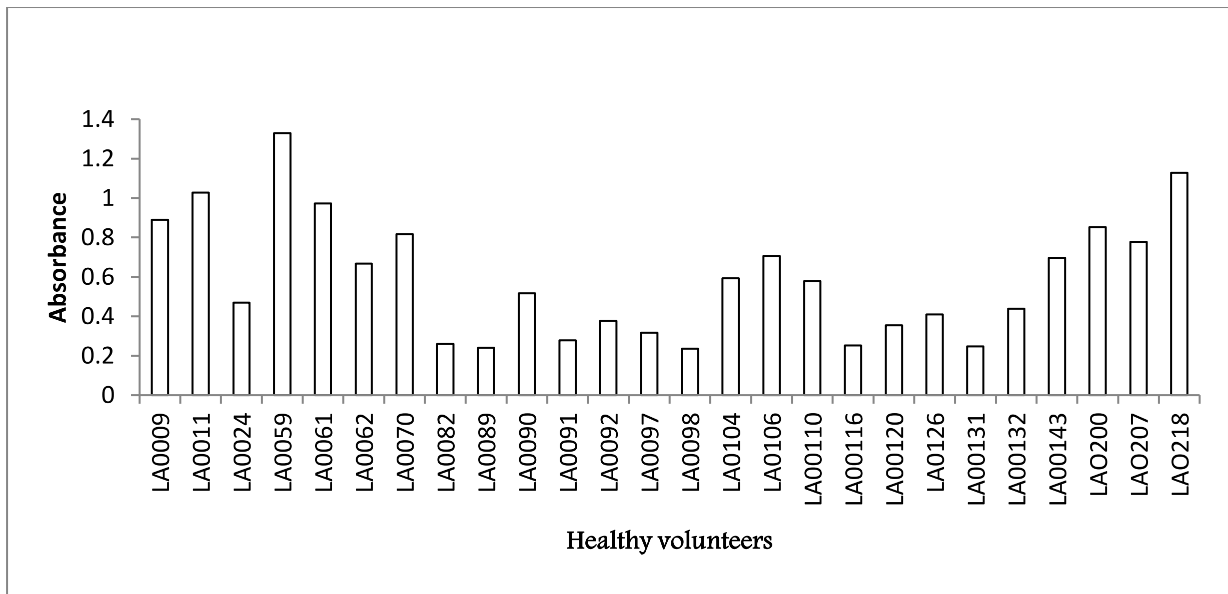
Screening of BR sera against *A.alternata* at 1in25 to establish the first dilution for antibody titration



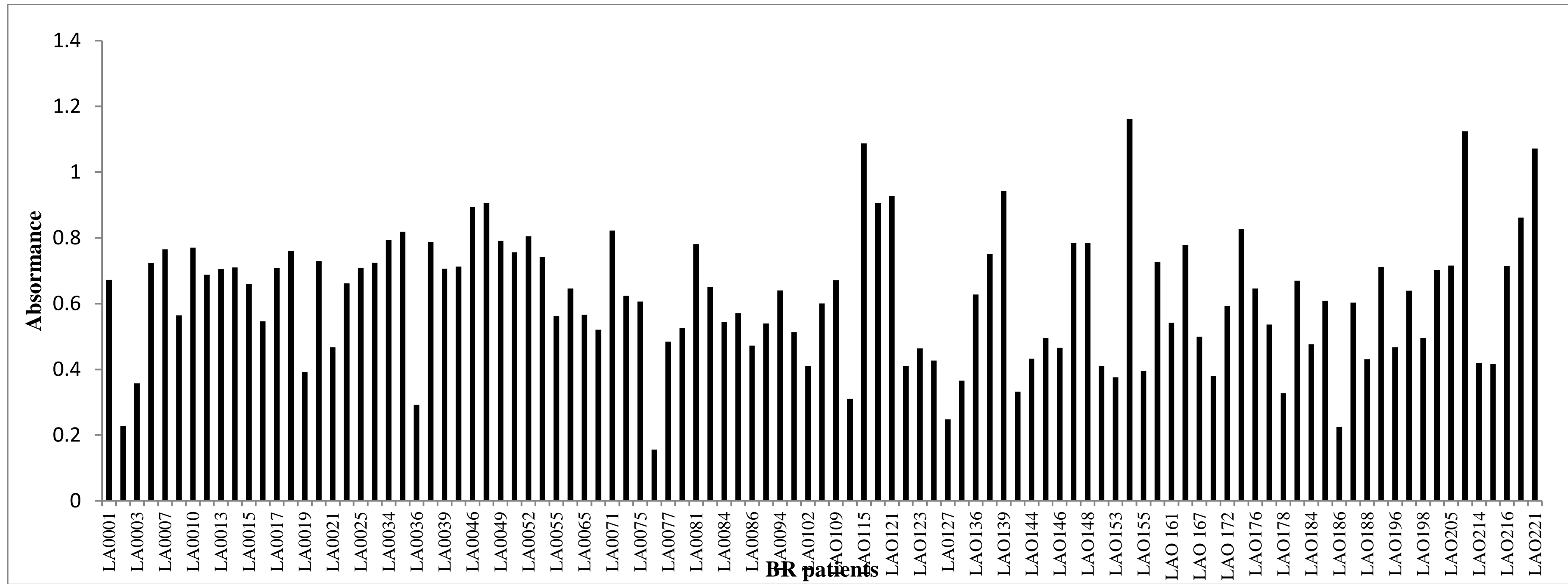
Screening of COPD sera against *A.alternata* at 1in25 to establish the first dilution for antibody titration.



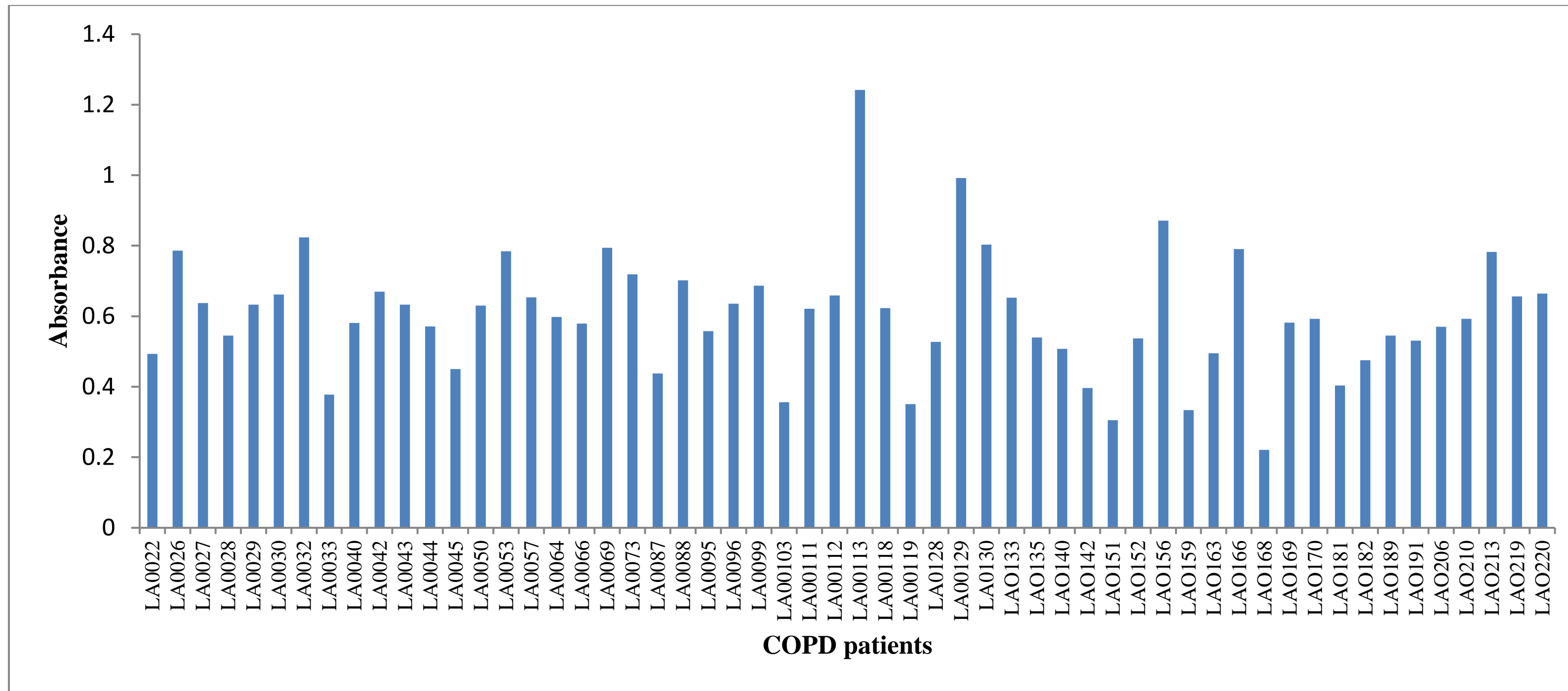
Screening of asthmatics sera against *A.alternata* at 1:25 to establish the first dilution for antibody titration.



