Immunomodulatory actions of vitamin D in the protection against acute respiratory infections

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Submitted in partial fulfilment of the requirements of the degree of Doctor of Philosophy

Dedication

To my Dad, Professor Lloyd Kelland,

Who I wish could have been here to see this.

Statement of Originality

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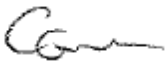
Signature: 

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Details of collaboration and publications:

- A549 cell culture work carried out in collaboration with Professor Jonathan Grigg's lab
    - First two experiments and associated RT-PCR and analysis, as well as A549 viability assay, carried out jointly by myself and Ms Reetika Suri (Blizard Institute, QMUL, UK)
    - Third experiment and associated RT-PCR and analysis carried out by myself
- Virus and bacteria stocks prepared and kindly provided by Professor Sebastian Johnston's lab (National Heart and Lung Institute, Imperial College London, UK)
    - Cytopathic effect assay carried out by myself and Ms Tatiana Kebadze
- All genetic interaction effect work and analysis carried out by Mr David Jolliffe (Blizard Institute, QMUL, UK) and staff at the Barts and the London Genome Centre (QMUL, UK).

- Study teams recruited all clinical trial participants, carried out all study visits, and collected all blood samples
    - I collected sputum samples with assistance from study teams
    - I processed all induced sputum samples, and carried out all differential cell counts
    - I processed all blood samples, except when on annual leave or ill, when Mr David Jolliffe performed the *ex vivo* stimulation of whole blood, aspirated supernatants and stored in the -80^{o}C freezer; I carried out all 30-plex ELISAs of supernatants
- Study design for all three trials and primary analysis of clinical trial results (time to exacerbation/URI etc) was performed by Dr Adrian Martineau (Blizard Institute, QMUL, UK)
- All coulter counts and serum 25(OH)D concentration tests were performed by staff at the Royal London Hospital and at Homerton Hospital respectively
- Flow cytometry on induced sputum samples was developed in collaboration with Professor Catherine Hawrylowicz and Dr Emma Chambers (MRC & Asthma UK Centre in Allergic Mechanisms of Asthma, King's College London), and they provided assistance in data analysis
- Multiplex ELISA data analysis was carried out with guidance from Dr Anna Coussens and Qlucore staff

Abstract

Introduction: Vitamin D is a micronutrient that possesses immunomodulatory actions. Higher vitamin D status has been associated with decreased incidence of acute respiratory infections (ARI) in a number of observational studies. However, mechanistic *in vitro* work investigating effects of vitamin D on the immune response to ARIs is lacking, especially for rhinovirus, which is the most common respiratory pathogen. Results of clinical trials of vitamin D supplementation in the prevention of ARIs have also been conflicting, in that some demonstrate a protective effect of this intervention against ARI, while others do not.

Methods: An immunological assay of *ex vivo* stimulation with TLR ligands and pathogens in blood samples from participants with asthma, COPD or neither condition in three randomised controlled trials of vitamin D supplementation for the prevention of ARI and exacerbations was developed. This assay was used in conjunction with cellular profiling of clinical trial blood and sputum samples, and a rhinovirus-infected human alveolar cell line (A549 cells) to determine the effects of vitamin D in the protection against acute respiratory infections.

Results: The main finding of cell culture experiments was that A549 cells pre-treated with physiological concentrations of 25-hydroxyvitamin D (25[OH]D, the major circulating vitamin D metabolite) had increased resistance to rhinovirus infection, which was associated with attenuation of rhinovirus-induced intercellular adhesion molecule-1 (ICAM-1) and platelet-activating factor receptor (PafR) expression. Immunological analysis of clinical trial samples did not demonstrate any consistent effect of bolus-dose vitamin D supplementation on circulating or pathogen-stimulated inflammatory profiles, or on inflammatory indices in induced sputum.

Conclusions: Co-incubation with 25(OH)D was associated with transient protection against rhinovirus infection in a respiratory epithelial cell line *in vitro*, but these findings did not translate to any changes in cellular profile or inflammatory mediator release in clinical trials samples following *in vivo* vitamin D supplementation.

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List of Abbreviations

$1,25(OH)_2D$	1,25-dihydroxyvitamin D; calcitriol
25(OH)D	25-hydroxyvitamin D; calcidiol
7-DHC	7-dehydrocholesterol
Ab	Antibody
Ag	Antigen
AHR	Airway hyper-responsiveness
aHR	Adjusted hazard ratio
AMP	Antimicrobial peptide
APC	Antigen presenting cell
APP	Acute phase protein
ARI	Acute respiratory infection
BAL	Bronchoalveolar lavage
CCL	Chemokine (C-C motif) ligand
CCR	Chemokine (C-C motif) receptor
CD	Cluster of differentiation
cDNA	Complementary DNA
CFU	Colony forming unit
CI	Confidence interval
COPD	Chronic obstructive pulmonary disease
CPE	Cytopathic effect
CpG ODN	Cytosine phosphate guanine oligodeoxynucleotide
CXCL	Chemokine (C-X-C motif) ligand
CYP24A1	Cytochrome p450, family 24, subfamily A, polypeptide 1; 24 hydroxylase
CYP27B1	Cytochrome p450, family 27, subfamily B, polypeptide 1; 1 alpha hydroxylase
DC	Dendritic cell
DNA	Deoxyribonucleic acid
ds	double-stranded
DTT	DL-Dithiothreitol
EGF	Epidermal growth factor
ELISA	Enzyme-linked immunosorbent assay
Eotaxin *	CCL11
FACS	Fluorescence-activated cell sorting
FCS	Foetal calf serum

FEV	Forced expiratory volume
FGF-basic	Basic fibroblast growth factor
Foxp3	Forkhead box P3
FVC	Forced vital capacity
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
G-CSF	Granulocyte colony-stimulating factor
GM-CSF	Granulocyte macrophage colony-stimulating factor
HBD	Human beta defensin
hCAP-18	Human cationic antimicrobial peptide of 18kDa
HGF	Hepatocyte growth factor
ICAM-1	Intercellular adhesion molecule-1
ICS	Inhaled corticosteroid
IFN	Interferon
Ig	Immunoglobulin
IkBa	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor alpha
IKK	Inhibitor of NF-κB kinase
IL	Interleukin
IL-8 *	Interleukin 8; CXCL8
IMP	Investigational medicinal product
iNOS	Inducible nitric oxide synthase
IP-10 *	Interferon gamma-induced protein 10; CXCL10
IQR	Interquartile range
IRF	Interferon regulatory factor
ISG	Interferon stimulated gene
ISRE	Interferon stimulated response element
IU	International units
LDH	Lactate dehydrogenase
LPS	Lipopolysaccharide
LRTI	Lower respiratory tract infection
LTA	Lipoteichoic acid
MAPK	Mitogen activated kinase-like protein
MCP-1 *	Monocyte chemotactic protein-1; CCL2
MDA5	Melanoma differentiation-associated gene 5
mDC	Myeloid dendritic cell
MDP	Muramyl dipeptide

MHC	Major histocompatibility complex
MIG *	Monokine induced by gamma interferon; CXCL9
MIP-1α *	Macrophage inflammatory protein-1α; CCL3
MIP-1β *	Macrophage inflammatory protein-1β; CCL4
mRNA	Messenger RN
MYD88	Myeloid differentiation primary response 88
NF-kB	Nuclear factor kappa B
NK	Natural killer
NLR	NOD-like receptor
NOD	Nucleotide oligomerisation domain
PafR	Platelet-activating factor receptor
Pam2	Pam2CSK4
Pam3	Pam3CSK4
PAMP	Pathogen-associated molecular pattern
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
pDC	Plasmacytoid dendritic cell
PEFR	Peak expiratory flow rate
PGN	Peptidoglycan
pIgR	Polymeric immunoglobulin receptor
polyI:C	Polyinosinic polycytidylic acid
PRR	Pattern recognition receptor
PTH	Parathyroid hormone
RANTES *	Regulated on activation, normal T cell expressed and secreted; CCL5
RIG-I	Retinoid acid-inducible gene-1
RLR	RIG-like receptor
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RSV	Respiratory syncytial virus
RT-PCR	Reverse transcriptase-polymerase chain reaction
RV	Rhinovirus
RXRA	Retinoid X Receptor
s.d.	Standard deviation
SEM	Standard error of the mean
SGRQ	St George's respiratory questionnaire

SNP	Single nucleotide polymorphism
ss	Single-stranded
STAT	Signal transducer and activator of transcription
TCID	Tissue culture infective dose
TGF	Transforming growth factor
TLR	Toll-like receptor
TNF	Tumour necrosis factor
Treg	Regulatory T-cell
URTI	Upper respiratory tract infection
UV	Ultraviolet
VDBP	Vitamin D binding protein
VDR	Vitamin D receptor
VDRE	Vitamin D response element
VEGF	Vascular endothelial growth factor
ViDiAs	Trial of vitamin D supplementation in asthma
ViDiCO	Trial of vitamin D supplementation in COPD
ViDiFlu	Trial of vitamin D supplementation for the prevention of influenza and other respiratory infections
WBA	Whole blood assay

** The use of the old chemokine nomenclature is used in the results chapters, due to this being used by the Invitrogen Human Cytokine Magnetic 30-plex kits. Therefore, the new nomenclature is noted in the abbreviations list above to minimise confusion.*

1. Introduction

In this chapter the burden of acute respiratory tract infections and the immune response to them is outlined, with specific focus on 3 vulnerable populations – asthmatics, COPD patients and the elderly. Vitamin D supplementation may alleviate some of the burden caused by acute diseases of the respiratory tract, and an overview of the literature pertaining to this is described here.

1.1 Respiratory tract infections

Acute respiratory tract infections are a leading cause of morbidity and mortality worldwide, and as such are a major global health problem. As well as causing significant morbidity and mortality, they are also responsible for a huge economic burden, precipitating considerable absence from work and school, and large numbers of visits to clinicians, with a quarter of the population visiting a GP every year for respiratory tract infections in England and Wales in 2000 [1]. Approximately 4.25 million deaths worldwide every year can be attributed to acute respiratory tract infections [2], with an associated disease burden of an estimated 94 million DALYs (disability adjusted life years) [3] making them the third leading cause of death worldwide, and the leading killer in developing countries [4]. Acute respiratory tract infections cause 18% of all deaths in children under five years of age worldwide [5], with respiratory syncytial virus (RSV) strongly associated with severe infections requiring hospitalization, and resulting in between 66,000 and 199,000 deaths annually [6, 7]. Furthermore, when also including non-classic acute respiratory infections such as the childhood diseases measles and pertussis, and chronic respiratory diseases such as asthma, COPD and tuberculosis, the number of deaths caused by diseases of the respiratory system is increased by a further 6 million every year [3]. Therefore, the respiratory tract is very important as a site of infection and disease, thus making an understanding of respiratory physiology and the immune response to respiratory pathogens vital in the development of preventative and therapeutic strategies, and to reduce the huge burden resulting from respiratory disease.

The pathogens most commonly associated with acute respiratory tract infections are the viruses influenza A and B, rhinoviruses, parainfluenza, RSV, adenoviruses and coronaviruses, and the bacteria *Streptococcus pneumonia*, *Haemophilus influenzae*, *Mycoplasma pneumoniae*, *Moraxella catarrhalis*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Legionella spp*. [8-11]. Fungal species such as *Histoplasma capsultum* can also cause respiratory tract infections, although these

are more common in immunocompromised individuals [12]. Two general categories of acute respiratory tract infection exist, with upper respiratory tract infections affecting the airways from the nose to the larynx, and including diseases such as the common cold and pharyngitis, and also the middle ear infection otitis media. Lower respiratory tract infections affect the airways downstream from the larynx into the bronchi and bronchioles of the lungs, and include diseases such as pneumonia and bronchitis. While lower respiratory tract infections are more severe and result in a higher proportion of mortalities, mostly due to pneumonia, upper respiratory tract infections are more common and represent a greater economic burden. The common cold, for example, is the most prevalent infectious disease in humans, resulting in an estimated 189 million school days and 196 million work days lost in the USA each year, and costing the country $40 billion annually [13]. However, there is also significant overlap between the two categories of acute respiratory tract infections, with many pathogens able to infect both the upper and lower respiratory tract, resulting in this distinction often being too generalised. For example, *S.pneumoniae* colonises the nasopharynx causing infection whilst also enabling its spread into lower parts of the respiratory tract [14], avian influenza A has been demonstrated to infect both the upper and lower respiratory tract [15], and rhinovirus has been shown to be able to replicate and cause bronchiolitis and pneumonia in the lower respiratory tract, as well as the common cold in the upper respiratory tract [16, 17].

1.2 Immune response to viral respiratory infection

When a virus is inhaled, it first binds to non-specific receptors (usually glycolipids or glycoproteins such as intercellular adhesion molecule [ICAM]-1) on the respiratory epithelium. Membrane fusion or endocytosis follows, thus internalizing the virus and enabling subsequent replication, transcription and translation of new viruses which can then be released to infect new cells. However, once a cell has been infected, pathogen associated molecular patterns (PAMPs) on the virus can be recognised by various intracellular innate pathogen recognition receptors (PRRs) such as the toll-like receptors (TLRs), retinoic-acid-inducible gene-I (RIG-I)-like receptors (RLRs) and nucleotide binding-oligomerisation domain (NOD)-like receptors (NLRs). Pulmonary epithelial cells have been shown to express all of the known human TLRs and RLRs which detect viruses, and ligands for these PRRs activate epithelial cells in order to initiate a rapid immune response against viral invasion [18]. In addition to direct infection of epithelial cells, intraepithelial dendritic cells (DCs) and those residing just below the respiratory epithelium continually sample particles in the airway lumen and can internalize them by phagocytosis and macropinocytosis [19]. This pathogen exposure then

results in activation of various PRRs, with conventional myeloid DCs (mDCs) expressing all known TLRs (except TLR9), as well as NLRs and RLRs, and TLRs 7 and 9 highly expressed in plasmacytoid DCs (pDCs) [19-22]. Similarly, tissue resident macrophages are also able to internalise pathogens and initiate an immune response following PRR ligation [23].

The intracellular TLRs 3, 7, 8 and 9 are mainly located on the endoplasmic reticulum (ER) membrane before UNC93B1-dependent (an ER multi-transmembrane-domain-containing protein) trafficking to the endolysosome following viral infection [24, 25]. These nucleic acid-sensing TLRs recognise single-stranded RNA (TLR 7/8) or unmethylated CpG double-stranded DNA motifs (TLR 9) of the viral genome, or the intermediary double-stranded RNA (TLR 3) produced during viral replication [26-29]. TLR3 has been demonstrated to be the most effective activator of the airway epithelial immune response [18, 30], although it has also been associated with viral pathogenesis with its stimulation resulting in detrimental over-activation of the pro-inflammatory response [31]. TLR4, while usually extracellular and implicated in the binding of bacterial lipopolysaccharide (LPS), has been demonstrated to traffic to the endolysosome and potentially play a role in viral recognition, as the fusion protein of RSV is a ligand for it [29, 32, 33]. However, a direct interaction between RSV particles and TLR4 has not been shown, and evidence pertaining to the ability of RSV to elicit a TLR4-dependent immune response is contradictory [34]. Additionally, TLR2 receptor complexes, which are also typically extracellular and associated with bacterial PAMP recognition, have been demonstrated to localise to endosomes and induce type I interferon (IFN) responses [35], with the TLR2/6 complex recognising RSV and activating a subsequent immune response [36]. Viruses which avoid recognition by TLRs, for example by entering epithelial cells by direct membrane fusion rather than via endosomes, can be recognised by RLRs which are present throughout the cytosol. RIG-I is activated by short dsRNA (up to 1kb), with the presence of a 5′-triphosphate end greatly enhancing the induction of type I IFNs, and it is important in the immune response to many RNA viruses [24, 37, 38]. The related RNA helicase melanoma differentiation-associated gene 5 (MDA5) on the other hand interacts with polyinosinic polycytidylic acid (polyI:C) and long dsRNA (more than 2kb), and is crucial in the recognition of picornaviruses [24, 39, 40]. Additionally, the cytosolic NLR NOD2, whilst normally associated with the recognition of bacterial muramyl dipeptide, has also been demonstrated to be involved in the recognition of the ssRNA genome of RSV [41]. Despite differences in viral genomes, replication strategies, and the types of PRRs activated, common signalling pathways are utilized. Thus recognition of viral pathogens elicit conserved outcomes, with the interferon regulatory factor (IRF)-mediated production of type I IFNs a central feature, along with nuclear factor kappa B (NF-κB)- and mitogen-activated protein kinase (MAPK)- mediated regulation of various inflammatory cytokines [29, 42] (Figure 1.1).