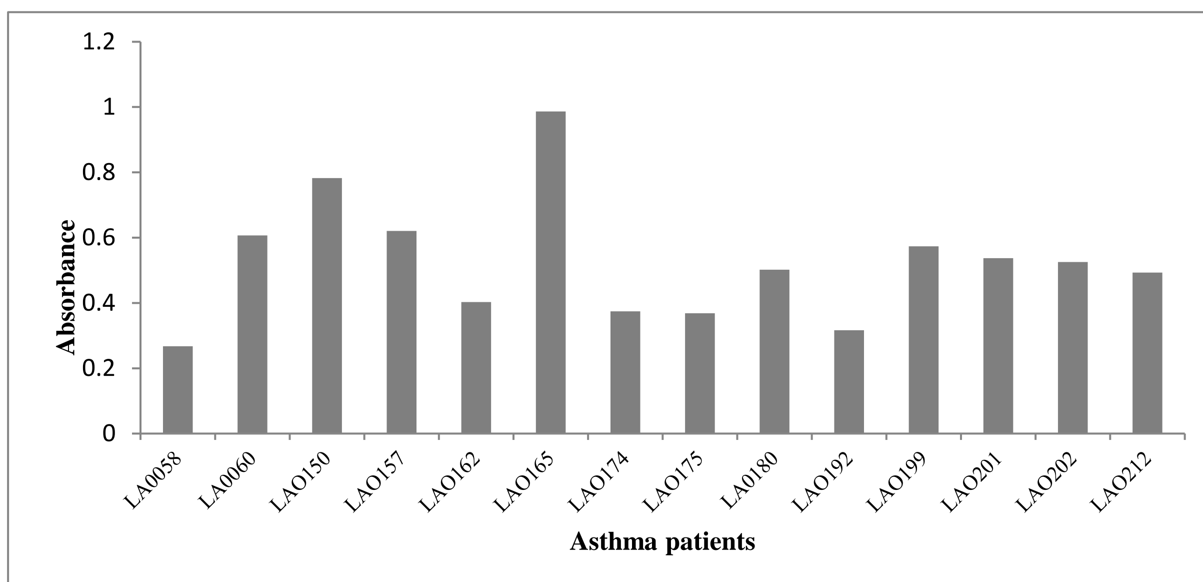
Screening of healthy volunteer’s sera against *A. alternata* at 1in 25 to establish the first dilution for antibody titration.



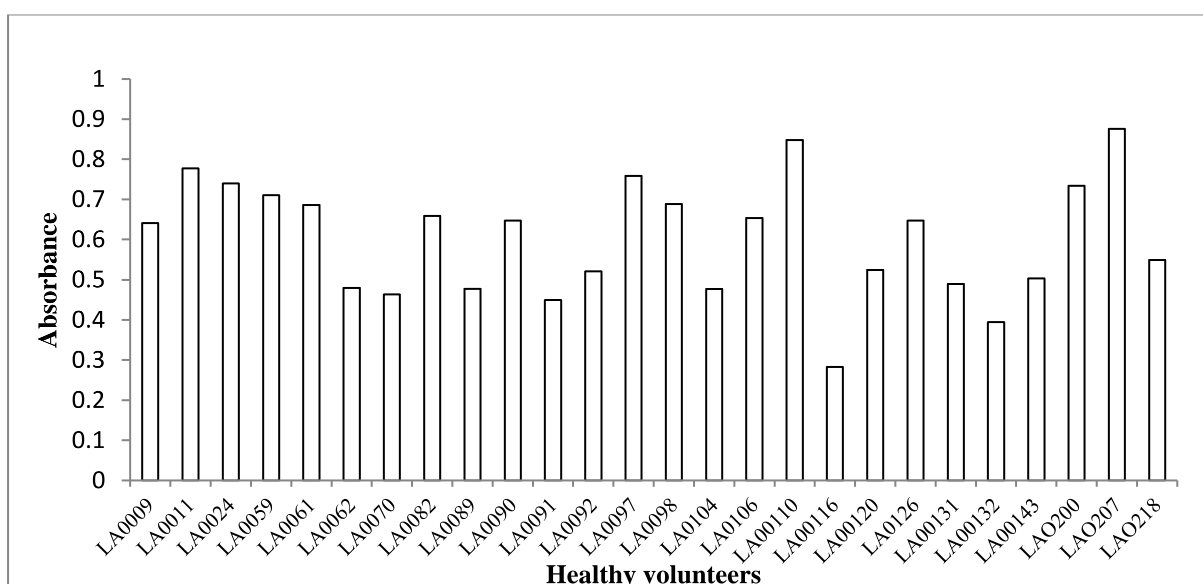
Screening of BR sera against *M. catarrhalis at* 1in 25 to establish the first dilution for antibody titration



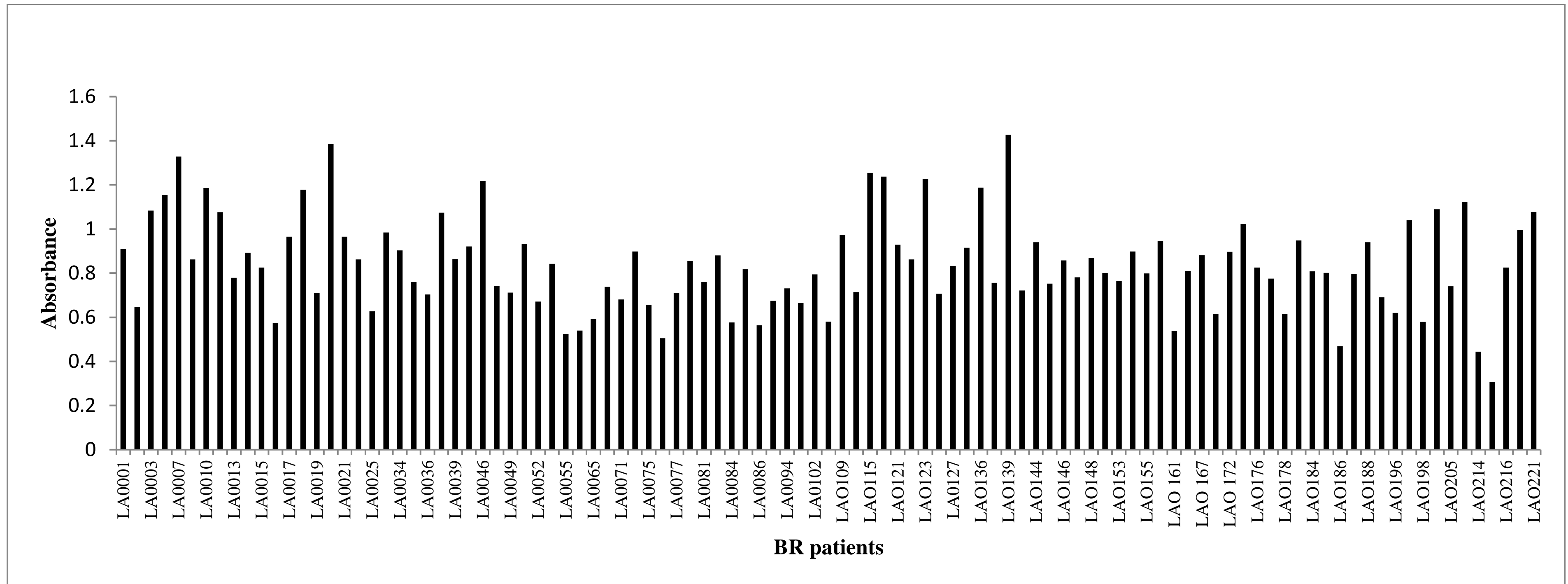
Screening of COPD sera against *M. catarrhalis at* 1in 25 to establish the first dilution for antibody titration