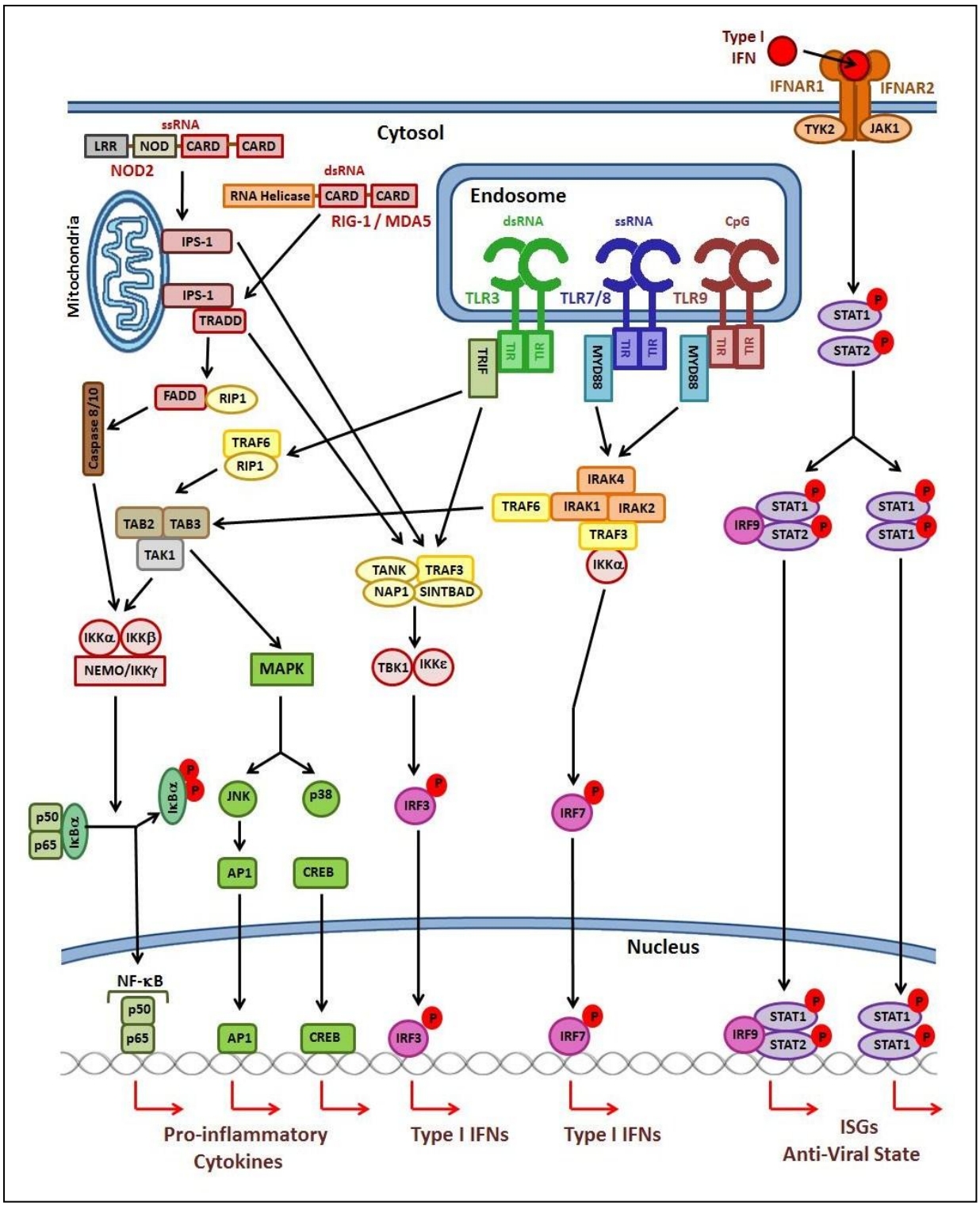


Figure 1.1: Pathogen recognition receptor signalling following viral infection. Ligand-induced dimerisation occurs following PAMP recognition by endosomal TLRs, with TLR7/8 forming a heterodimer, and the rest forming homodimers. This engages the Toll-IL-1 receptor (TIR) domains present in each TLR to initiate adaptor molecule recruitment and signal transduction. MYD88-dependent signalling results in the formation of an IRAK/TRAF6 complex, with TRAF6 subsequently becoming ubiquitinated and activating a complex of TAK1 and TAB2/3. This complex phosphorylates NEMO to activate the IKK complex, which results in the phosphorylation and degradation of IκBα, leaving NF-κB free to translocate into the nucleus where it drives the transcription of pro-inflammatory cytokine genes. Simultaneously, TAK1 activates MAPK cascades, resulting in the activation

of AP-1 and CREB which drive further transcription of pro-inflammatory cytokines. TRIF-dependent signalling following endosomal TLR3 ligation can also activate NF-κB, AP-1 and CREB via recruitment of TRAF6 and RIP1. Alternatively, TRAF3 is recruited, which requires NAP1 and SINTBAD to activate TBK1/IKKε. This results in the phosphorylation of IRF3 which translocates into the nucleus to induce expression of type I IFNs. TLR7/8 and TLR9 initiated recruitment of the TRAF/IRAK/IKKα complex can also phosphorylate IRF7. IRF7 thus translocates into the nucleus and causes the transcription of type I IFN genes. Upon dsRNA recognition, RIG-I and MDA5 interact with IPS-1 localized on the mitochondrial membrane through homophilic interactions between their CARD domains. This allows IPS-1 to interact with TRADD and form a complex with FADD and caspase 8/10 to activate NF-κB. Additionally, TRADD can interact with TRAF3 to activate TBK1/IKKε, resulting in the phosphorylation and translocation of IRF3 to induce production of type I IFNs. Similarly, upon recognition of ssRNA by NOD2, CARD domains interact with IPS-1 localized on the mitochondrial membrane, thus facilitating interaction with TRAF3 to activate TBK1/IKKε and result in transcription of type I IFN genes. The type I IFNs produced bind to their receptor, thus activating the associated TYK2 and JAK1 molecules and resulting in phosphorylation of STAT1 and STAT2. The STAT1/STAT2/IRF9 complex (ISGF3: IFN-stimulated gene factor 3) translocates to the nucleus and binds to IFN-stimulated response elements (ISREs) in the DNA to initiate gene transcription. STAT1 homodimers translocate and bind to GAS (IFNγ activated site) elements in the promoter region of ISGs, initiating transcription of these genes.

Abbreviations used: AP-1: activator protein 1; CARD: caspase recruitment domain; CpG: cytosine phosphate guanine; CREB: cyclic adenosine monophosphate (cAMP) responsive element-binding protein; ds: double-stranded; FADD: FAS associated death domain; GAS: IFN-γ activated site; IFN: interferon; IFNAR1: IFN alpha receptor 1; IκBα: nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor alpha; IKK: inhibitor of NF-κB kinase; IPS-1: IFN-β promoter stimulator 1; IRAK: interleukin-1-receptor-associated kinase 1; IRF: interferon regulatory factor; ISG: interferon stimulated gene; ISGF3: interferon-stimulated gene factor 3; ISRE: interferon stimulated response element; JAK: janus kinase; JNK: c-jun N-terminal kinase; LRR: leucine rich repeat; MAPK: mitogen activated kinase-like protein; MDA5: melanoma differentiation-associated protein kinase; MYD88: myeloid differentiation primary response 88; NAP: NF-κB activating kinase (NAK)-associated protein; NEMO: NF-κB essential modulator; NF-κB: nuclear factor kappa B; NOD: nucleotide-binding oligomerisation domain; PAMP: pathogen-associated molecular pattern; PRR: pattern recognition receptor; RIG-1: retinoic acid-inducible gene-1; RIP1: receptor interacting protein; SINTBAD: similar to NAP-1 TBK adaptor; ss: single-stranded; STAT: signal transducer and activator of transcription; TAB: TGF-β activated kinase; TAK: TGF-β activated kinase; TANK: TRAF family member associated NF-κB activator; TBK: TANK-binding kinase; TIR: Toll/IL-1 receptor; TLR: Toll-like receptor; TRADD: TNF receptor associated death domain; TRAF: TNF receptor associated factor; TRIF: TIR-containing adaptor inducing IFN-β; TYK: tyrosine kinase. Circled P's represent phosphorylation.

Upon PAMP-PRR interaction and the activation of signalling transduction pathways, the type I IFNs (IFN-α and IFN-β) are some of the earliest cytokines to be produced. Their transcription and subsequent binding to the IFN receptor induces expression of a variety of interferon-stimulated genes (ISGs), with their products altering antiviral and immunomodulatory actions to limit and clear infection [42]. Features of the induced anti-viral state include resistance to viral replication in all

cells, induction of apoptotic cell death in infected cells, increased major histocompatibility complex (MHC) class I expression to enhance antigen presentation, activation of dendritic cells (DCs) and macrophages, and stimulation of natural killer (NK) cells to enhance their cytolytic activity [43]. The inflammatory cytokines TNF-α, IL-1β, IL-6 and IL-12 are also produced at an early stage of the innate immune response. These cytokines promote leukocyte extravasation by increasing endothelial expression of adhesion molecules such as ICAM-1 and VCAM-1, increase vascular permeability, induce synthesis of acute phase proteins, and contribute to recruitment and activation of cells of the adaptive immune response. Additionally, IL-1β and TNF-α amplify the inflammatory response by triggering further NF-κB and MAPK activation [44]. PRR signalling also results in chemokine production, with IL-8, CXCL9 / 10 and IL-15 recruiting neutrophils and NK cells respectively [45, 46]. While neutrophils are the most rapidly recruited cells to the site of infection during the innate immune response and have a well established role in bacterial infections, their function in antiviral immunity is less clear and is likely to be minimal, with inhibition of neutrophil recruitment to the lung demonstrated to have no effect on the course of influenza infection [47].

PRR signalling also facilitates the maturation and trafficking of dendritic cells, with the release of the chemokines CCL2 and CCL20, and increased expression of CCR7 [48, 49]. Thus, around 72 hours after infection, DCs with antigen-MHC complexes migrate through the afferent lymph vessels to secondary lymph nodes where they form interactions with naive $CD4^+$ and $CD8^+$ T lymphocytes. These T-lymphocytes are activated, proliferate, differentiate into effector T-cells and migrate via efferent lymph vessels into the circulation. Multiple chemokines, such as CCL3, CXCL9 and CXCL10, are expressed in the respiratory epithelium and result in changes in integrin affinity, allowing effector T-cells to bind to the endothelium and migrate into the infected tissue [29, 50-53]. For efficient and effective viral clearance Th1 effector T-cells are required, which produce IL-2, TNF-α and IFN-γ to activate NK cells and induce generation of cytolytic molecules. $CD8^+$ effector T-cells and NK cells can then induce apoptosis of infected cells via the release of cytolytic granules or by direct interaction between surface Fas receptor and Fas ligand (FasL) [29]. B-cells have also been demonstrated to play an important role in the immune response to highly pathogenic viral infections. Contact between $CD4^+$ T-cells and naive B-cells in secondary lymphoid tissues results in their proliferation and antibody class-switching, with neutralizing virus-specific antibodies crucial for optimal viral clearance [29, 54, 55]. Combined, these effector mechanisms of the adaptive immune response rapidly clear the viral infection.