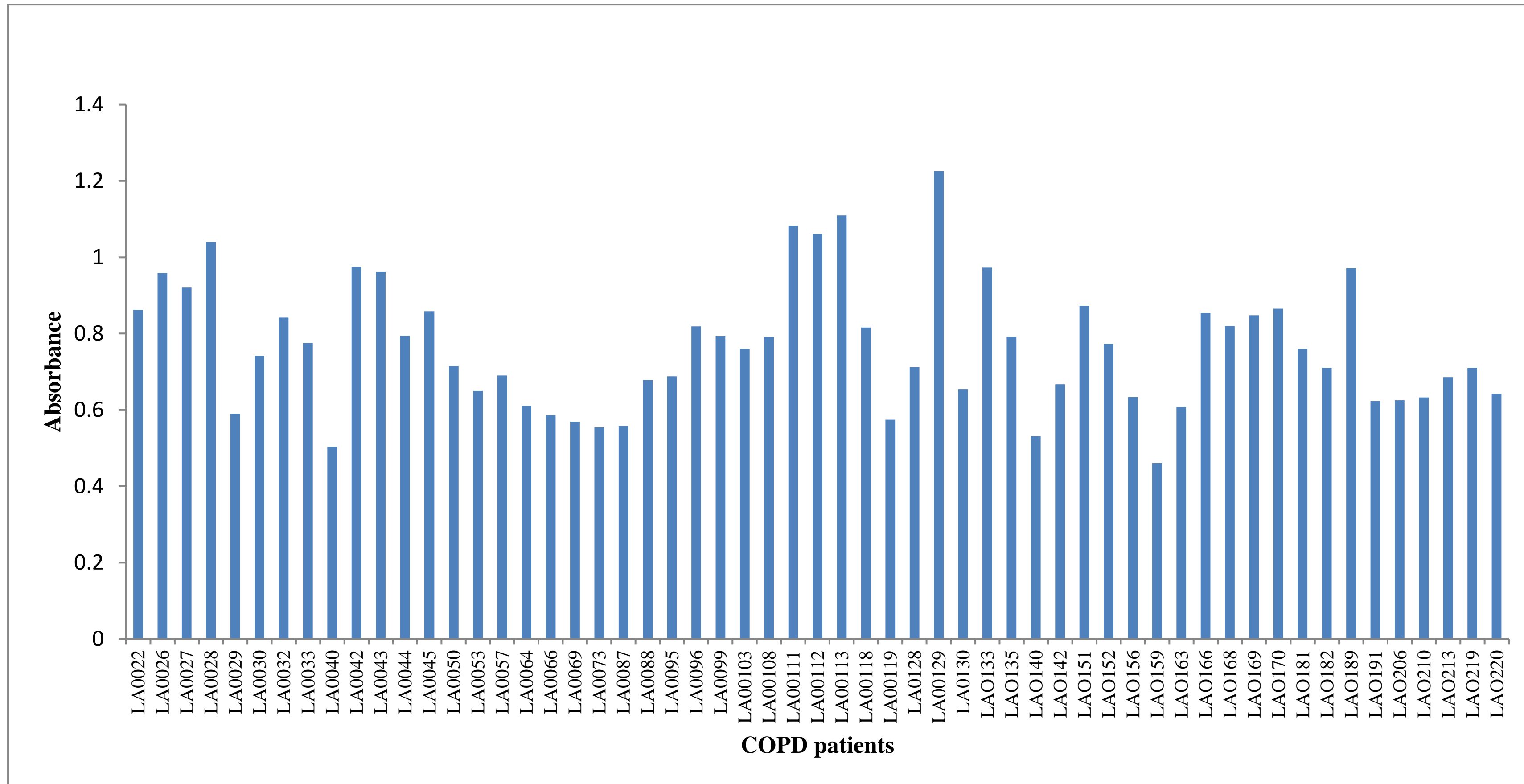
Screening of asthmatics sera against *M. catarrhalis at* 1in 25 to establish the first dilution for antibody titration.



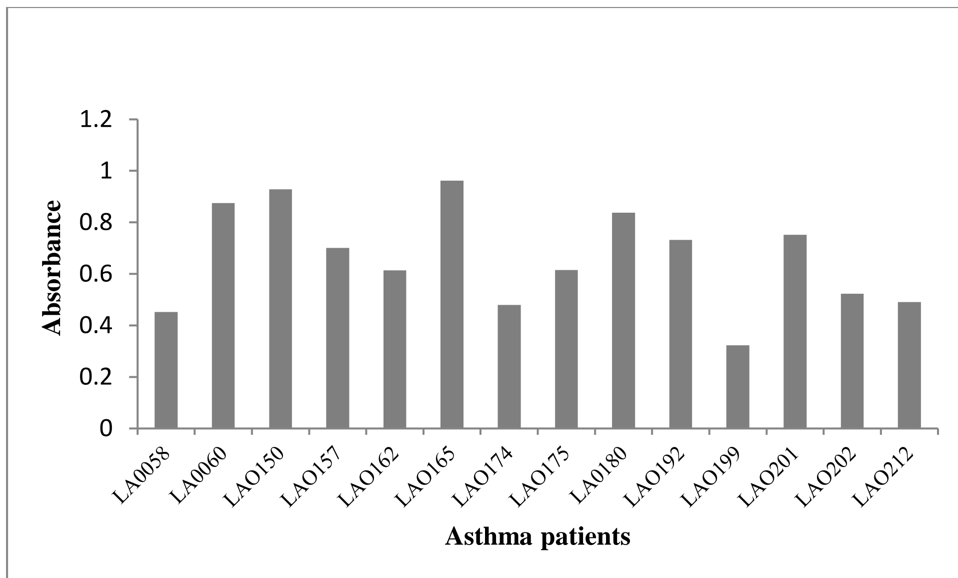
Screening of healthy volunteers sera against *M. catarrhalis at* 1in 25 to establish the first dilution for antibody titration.



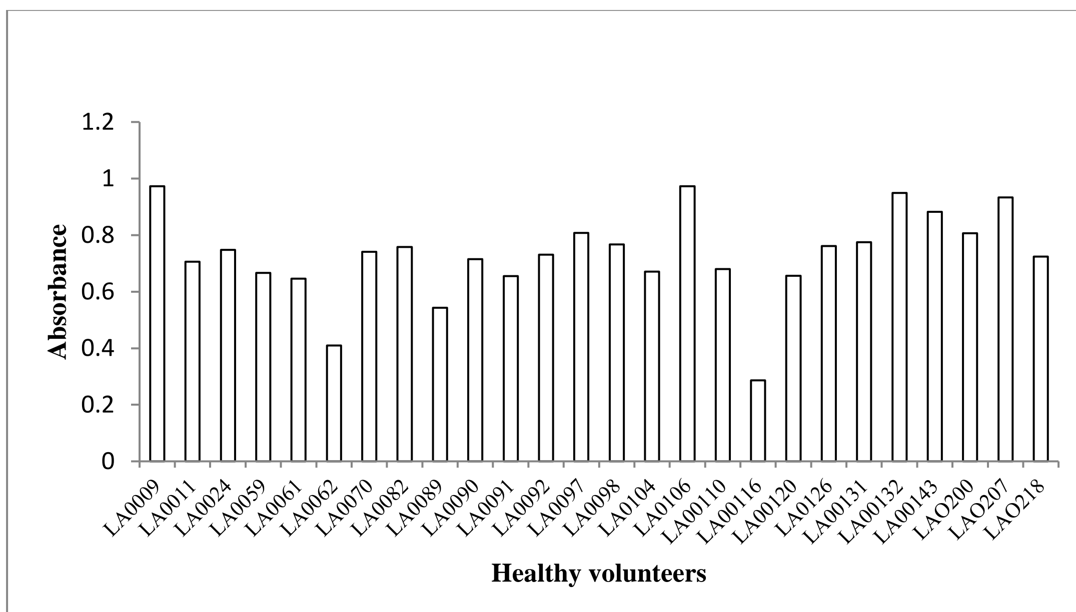
Screening of BR sera against NT*H.influenzae at* 1in25 to establish the first dilution for antibody titration



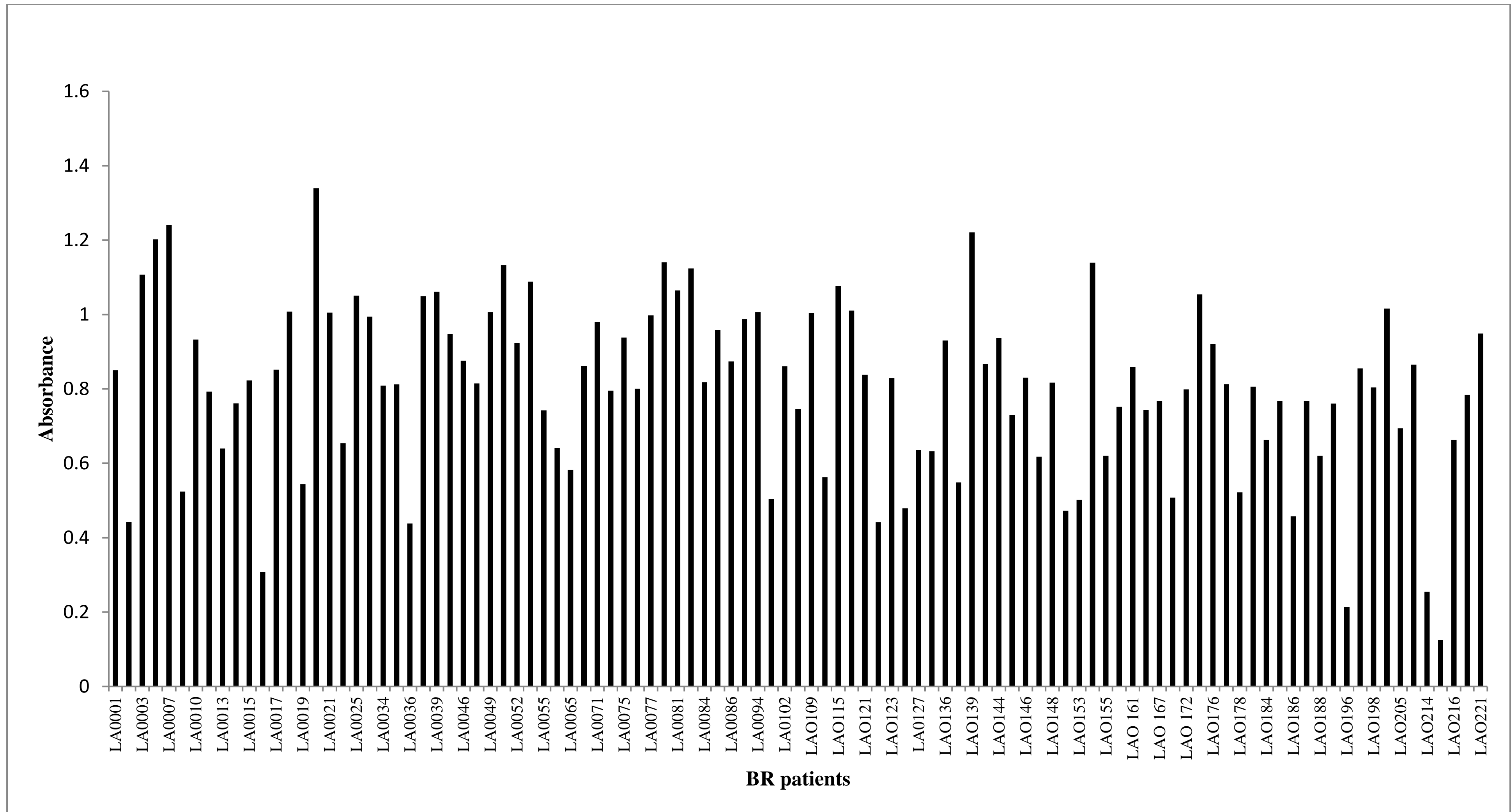
Screening of COPD sera against NT.*H.influenzae* at 1:25 to establish the first dilution for antibody titration



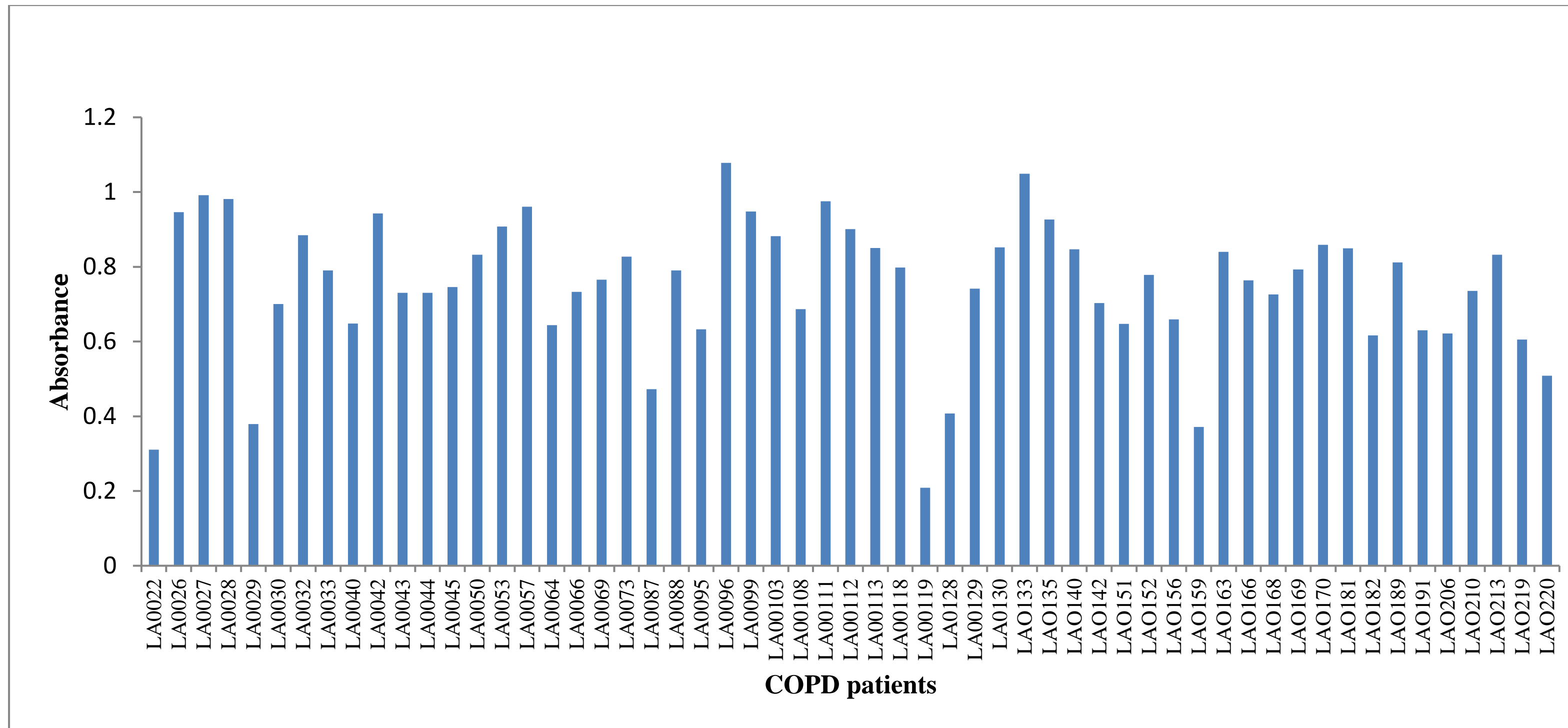
Screening of asthmatics sera against NT*H.Influenzae at* 1in25 to establish the first dilution for antibody titration.



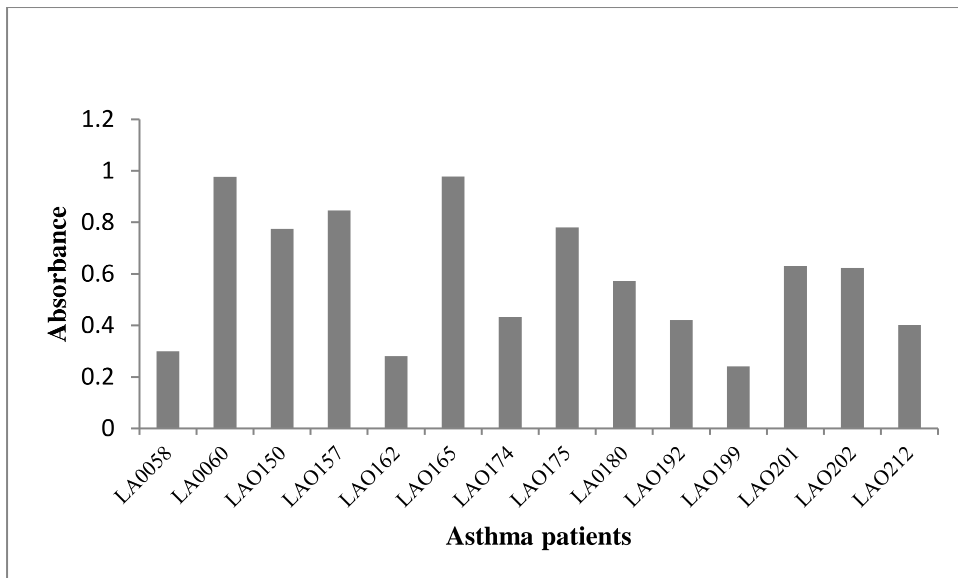
Screening of healthy volunteers sera against NT*H.Influenzae at* 1in 25 to establish the first dilution for antibody titration.