1.3 Immune response to bacterial respiratory infection

Similarly to viruses, when bacterial respiratory pathogens are inhaled they are recognised by PRRs on the respiratory epithelial surface. TLR2, which forms heterodimers with TLR1 and TLR 6 and requires the cofactor CD14, is a key receptor in recognising PAMPs from both *S.pneumoniae* and *H.influenzae,* two major respiratory bacterial pathogens. As well as its extracellular location, TLR2 receptor complexes are also found in endosomes [35, 56], whereby they induce type I IFN production, allowing recognition and immune response to viruses. This internalisation may also be important in the innate immune response to bacteria, with the endosomal complex of TLR2/6 and CD14 involved in the recognition and subsequent immune response to lipoteichoic acid (LTA) and *Listeria monocytogenes* infection [57, 58], although other studies have demonstrated that bacterial ligands are unable to stimulate type I IFN production [59]. Similarly, TLR4 is found both on the plasma membrane and in endosomes, eliciting different signal transduction pathways in each location [60]. The LPS found in the outer membrane of Gram-negative bacteria such as *H.influenzae* is the major ligand of TLR4, requiring association with the recognition protein MD2 and the cofactor CD14 for ligation and subsequent signalling, with CD14 also involved in the internalisation of the TLR4 receptor complex [61]. The LPS-induced trafficking of TLR4 to endosomes may be an important factor in limiting the immune response to LPS and thus avoiding septic shock [62], and also in modulating the immune response by reducing NF-κB- and MAPK-induced chemokine production, neutrophil infiltration and tissue damage [63]. Additionally, TLR9 and TLR3, which are traditionally thought of as PRRs for viruses, may have a role in the innate immune response to bacteria, with the unmethylated CpG DNA (DNA containing motifs of cytosine followed by guanine) of *S.pneumoniae* demonstrated to be a ligand for TLR9 [64], and TLR3 demonstrated to be located on the surface of epithelial cells, away from its normal niche of endosomes, from where it has been shown to be able to recognise *H.influenzae* [65]. Cytosolic NLRs may also play a role in respiratory microbial recognition, either by direct sensing of intracellular bacterial PAMPs, or by PAMPs that are transported into the cytoplasm by type III or IV secretion systems [66]. NOD2 is primarily expressed in antigen-presenting cells (APCs) and recognises muramyl dipeptide (MDP), the largest component of the peptidoglycan (PGN) bacterial cell wall, while NOD1 is ubiquitously expressed and recognises γ-D-glutamyl-mesodiaminopimelic acid (iE-DAP), another substructure of peptidoglycan [66, 67]. NOD-mediated NF-κB activation has been shown to be essential in the protection against *S.pneumoniae* [68], and the respiratory bacteria *P.aeruginosa*, *S.aureus* and *M.tuberculosis* have also all been demonstrated to be detected by NOD 1 or NOD2 [69-72].

As with viral infection, PRR ligation by bacterial PAMPs initiates signal transduction pathways, resulting in the activation of downstream transcription factors (Figure 1.2). The inflammatory cytokines produced following MAPK and NF-κB activation are central in the innate immune response to respiratory bacteria. While typically associated with their crucial role in the immune response to viruses, as previously described, type I IFNs can also be produced in response to bacterial infection, with actions such as IFN-β-mediated macrophage production of cytokines and nitrogen radicals demonstrated to be important in host resistance to various bacterial species [73, 74].

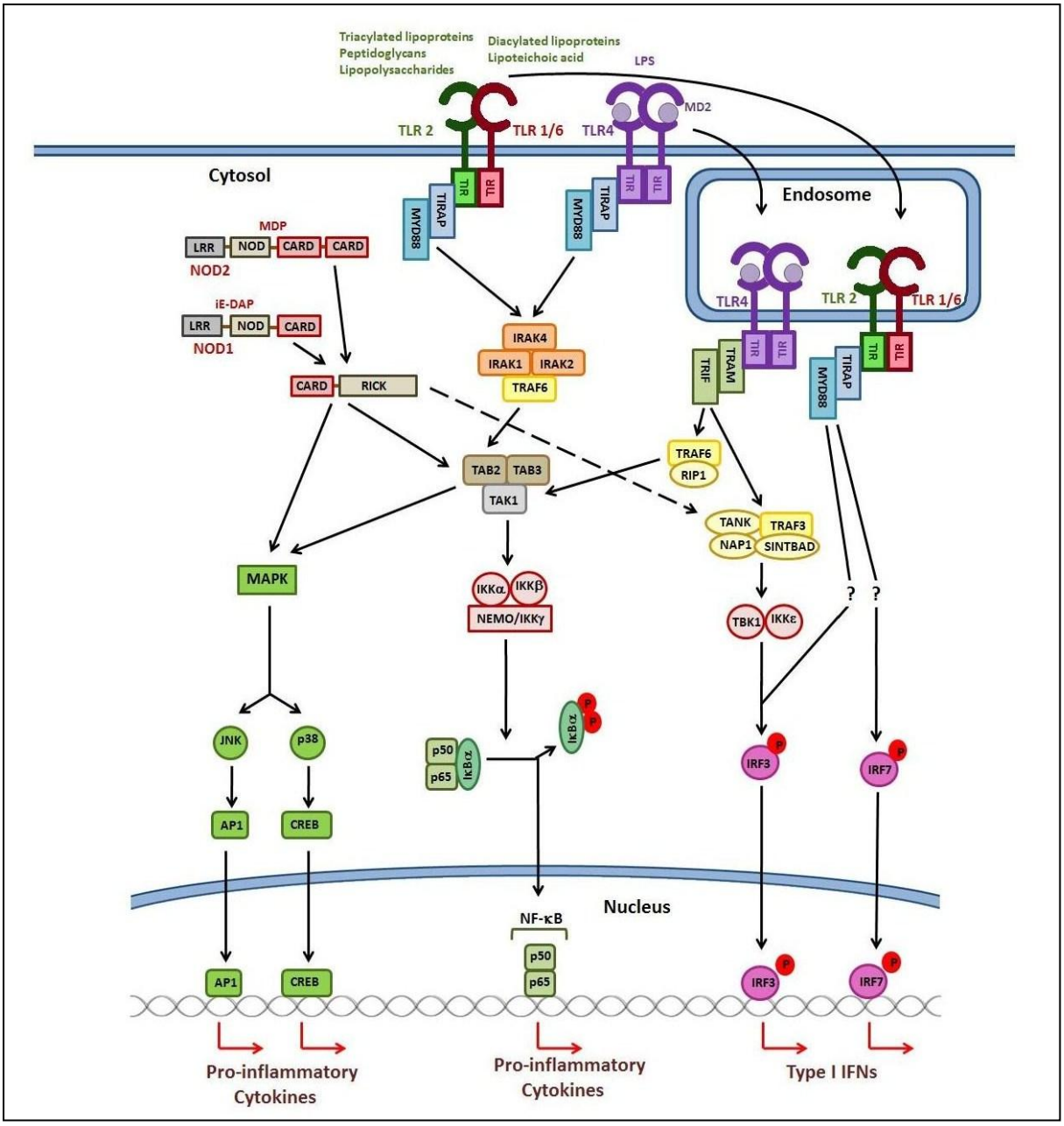


Figure 1.2: Pathogen recognition receptor signalling following bacterial infection. Ligand-induced dimerisation occurs following PAMP recognition by TLRs, with the TLR4/MD2 complex forming a homodimer, and TLR 2 forming heterodimers with either TLR1 or TLR6. This engages the Toll-IL-1 receptor (TIR) domains present in each TLR to initiate adaptor molecule recruitment and signal transduction. MYD88-dependent signalling results in the formation of an IRAK/TRAF6 complex, with TRAF6 subsequently becoming ubiquitinated and activating a complex of TAK1 and TAB2/3. This complex phosphorylates NEMO to activate the IKK complex, which results in the phosphorylation and degradation of IκBα, leaving NF-κB free to translocate into the nucleus where it drives the transcription of pro-inflammatory cytokine genes. Simultaneously, TAK1 activates MAPK cascades, resulting in the activation of AP-1 and CREB which drive further transcription of pro-inflammatory cytokines. TLR ligation can also induce translocation of TLR4 homodimers and TLR2 heterodimers to the endosome. TLR4 TRIF-dependent signalling can activate NF-κB, AP-1 and CREB via recruitment of TRAF6 and RIP1. Alternatively, TRAF3 is recruited, which requires NAP1 and SINTBAD to activate TBK1/IKKε. This results in the phosphorylation of IRF3 which translocates into the nucleus to induce expression of type I IFNs. Endosomal

TLR2/6 and TLR2/1 signalling have been demonstrated to facilitate phosphorylation of IRF7 and IRF3 and subsequent transcription of type I IFN genes, but the intermediary signalling components are currently unknown. Upon recognition of bacterial PAMPS, the CARD domains of NOD2 and NOD1 interact with RICK, which in turn is able to activate MAPK signalling, activate NF-κB via the TAK1 TAB2/3 complex, and recruit TRAF3 to activate TBK1/IKKε and induce transcription of type I IFN genes.