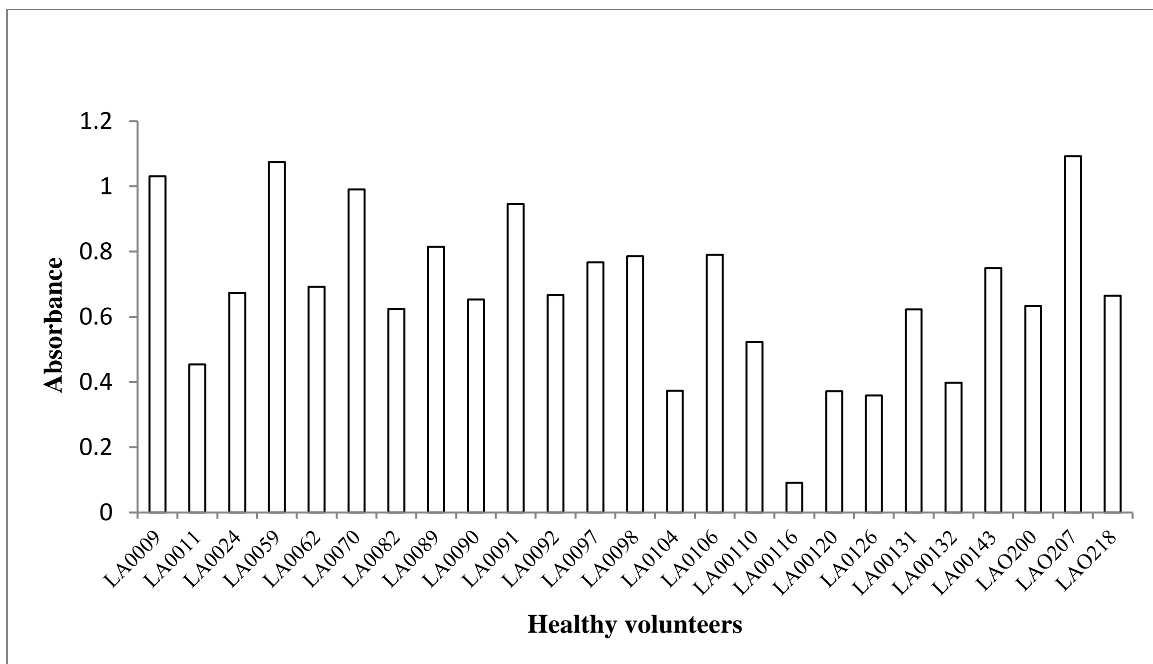
Screening of BR sera against *S.maltophilia* at 1:25 to establish the first dilution for antibody titration.



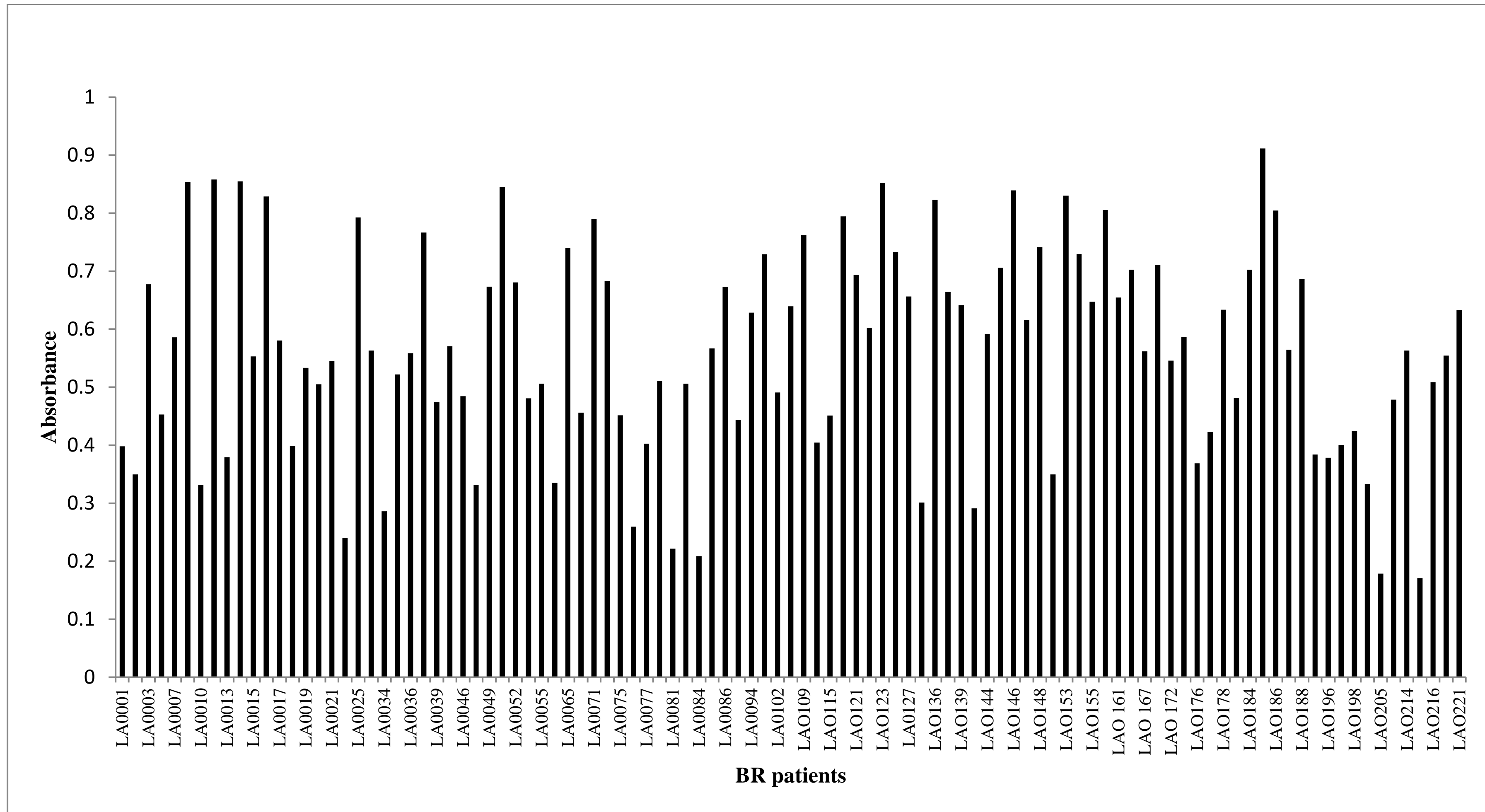
Screening of COPD sera against *S.maltophilia* at 1in 25 to establish the first dilution for antibody titration.



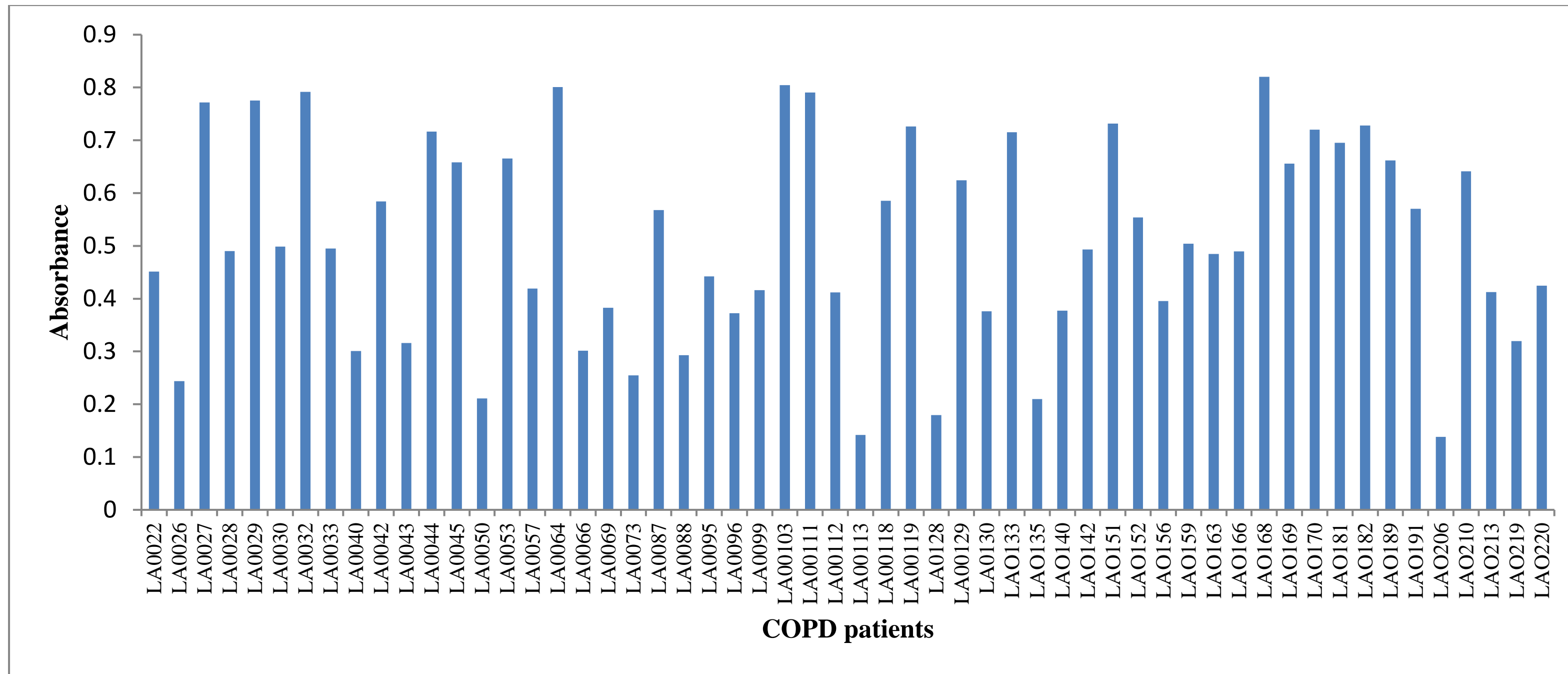
Screening of Asthmatic sera against *S.maltophilia* at 1:25 to establish the first dilution for antibody titration.



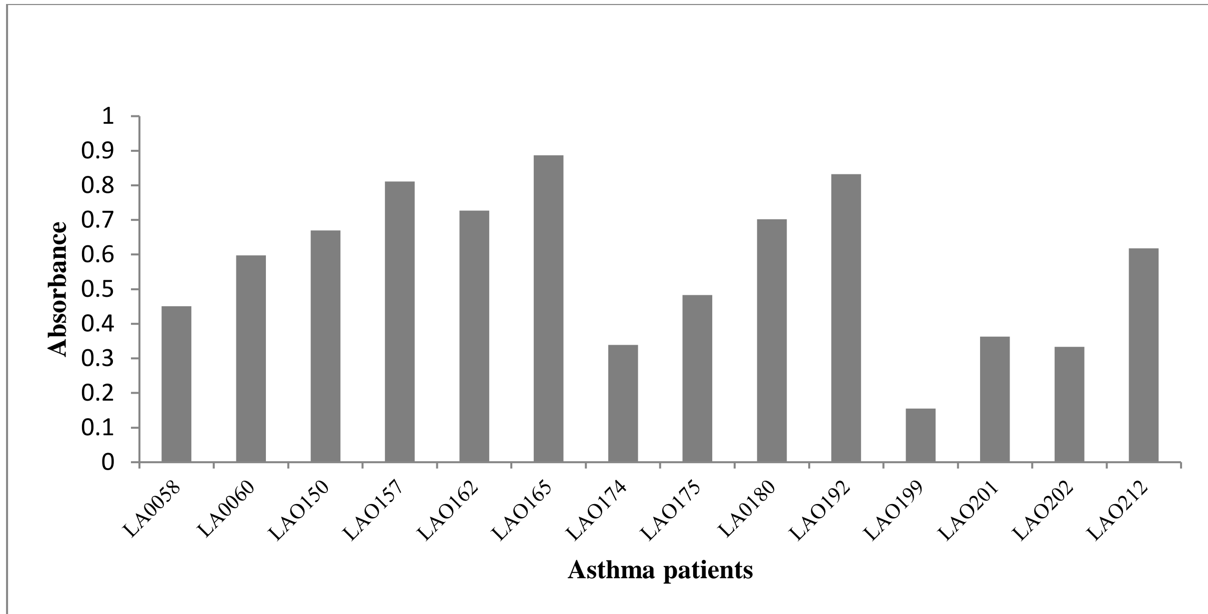
Screening of healthy volunteers sera against *S.maltophilia* at 1in 25 to establish the first dilution for antibody titration.



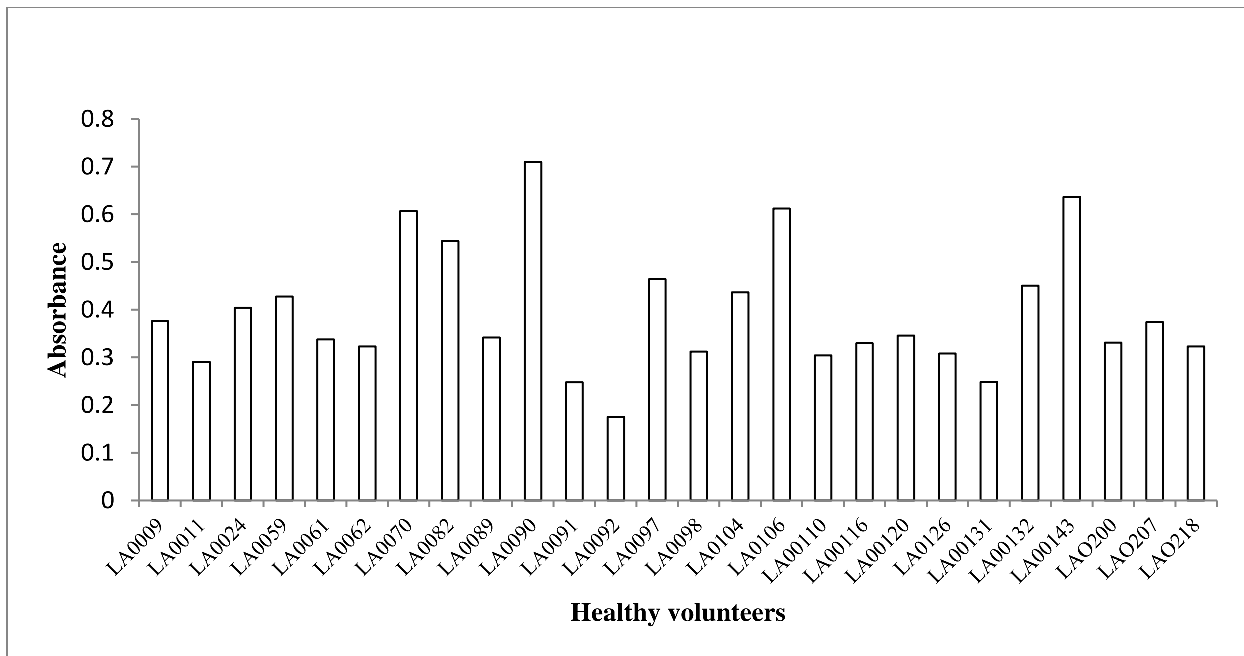
Screening of BR sera against *S.pneomoniae* polysaccharide at 1in 25 to establish the first dilution for antibody titration