Abbreviations used: AP-1: activator protein 1; CARD: caspase recruitment domain; CREB: cyclic adenosine monophosphate (cAMP) responsive element-binding protein; iE-DAP: γ-D-glutamyl-mesodiaminopimelic acid; *IFN: interferon; IκBα: nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor alpha; IKK: inhibitor of NF-κB kinase; IRAK: interleukin-1-receptor-associated kinase 1; IRF: interferon regulatory factor; ISG: interferon stimulated gene; ISRE: interferon stimulated response element; JNK: c-jun N-terminal kinase; LPS: lipopolysaccharide; LRR: leucine rich repeat; MAPK: mitogen activated kinase-like protein; MD2: myeloid differentiation protein-2; MDP:* muramyl dipeptide; *MYD88: myeloid differentiation primary response 88; NAP: NF-κB activating kinase (NAK)-associated protein; NEMO: NF-κB essential modulator; NF-κB: nuclear factor kappa B; NOD: nucleotide-binding oligomerisation domain; PAMP: pathogen-associated molecular pattern; PRR: pattern recognition receptor; RICK: receptor-interacting CARD-containing serine/threonine kinase (also known as RIP2 or CARDIAK); RIP1: receptor interacting protein; SINTBAD: similar to NAP-1 TBK adaptor; TAB: TGF-β activated kinase; TAK: TGF-β activated kinase; TANK: TRAF family member associated NF-κB activator; TBK: TANK-binding kinase; TIR: Toll/IL-1 receptor; TIRAP: TIR domain containing adaptor protein; TLR: Toll-like receptor; TRAF: TNF receptor associated factor; TRAM: TRIF-related adaptor molecule; TRIF: TIR-containing adaptor inducing IFN-β. Circled P's represent phosphorylation.*

Many of the key processes in the immune response against bacteria are the same as with viruses: after PRR ligation and signal transduction, pro-inflammatory cytokines are released, chemokines attract immune cells to the site of infection, vascular permeability is increased and sentinel macrophages and DCs are activated. However, there are some key differences in the course of the immune response to the two classes of respiratory pathogens.

Bacteria may remain extracellular, and can thus be targeted by antibodies, complement proteins and phagocytosis. Neutrophils and macrophages are vital in the innate response to microbes, as both can phagocytose and degrade pathogens. In addition to this, neutrophils can release cytokines such as IL-18 to activate NK cells, and can form neutrophil extracellular traps (NETs) to bind and destroy bacteria [75, 76]. Macrophages can produce antimicrobial peptides (AMPs) such as LL-37, and TNF-α which is very important in orchestrating the immune response to microbes, as it stimulates acute-phase protein (APP) synthesis by the liver, as well as enhancing leukocyte migration from the blood, activating endothelial cells to trigger blood clotting and containment of infection, stimulating DC maturation, and stimulating IL-1 and IL-6 production [77].

The acute phase response is the process by which the concentration of plasma proteins increases during the inflammatory state. Notably, lactoferrin sequesters iron to deplete this essential nutrient for bacterial utilisation, and also has bactericidal activities on direct contact with microbes [78]. Other APPs include C-reactive protein and mannose-binding lectin, which can bind to the bacterial surface and then associate with the protein C1q, thus initiating the complement cascade. Complement can also be activated directly via the pathogen surface, but all pathways ultimately result in release of inflammatory mediators, opsonisation of pathogens, and formation of a membrane-attack complex which disrupts the lipid bilayer of the bacteria and results in its eventual destruction [79].

Similarly to complement proteins, antibodies can opsonise pathogens and facilitate phagocytosis. They are produced by activated plasma cells, which are the effector B-cells produced after interaction with T-cells, or sometimes after direct binding to certain bacterial surface molecules. Antibodies also initiate antibody-dependent cell-mediated cytotoxicity (ADCC), whereby CD16 on NK cells recognises the Fc portion of antibodies bound to the bacterial surface and kills the target cell.

S.pneumoniae, H.influenzae and *M.catarrhalis* are classically thought of as extracellular pathogens, but have all been shown to be able to invade cells and survive intracellularly to cause infection [80-83]. As such, the cytotoxic response of T lymphocytes and NK cells is relevant in bacteria, as well as viruses.

1.4 Bacterial colonisation of the respiratory tract

Both colonization and infection first require adherence to the epithelial surface of the respiratory tract. *S.pneumoniae* has a polysaccharide capsule, reducing entrapment by mucus and inhibiting opsonophagocytosis, thus enabling access to the epithelium. The capsule, while effectively preventing an immune response being mounted against the pathogen, also inhibits adherence, as the adhesins are hidden on the cell wall. Thus, phase variation occurs between the opaque and transparent (thinner capsule) forms of the bacteria and once the cell wall is exposed, adherence can occur. Many host receptors and bacterial components have been implicated in this, and it has been suggested that the initial contact occurs via non-specific physicochemical interactions and pneumococcal surface adhesion A (PsaA) binding to N-acetyl-glycosamine on resting epithelial cells. In non-encapsulated bacteria, such as nontypeable *H.influenzae*, adherence is achieved via various

adhesins, such as those associated with fimbriae. Due to the lack of a protective capsule, innate clearance must be avoided, and this is achieved by mechanisms such as secreting IgA proteases and phase variation, which allows selection of the most suitable derivative for the immediate environment [84]. This initial colonization is asymptomatic, and is usually cleared by the immune system, however, the epithelium can be altered by the local generation of inflammatory factors to have different types and numbers of receptors on their surface. Via the binding of bacterial cell-surface proteins such as phosphorylcholine (ChoP) to the platelet-activating-factor receptor (Pafr), internalisation can occur, resulting in invasive infection [85, 86]. Other epithelial receptors implicated in respiratory bacterial adhesion include polymeric immunoglobulin receptor (pIgR), ICAM-1 and carcinoembryonic adhesion molecule 1 (CEACAM1) [87-89].

The upper respiratory tract is a dynamic environment, with constant turnover and acquisition of new strains of bacteria. While some are pathogenic and invade the epithelium to cause disease, some bacteria remain on the surface of the epithelium resulting in long-term asymptomatic colonization. As such, the upper respiratory tract acts as a reservoir for a diverse range of commensals and potentially pathogenic bacteria (termed pathobionts), including *S.pneumoniae*, *H.influenzae* and *M.catarrhalis*, resulting in a complex microbial community [90]. The microbiome can offer protection to the host from invading pathogens by colonization resistance, whereby competition for sites of attachment and nutrients limits infection. It has also been implicated in controlling maturation and regulation of the immune system, by promoting immune homeostasis, aiding the development of secondary lymphoid tissues, contributing to IgA and antimicrobial peptide production, and mediating correct inflammasome activation [91, 92]. However, when the balance of the microbiome is disrupted it can contribute to acquisition and increased carriage of pathogenic bacteria and viral co-infections, resulting in overgrowth and invasion of bacterial pathogens and the driving of inflammation and disease [90].

Microbial secretion systems enable a dynamic exchange of molecules, thus facilitating host-bacterial interactions, whether beneficial or harmful. In the gut for example, the bacterial type VI secretion system (T6SS) of the pathobiont *Helicobacter hepaticus* has been demonstrated to limit colonization and inflammation by shaping an immunologically tolerant environment. Therefore, a non-pathogenic state is actively maintained, promoting a long-term balanced symbiotic relationship between microbe and host. However, disturbances in interactions such as these can promote inflammation, with dysbiosis associated with inflammatory disorders such as inflammatory bowel disease (IBD) [93]. The exact mechanisms by which these disturbances occur and homeostasis is disrupted are unclear, although it has been demonstrated that antibiotic use can disrupt the gut

microbiota and result in inflammasome activation by resident pathobionts, thus leading to inflammatory disease [94]. While the majority of research into the microbiota has been carried out in the gut, similar relationships are likely to exist in the respiratory tract with related mechanisms driving dysbiosis and pathobiont-related disease. Additionally, the gut microbiota has been implicated in diseases at distant sites, with a role emerging in atopic diseases such as eczema, allergic rhinoconjunctivitis and asthma due to its effects on perinatal programming [95, 96], suggesting that the gut microbiome could directly influence diseases of the respiratory tract.

Viruses are also commonly found in the upper respiratory tract of asymptomatic individuals, adding to the reservoir of commensals and pathobionts present there [97]. Collectively, this niche of potential viral and bacterial pathogens has been implicated in host-to-host transmission, thus demonstrating the importance of asymptomatic carriage [98]. While the mechanisms underlying how and why asymptomatic pathobiont colonization can result in infectious disease are unclear, the microbiome is likely to be an important component in respiratory diseases.

1.5 Dual viral and bacterial infections of the respiratory tract

Viral infections are commonly seen to predispose patients to an increased incidence and severity of secondary bacterial infections. The catastrophic influenza A pandemic of 1918 was the origin of the theory of viral-bacterial synergism, with many of the 40-50 million deaths attributable to bacterial pneumonia [99]. Influenza A, RSV, rhinovirus, parainfluenza, coronavirus and adenovirus infections have all been shown to predispose to bacterial infections, with *S.pneumoniae*, *H.influenzae* and *S.aureus* being increased most often [88, 100-103]. Additionally, mouse models have suggested that this viral predisposition to bacterial infection not only occurs during simultaneous infection, but can also take place a week after initial infection, and even after full recovery of viral infection [90, 104, 105]. The synergism between virus and bacteria also varies in a species- and cell type- dependent manner, with influenza A and *S.pneumoniae* being the most commonly associated pathogens with dual infections [106].