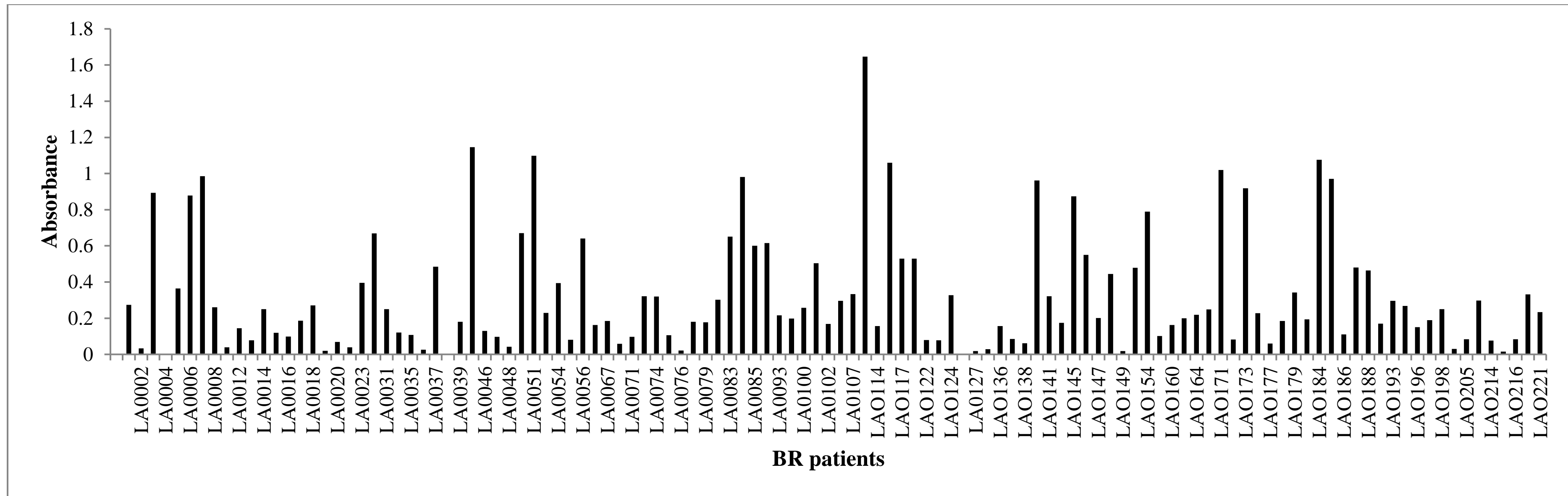
Screening of COPD sera against *S.pneomoniae* polysaccharide at 1in 25 to establish the first dilution for antibody titration



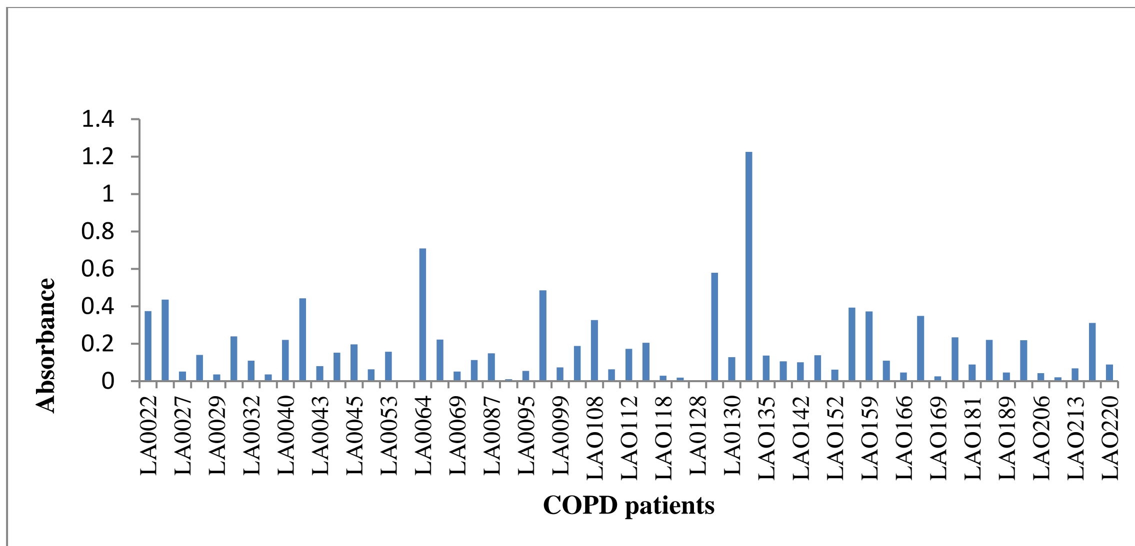
Screening of Asthmatic sera against *S.pneomoniae* polysaccharide at 1in 25 to establish the first dilution for antibody titration.



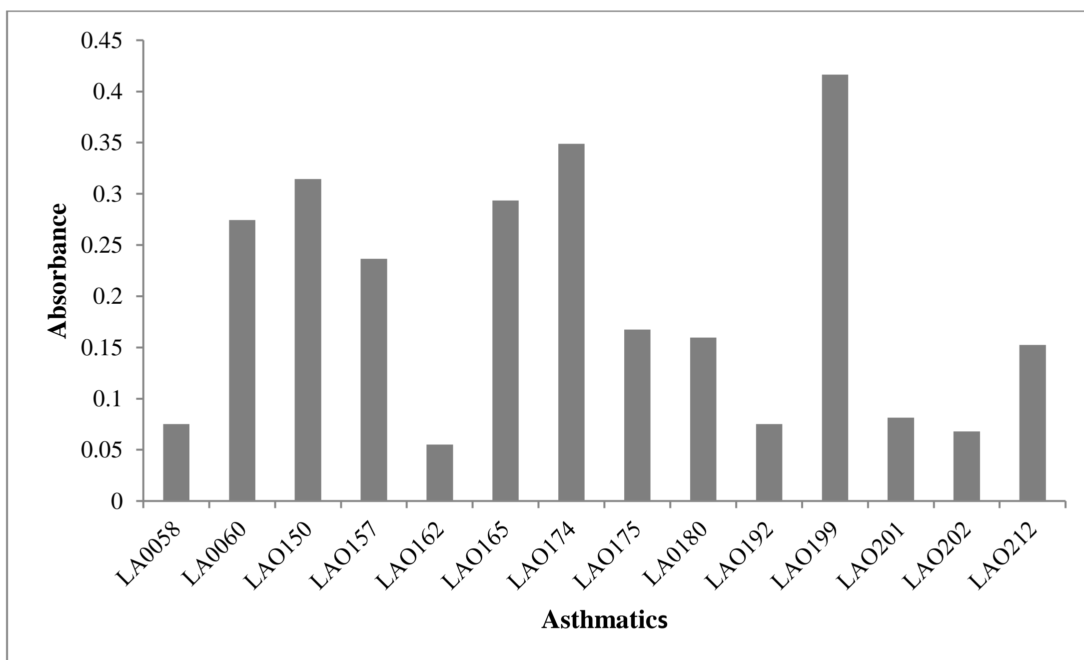
Screening of healthy sera against *S.pneomoniae* polysaccharide at 1:25 to establish the first dilution for antibody titration



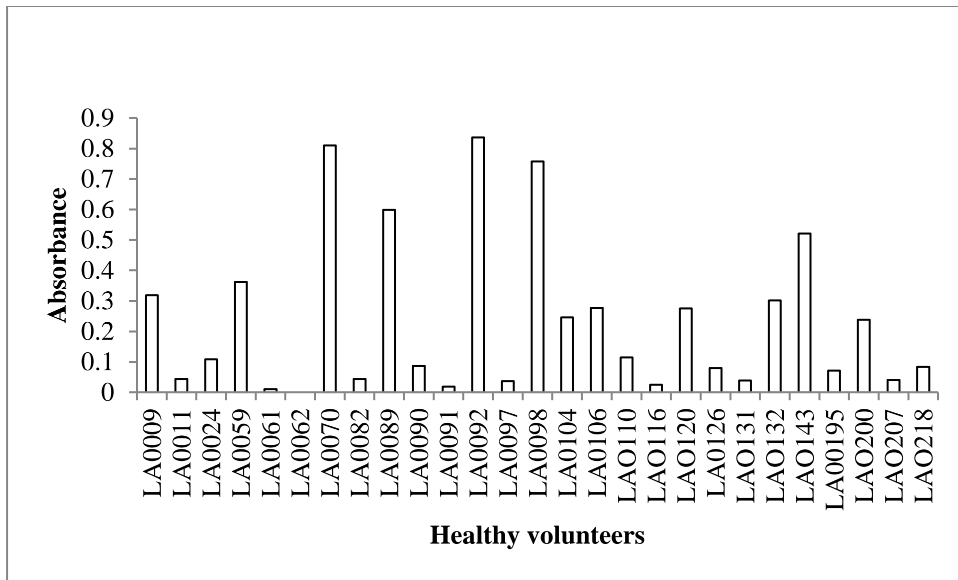
Screening of BR sera against *P.aeruginosa* (LPS) at 1in 25 to establish the first dilution for antibody titration.



Screening of COPD sera against *P.aeruginosa* (LPS) at 1in 25 to establish the first dilution for antibody titration.

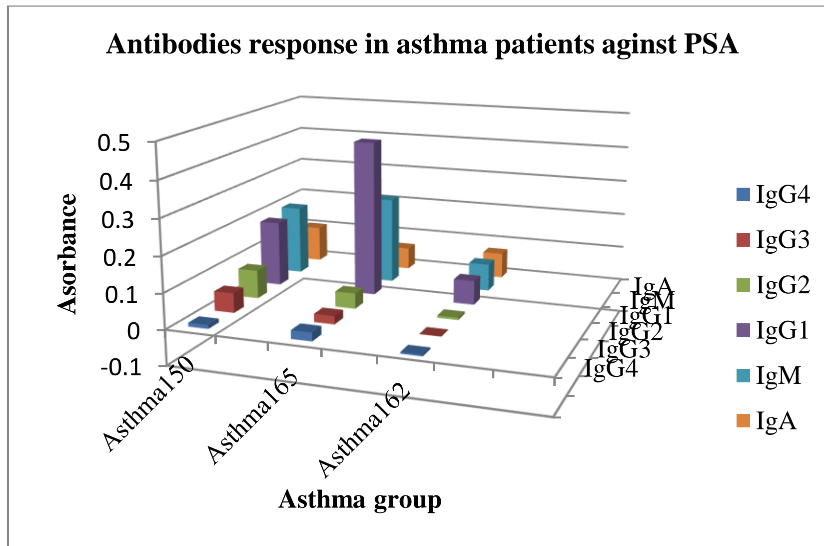


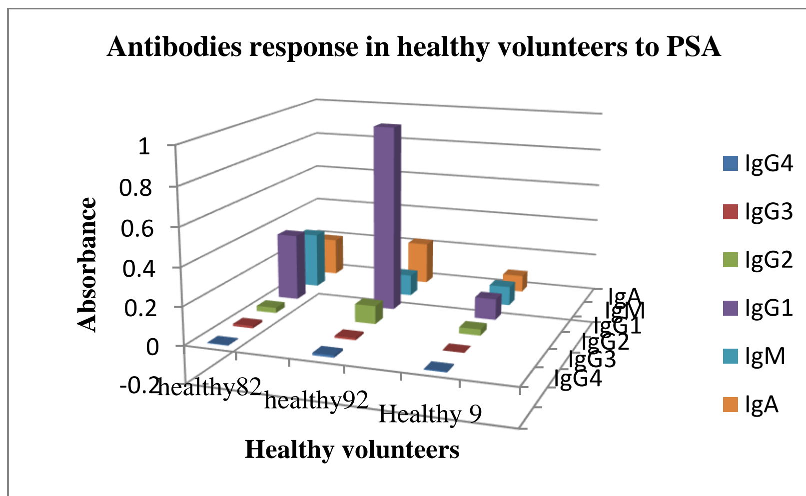
Screening of asthmatics sera against *P.aeruginosa* (LPS) at 1in 25 to establish the first dilution for antibody titration.



Screening of healthy sera against *P.aeruginosa* (LPS) at 1in 25 to establish the first dilution for antibody titration.

Appendix 10: Isotypes for asthma and healthy controls





Appendix 11: BSI exacerbation scores

Exacerbation score 5=hospitalised, 2=3 or more outpatient exacerbations, 0=<3 exacerbations per year

Numbers	BSI	Aetiology	Exacerbation score
LA0001	14	COPD	5
LA0002	13	ASTHMA	5
LA0003	14	IMMUNE DIFICIENCY	2
LA0004	15		5
LA0005	8	POST INFECTIOUS	2
LA0006	11	IDIOPATHIC	2
LA0007	10	POST INFECTIOUS	5
LA0008	8	POST INFECTIOUS	2
LA0010	9	IDIOPATHIC	0
LA0012	7	IDIOPATHIC	2
LA0013	15	IDIOPATHIC	5
LA0014	13	IDIOPATHIC	5
LA0015	4	INFLAMATORY BOWEL DISEASE	2
LA0016	6	IDIOPATHIC	2
LA0017	2	IDIOPATHIC	0
LA0018	15	ASTHMA	5
LA0019	12	RA/POST INFECTIOUS	5
LA0020	6	IDIOPATHIC	0
LA0021	13	COPD	2
LA0023	3	IDIOPATHIC	0
LA0025	5	POST INFECTIOUS	2
LA0031	11	IDIOPATHIC	2
LA0034	6	IDIOPATHIC	2
LA0035	12	IDIOPATHIC	2
LA0036	3	IDIOPATHIC	0
LAO 037	3	POST INFECTIOUS	0
LAO038	6	IDIOPATHIC	0
LA0039	8	POST INFECTIOUS	2
LA0041	6	IDIOPATHIC	0
LA0046	3	POST INFECTIOUS	0
LA0047	15	IMMUNE DIFICIENCY	5
LA0048	9	PINKS DISEASE	
LA0049	7		2
LA0051	5	IDIOPATHIC	2
LA0052	7		2
LA0054	14	PINKS DISEASE	5
LA0055	4	IDIOPATHIC	2
LA0056	2	IDIOPATHIC	0

LA0067	3	Immune Deficiency CVID	0
LA0065	9	IDIOPATHIC	
LA0068	2	IDIOPATHIC	0
LA0071	8	IDIOPATHIC	
LA0072	9	RA	
LA0074	3	IMMUNE DIFICIENCY	2
LA0075	9	IDIOPATHIC	
LA0076	8	RA	0
LA0077	9	IDIOPATHIC	2
LA0079	3	INFLAMATORY BOWEL DISEASE	
LA0081	4	IDIOPATHIC	2
LA0083	6		2
LA0084	5	IDIOPATHIC	0
LA0085	11	IDIOPATHIC	0
LA0086	11	IDIOPATHIC	0
LA0093	11	IDIOPATHIC	2
LA0094	7	IDIOPATHIC	
LA0100	5	IMMUNE DIFICIENCY	2
LA0101	16	RA	5
LA0102	7	IDIOPATHIC	
LA0105	7	IDIOPATHIC	
LAO107	1	IMMUNE DIFICIENY CVID	
LA0109	9	POST INFECTIOUS	2
LAO114	6	ASTHMA	
LAO115	9	POST INFECTIOUS	
LAO117	11	IDIOPATHIC	2
LAO121	8	IMMUNE DIFICIENY	5
LAO122	4	IDIOPATHIC	
LAO123	5	ASTHMA	2
LAO124	7	IMMUNE DIFICIENCY	
LAO125	2	POST INFECTIOUS	
LAO127	5	IDIOPATHIC	
LAO134	9		0
LAO136	4	POST INFECTIOUS	0
LAO137	12	IDIOPATHIC	
LAO138	4	POST INFECTIOUS	0
LAO139	8		2
LAO141	9	ASTHMA	
LAO144	8	IMMUNE DEFICIENCY	2
LAO145	4	IDIOPATHIC	0
LAO146	6	POST INFECTIOUS	
LAO147	5	IDIOPATHIC	
LAO148	9		2
LAO149	3		0
LAO153			5

LAO154	10	IDIOPATHIC	2
LAO155	4		0
LAO161	1		0
LAO164	2	INFLAMATORY BOWEL DISEASE	0
LAO171	6		0
LAO172	9	IDIOPATHIC	2
LAO176	17	IDIOPATHIC	2
LAO177	15	asthma (Deceased)	5
LAO178	2	IDIOPATHIC	
LAO179	5	Immunodef	0
LAO183	2		0
LAO184	3		0
LAO185	6		0
LAO186	4		0
LAO187	12	IDIOPATHIC	5
LAO188	13		2
LAO190	11	ASTHMA	5
LAO193	2	IMMUNE DEFICIENCY	0
LAO194	4	IMMUNE DEFICIENCY	
LAO196	10		
LAO197	14	ASTHMA	5
LAO198	18	ASTHMA	5
LAO203	7	IDIOPATHIC	0
LAO204	11	PINKS DISEASE	5
LAO205	5	COPD	0
LAO211	7	IDIOPATHIC	
LAO214	18	RA	5