S.pneumoniae is commonly involved in causing invasive diseases such as pneumonia, meningitis and sepsis, and is part of the commensal flora of the upper respiratory tract, creating a carrier state of asymptomatic colonization. The mechanisms by which asymptomatic colonization of the respiratory tract can lead to invasive and often life-threatening disease are largely unknown, but the potential

role of viruses has been investigated extensively. It has been postulated that viral infections can facilitate bacterial infection by up-regulating expression of epithelial receptors, impairing mucociliary function, altering host immune responses, damaging the epithelial layer and by facilitating direct binding to viral glycoproteins [88, 89, 106]. Additionally, influenza virus has been demonstrated to impair the ability to tolerate tissue damage, with co-infected lungs presenting with a greater degree of airway epithelial necrosis, resulting in increased mortality [107]. IL-10 has also been implicated in precipitation of dual infections, with primary influenza infection associated with elevated levels of IL-10 in the lungs and reduced neutrophil function, resulting in greater susceptibility to secondary *S.pneumoniae* infection, enhanced bacterial outgrowth and increased lethality [105]. Finally, it has been demonstrated in COPD patients that rhinovirus infection results in an outgrowth of *H.influenzae* from the existing microbiota, thus precipitating secondary bacterial infections [108]. It is likely that a combination of all of these factors contributes to the synergism experienced between virus and bacteria, but most focus has been directed towards the increased expression of the epithelial receptors required for bacterial adhesion and colonization.

PafR has been shown to be a receptor for *S.pneumoniae* and *H.influenzae*, the two most common bacterial respiratory pathogens, and its expression has been shown to be increased by RSV, RV-14, influenza and coronavirus infections [88, 101, 102]. One possible mechanism by which viral infection increases the expression of PAF-r is via the transcription factor NF-κB which is activated in epithelial cells after viral recognition [102, 109]. The expression of the bacterial receptors pIgR, CEACAM1 and ICAM-1 have also been shown to be increased by NF-κB activation [87, 110, 111], suggesting that this may be a common mechanism for all virus-induced up-regulation of bacterial adhesion.

While viral-bacterial co-infections are the most commonly implicated in increased morbidity and mortality, dual bacterial infections can also occur. Co-colonization can be promoted, with, for example, *M.catarrhalis* releasing membrane blebs containing ubiquitous surface proteins which can deactivate complement factor C3 and thus protect *H.influenzae* from complement-mediated killing [90, 112]. Additionally, bacterial infections have been shown to increase susceptibility to viral infections, with *H.influenzae* stimulating ICAM-1 expression to provide a receptor for rhinovirus [113], a disturbed balance in the microbiota creating opportunity for viral infection, and the viral utilization of the microbial environment to escape immune clearance [114]. Therefore, respiratory viruses and microbiota may influence each other to affect the pathogenicity and development of infection, as has already been demonstrated in the gut [90, 115].

Finally, dual viral infections have also been implicated in increased disease severity and higher rates of hospitalization and mortality, with rhinovirus, influenza viruses, RSV and adenoviruses the most common pathogens [116-118]. However, this is still under debate, with many studies showing no effect [119, 120], or even a protective effect of dual viral infections [121].

Therefore, dual infections may result in more severe disease and thus a greater rate of hospitalization and mortality. As such, the mechanisms by which viral and bacterial species predispose to infection by other pathogens may be an important determinant in the frequency and severity of respiratory disease.

1.6 Determinants of vulnerability to respiratory tract infections

While acute respiratory tract infections are a burden in terms of morbidity and economics in otherwise healthy immunocompetent individuals, hospitalization and mortality rates are relatively low [122-125]. However, certain populations are more vulnerable to contracting acute respiratory tract infections and more prone to the associated complications, resulting in higher mortality rates. For example, HIV-positive children have been shown to have a higher disease burden and mortality rate associated with lower respiratory tract infections [126, 127], rhinovirus infections have been associated with considerable morbidity and mortality in myelosuppressed adult cancer patients [128], crisis-affected populations have been demonstrated to have high excess mortality due to acute respiratory infections [129], and high levels of air pollution and urban particulate matter have been linked with respiratory tract infection-associated mortality, showing enhanced pneumococcal adhesion to human epithelial cells [130, 131]. Additionally, vaccine and antibiotic use can disrupt the microbiome and alter susceptibility to infection. For example, the widespread use of pneumococcal conjugate vaccines has been demonstrated to increase incidence of *M.catarrhalis* and *H.influenzae* infections [132-134], and antibiotics decrease microbial diversity, which is associated with *Pseudomonas aeruginosa* colonization in cystic fibrosis patients, and an attenuated immune response to influenza virus in mouse models [92, 135, 136]. Thus there are many examples of such vulnerable populations, but this study will just be focussing on people with asthma or COPD, and older adults living in sheltered accommodation.

1.6.1 Asthma and COPD

Asthma is defined as a chronic respiratory condition caused by inflamed and hypersensitive bronchi which become easily irritated, causing periodic bronchoconstriction [137, 138]. Chronic obstructive pulmonary disease is the term now used to encompass emphysema and chronic bronchitis, and is defined as chronic airway obstruction due to chronic inflammation caused by airway and parenchymal damage, usually as a result of cigarette smoking [139]. While asthma is a disease typically consisting of reversible airway obstruction, involving eosinophil and Th2 cell infiltration into the airway submucosa, in COPD airway obstruction is classically irreversible, with higher numbers of neutrophils in the airway lumen and increased numbers of macrophages and $CD8^+$ T-cells in the airway wall [140]. Both diseases have characteristic mucus hypersecretion, and airway obstruction, remodelling and hyperresponsiveness, but the clinical course is also intermittently punctuated by episodes of acute worsening of symptoms known as exacerbations. As well as dyspnea, cough, wheezing and chest tightness, exacerbations in COPD are also accompanied by increased sputum volume and purulence and general fatigue, and such events alter the progression of the disease by accelerating the decline in lung function [141]. In 2004 it was estimated that 7% of the UK population suffered from asthma, with the disease causing 3 deaths every day and costing the UK over £2.3 billion every year [142]. Worldwide, asthma is thought to affect 300 million individuals [143]. COPD is also a major public health problem, and is the fourth most common cause of death worldwide. It was estimated to affect 5% of the UK population in 2000, costing the NHS £800 million every year [144]. Thus both diseases are a major burden in terms of morbidity, mortality and economics, with exacerbations being the major cause of hospitalisations and deaths.

1.6.2 Asthma and COPD exacerbations

Exacerbations in asthma and COPD can be caused by pollutants, with exacerbations in asthmatics additionally able to be caused by allergens and cold air, but most are precipitated by respiratory viruses [145]. While bacterial infections are also found in a significant number of COPD exacerbations, with lower airway colonization correlated to disease and exacerbation severity, they are also common in stable COPD, suggesting they are not the only causative trigger for exacerbations, and frequently there is found to be co-infection with respiratory viruses [146]. However, the acquisition of new strains to the dynamic niche of colonized bacteria in the respiratory tract has been demonstrated to result in exacerbations [147]. The most prevalent exacerbation-

precipitating pathogens are rhinoviruses, but RSV, influenza, coronaviruses, *H. influenzae* and *S.pneumoniae* are also commonly implicated [148, 149].

The respiratory viruses implicated in exacerbations have a range of complexities. Group V contains negative-sense, single-stranded RNA viruses, and includes the families Paramyxoviridae and Orthomyxoviridae. The Paramyxoviridae family can be split into 2 sub-families: paramyxovirinae, containing parainfluenza viruses, and pneumovirinae, containing metapneumovirus and RSV. These viruses all have a genome of 15 – 19 kilobases, and are enveloped. Similarly, the Orthomyxoviridae family, containing Influenzaviruses A, B and C, is made up of viruses with a genome of 13kb which are enveloped. Group IV, on the other hand, contains positive-sense, single-stranded RNA viruses, and includes the families Coronaviridae and Picornaviridae. The coronavirinae subfamily of the Coronaviridae family contains 3 types of coronavirus, as well as the SARS (severe acute respiratory syndrome) coronavirus, all of which are enveloped and have a very large 30kb genome. In comparison, the enterovirus genus in the Picornaviridae family, contains all three rhinovirus species, which are very small (7.5kb), non-enveloped viruses [150]. The differences between these respiratory viral species can potentially affect the immune response generated against them and the severity of symptoms caused in infected people, with, for example, non-enveloped viruses hardier and able to survive for longer periods of time, smaller viruses associated with more frequent outbreaks of disease, and larger viruses associated with a higher fatality rate [151, 152].

Additionally, within the genus enterovirus there are a number of rhinovirus species. More than 100 rhinovirus serotypes have been discovered, which are divided into major and minor groups based on their receptor usage, and into A and B groups based on antiviral sensitivity and nucleotide sequence [153]. Additionally, there are a further 50 – 60 more recently discovered species that have been defined as group C rhinoviruses, based on sequence data alone [153, 154]. These newly discovered group C rhinoviruses (HRV-C) have been demonstrated to account for the majority of asthma attacks in children presenting to hospital, causing more severe symptoms than other rhinovirus groups or other respiratory viruses [155, 156]. Group C rhinoviruses have also been associated with atopy, with hospital admission due to HRV-C infection increased in atopic subjects, but this interaction not observed for other rhinovirus species [157]. Therefore, group C rhinoviruses are likely to be a very important cause of asthma and COPD exacerbations, as well as having implications in atopic diseases, which includes certain phenotypes of asthma.

However, in spite of the burden they cause for people with asthma and COPD, the mechanisms behind virus-induced exacerbations are incompletely understood, and are likely different for the diverse range of pathogens which can cause them.

1.6.2.1 Exacerbation susceptibility

It is unclear whether sufferers of asthma and COPD are more susceptible to viral infections, and also why such individuals react differently to these pathogens compared to healthy people. In asthma there is no evidence for an increased risk of contracting a respiratory viral infection, but once infected it is more likely to reach the lower respiratory tract, resulting in more severe and longer-lasting symptoms [158]. In COPD exacerbation frequency increases with increased severity of the disease [159, 160], while it has also been suggested that a subgroup of patients may have a distinct phenotype of exacerbation susceptibility, independent of GOLD stage, suggesting an underlying intrinsic susceptibility to exacerbation-causing triggers such as viral infection [160]. E1A protein from latent adenovirus infection, which is common in COPD, has been shown to increase ICAM-1 expression [161, 162], the main receptor for rhinoviruses, thus suggesting that COPD sufferers may be more susceptible to viral infection. Also, airway epithelial cells cultured from COPD patients have been demonstrated to have defective anti-viral responses, with disproportionate cytokine production, a compromised epithelial barrier, impaired mucociliary clearance, and high viral load [163]. In addition to this, COPD patients commonly have bacterial colonisation of their lower airways, partly due to the effects of cigarette smoking on epithelial cilia and mucus production, and together these factors may further increase ICAM-1 expression and alter the immune response to any viruses that may enter the airways [113, 164, 165]. Finally, it has been suggested that the respiratory microbiome of people with asthma and COPD may differ from healthy people, with a reduction in Bacteroidetes, and specifically *Prevotella* spp. which have been shown to directly inhibit the growth of other bacteria, and an increase in Proteobacteria, especially *Haemophilus* spp. [143]. Whether this difference is a consequence of having a chronic respiratory disease such as asthma or COPD, or the result of previous antibiotic usage, remains to be shown. Additionally, this altered respiratory microbiome could play a role in the susceptibility to infection or be a causative mechanism for asthma or COPD, but this is also yet to be determined.

Therefore it seems that COPD patients may be more susceptible to contracting respiratory viral infections, while asthmatics may just experience more severe symptoms.

1.6.2.2 Mechanisms of asthma exacerbations

Respiratory viral infections clearly affect asthmatic and COPD sufferers differently to healthy people, with a disrupted immune response resulting in more severe disease and longer-lasting symptoms. However, the underlying pathology of the two diseases differs markedly, so mechanisms of virus-induced exacerbations are likely to be different in each disease.

In studies comparing rhinovirus infections in healthy subjects to those with asthma, there have been conflicting results regarding the levels of cytokines and chemokines such as CXCL8, IL-1β, IL-6 and IL-11, in the airways, with some studies showing an increase and some showing no difference [166-168]. CXCL8 (also known as IL-8) is a chemotactic factor for neutrophils, and these cells have been shown to contribute to the increased inflammatory state during asthma exacerbations. Neutrophils release elastase which can damage host tissue, thus increasing airway sensitivity, and proteases which induce mucus production, contributing to airway obstruction [169]. IL-11 may also be important in virus-induced asthma exacerbations, as it has been shown to be correlated with a detectable wheeze in children, and it may directly increase airway responsiveness [170]. Viral infections also trigger increased recruitment of eosinophils in both healthy and asthmatic people, but in asthmatics this eosinophilia persists for over six weeks after infection [171] which can contribute to airway hyper-responsiveness (AHR).

It has also been shown that the innate immune response is deficient in asthmatic people. IFN-β is vital in limiting viral replication and inducing rapid apoptosis of infected cells, but mRNA and protein levels of this cytokine induced after viral infection are reduced in asthmatic bronchial epithelial cell cultures. As a result of this, cytotoxic cell death is increased, causing the release of intact viral particles and inflammatory mediators, thus further increasing inflammation [172]. This epithelial necrosis can also result in exposure of sensory nerve fibres which can be stimulated by the increased numbers of irritants and particles able to cross the damaged epithelial barrier, thus increasing AHR and altering parasympathetic bronchoconstriction and the production of mediators of bronchodilation [170]. In addition to innate effects, the typically Th2 adaptive immune response in asthma patients may increase ICAM-1 levels [173], thus enhancing viral entry, and inhibiting the Th1 response required for viral clearance. Th2 cytokines have also been shown to further inhibit epithelial repair which is characteristic of asthmatics and correlated with AHR [174]. Due to this Th2 environment, asthmatic subjects produce significantly lower levels of the pro-inflammatory Th1 cytokines required for viral clearance than healthy subjects, and administering IFN-γ has been shown to fail in over-riding these Th2 effects on the airways [170].

1.6.2.3 Mechanisms of COPD exacerbations

Fewer studies have been carried out comparing virus-induced infections in COPD patients versus healthy controls to characterise the immune response in exacerbations. COPD is associated with mucosal neutrophil infiltration in the lungs and airways, with BAL and sputum samples containing increased levels of pro-inflammatory cytokines such as TNF-α and IL-6. The extent of the neutrophilic inflammation and the levels of cytokines present are increased with disease severity, relating to a reduced FEV_1, and are also amplified during respiratory infection [175]. Exacerbations have been suggested to be correlated with NF-κB activation and various cytokines and chemokines (TNF-α, CXCL8, epithelial-derived neutrophils attractant (ENA)-78 and CCL5), so these may be central components in the augmented inflammatory response experienced. There are conflicting results surrounding which cells contribute to the increased inflammation of COPD exacerbations, with neutrophils, lymphocytes and eosinophils implicated. These cells may release elastase and reactive oxygen species, thus causing tissue damage and also stimulating increased mucus production [149].

1.6.3 Existing treatments and prevention of asthma and COPD exacerbations

Treatment for asthma and COPD currently relies on inhaled bronchodilators and corticosteroids, but there is no definitive cure for either disease and such therapies are of limited effectiveness in severe cases [176]. Additionally, while exacerbation rates can be reduced by them, they are not completely prevented [177, 178]. This potentially relates to the observation that corticosteroids are ineffective at reducing neutrophilic inflammation, with asthma exacerbations commonly associated with neutrophilia rather than the eosinophilic inflammation of stable patients, and likewise COPD exacerbations associated with increased neutrophil inflammation [175, 179-181]. In addition to this, many people are insensitive to corticosteroids, there is a large amount of non-compliance, tachyphylaxis can cause complications, and adverse effects can include oral candidiasis and glaucoma [182].

Treatment for the respiratory pathogens commonly associated with asthma and COPD exacerbations is also limited. Vaccines against the common exacerbation-precipitating pathogens are only available and routinely administered in the UK against seasonal influenza, *S.pneumoniae* and *H.influenzae* Type B [183], but not against nontypeable *H.influenzae* which is more commonly implicated in adult respiratory tract infections [184]. The availability of effective antiviral drugs is

also limited, with no clinical treatments for rhinoviruses, the main cause of exacerbations, although the drug Pleconaril (Schering-Plough) which inhibits viral capsid uncoating to prevent attachment to host receptors such as ICAM-1 [185] is currently in its late clinical trials stage [186]. Treatment of bacterial infections is better established, but concerns are emerging about clinical efficacy being increasingly limited by antimicrobial resistance to antibiotics [187]. In addition to this, antimicrobial agents are frequently prescribed for viral infections, and this inappropriate and widespread use has contributed to concerns about emerging antimicrobial resistance among bacteria commonly involved in respiratory tract infections [185]. As such, new treatments and therapies are much needed in the fight against these two diseases, especially in the prevention of exacerbations.

1.6.4 Respiratory tract infections in older adults

Elderly adults (typically defined as those older than 65 years of age) are more susceptible to acute respiratory tract infections, with prolonged recovery times, increased health-care use, frequent complications and increased mortality [188]. The elderly population is also increasing, representing the fastest growing population group in the western world, with 9.4 million people in the UK in 2001, and 10.4 million in 2011 [189], thus further adding to this burden.

Various respiratory diseases have been shown to be more prevalent in the elderly. Community-acquired pneumonia is the leading infectious driver of morbidity and mortality in the elderly, with this age-group accounting for 81% of all cases [190]. A study in the USA showed that the mortality rate associated with community-acquired pneumonia doubles as age increases from 65, to 69, to >90 years old, with an average length of stay in hospital of 7.6 days, and the overall annual hospital costs associated with community-acquired pneumonia in the elderly reaching $4.4 billion [191]. *S.pneumoniae* is the most common cause, followed by *H.influenzae* and *S.aureus*, and infections with these bacteria have been shown to occur more frequently in the elderly compared to younger age groups [192]. Influenza also impacts the elderly more than other age groups, with increased rates of hospitalization and an estimated 85% of influenza-related deaths occurring in this age-group in the USA [193]. Collectively in the USA 59,000 deaths are attributed to pneumonia and influenza in the elderly every year [194]. While traditionally thought of as a pathogen only affecting young children, RSV is also now considered an important cause of disease in the elderly, with a diminished Th1 cytokine response to RSV infection seen in this age group, and progression to pneumonia in 2-7% of infected individuals [195, 196].

While the increased rate of hospitalization and fatalities amongst the elderly in the winter months associated with influenza is well established, it is emerging that viruses associated with typically mild upper respiratory tract infections such as rhinoviruses and coronaviruses may also be associated with severe respiratory illnesses in this population [10, 197]. Rhinovirus accounts for 25-50% of respiratory illnesses in the elderly, with documented outbreaks resulting in significant morbidity and mortality [198, 199].

Finally, elderly adults living in sheltered accommodation, nursing homes, or residential care homes, as well as the staff working in such facilities, are at increased risk of contracting acute respiratory tract infections due to the confined space they inhabit. Outbreaks of such infections can occur year-round, with studies reporting rates of 0.42 – 1.2 infections per 1000-resident days, and influenza has been implicated as the most commonly reported cause of such outbreaks [200-202]. Additionally, the management of infectious diseases is often limited in such facilities, with chronic co-morbidities potentially obscuring signs of infection, impaired cognition and hearing impeding expression of symptoms, and atypical presentation of diseases such as confusion and afebrile infection in elderly people [203].

1.6.5 Susceptibility to acute respiratory infection in the elderly

Collectively termed immunosenescence, numerous changes in immune response and immune regulation have been shown to change with advanced age and may cause this increased susceptibility to respiratory tract infections [204]. Neutrophils are more prone to apoptosis, with a decreased ability to produce reactive oxygen species and kill phagocytosed organisms [205-207], dendritic cells have a decreased ability to present antigens [208], macrophages are less able to produce superoxide anion to kill intracellular bacteria [209], NK cells are less capable of cytolytic activity [210], T-cells decrease in number with memory T-cell responses waning and a decline in Fas-mediated T-cell apoptosis [211, 212], there is a shift from Th1 to Th2 cytokine production [213], B-cell production decreases [214], and antibodies have a lower affinity and avidity for antigens [215]. Functional changes to the respiratory system also occur in elderly people. There is a decrease in elastic recoil [216, 217], lung compliance increases due to the loss of structural extracellular matrix proteins such as elastin and collagen [204], and there is a decline in mucociliary clearance associated with microtubular abnormalities [218].

In addition to immunosenescence and functional respiratory changes, there is also thought to be age-associated inflammation in the lungs of the elderly. This low-grade chronic inflammatory state is due to a combination of underlying medical conditions, cell damage, dysregulation of cellular processes and exposure to infectious agents. It has been demonstrated to cause augmented NF-κB levels and also increased expression of PafR and pIgR, thus potentially increasing bacterial adhesion in the airways [87]. Dendritic cells from older adults have also been shown to display an enhanced level of activation, with spontaneous secretion of pro-inflammatory cytokines such as IL-6 and TNF-α, and altered cross-talk with epithelial cells [219]. Additionally, as with asthma and COPD patients, the elderly appear to have an altered microbiome, which could affect their susceptibility to infection. While the respiratory tract has not yet specifically been studied, work emerging from the gut has shown that the elderly have a decreased *Firmicutes*/*Bacteroidetes* ratio, a reduction in the numbers and diversity of protective commensals such as *Bifidobacteria*, and an increase in colonization of the pathobiont *Escherichia coli* when compared to younger adults [220, 221].

Underlying factors relating to age and co-morbidities may also play a role in the susceptibility to infection in elderly people. Xerostomia (impaired salivation), a side-effect of many medications, and dysphagia (difficulty swallowing) are common in the elderly and can contribute to impaired clearance of organisms, thus enabling oropharyngeal colonization which is a risk factor for developing pneumonia [204, 222]. Silent aspiration, whereby gastric contents and food enter the bronchial tree, is also common in the elderly, and has been associated with chronic bronchiolar inflammation and reduced bactericidal activity due to exposure of respiratory epithelial cells to an acidic pH [223, 224]. Malnutrition may also be important, with a low body mass index linked to a decreased ability to resist infection, hypoalbuminemia shown to be a risk factor for pneumonia, and depressed leptin levels associated with impaired bacterial clearance [204, 225-227].

Therefore, a combination of these factors is likely to be what makes the elderly more susceptible to respiratory infections, and makes such infections more severe. However, there is a large amount of variability between individuals, and people age at different rates in terms of their fitness, frailty and functional impairment [228], with even centenarians having been shown to mount a robust immune response to a variety of stimuli [229].

1.6.6 Existing treatments and prevention of respiratory infections in the elderly

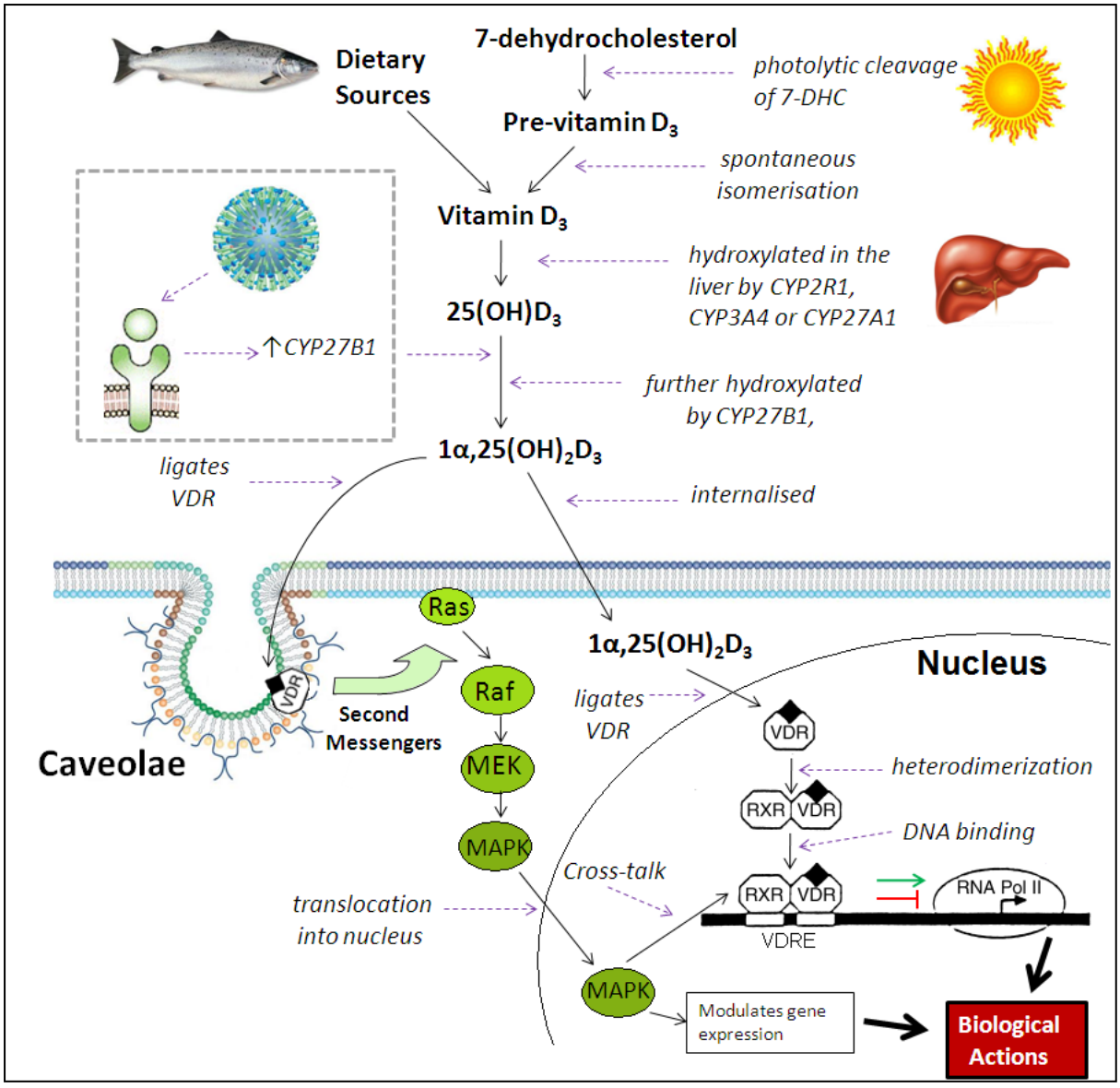
As with asthma and COPD exacerbations, treatment of respiratory tract infections in the elderly usually consists of symptom-relieving therapies and the use of antibiotics, with which there are concerns about emerging resistance. Annual influenza vaccines and the pneumococcal polysaccharide capsule vaccine are also provided to people aged over 65, and while vaccine responses tend to be attenuated in the elderly due to waning immune responses, both vaccines have been demonstrated to be safe and effective in this age group [204, 230-233]. However, neither vaccine protects against all strains of pathogen, vaccine-induced antibodies have been demonstrated to wane over time, thus questioning how long these vaccinations offer protection for, and the use of the pneumococcal polysaccharide capsule vaccine has also been questioned [204, 231, 234, 235].

Therefore, an agent that would help in preventing respiratory tract infections in vulnerable populations such as the elderly and people with chronic respiratory conditions would be hugely beneficial, potentially reducing hospitalizations, morbidity and mortality.

1.7 Vitamin D

Vitamin D has long been known to have effects on calcium homeostasis, with the discovery in the early 20th Century that rickets (a disease of vitamin D deficiency) could be cured or prevented with cod liver oil, exposure to sunlight and food irradiation [236, 237]. Since then much research has been focussed on vitamin D, resulting in its reclassification as a steroid, and the discovery of its mode of action via metabolism of the active form $1\alpha,25(OH)_2D_3$ (calcitriol) and subsequent binding to vitamin D receptors (VDRs). Vitamin D (cholecalciferol) is acquired from dietary sources, primarily oily fish, or from UV-mediated synthesis in the skin, before it is metabolised in the liver to form $25(OH)D_3$. This $25(OH)D_3$ is the circulating form of vitamin D, with a half-life estimated to range from 2 weeks to 2 months [238-240], and its serum concentration is used to define vitamin D status. Further hydroxylation results in the synthesis of $1\alpha,25(OH)_2D_3$ and ligation of nuclear VDRs, allowing subsequent binding to vitamin D responsive elements in promoter regions of specific genes, resulting in the repression or induction of gene transcription [241] (Figure 1.3). The location of the VDR was initially thought to be only in the nucleus, but in the 1980s $1\alpha,25(OH)_2D_3$-mediated non-genomic rapid responses were discovered. These responses are too fast to be explained by the

ligation of nuclear VDRs and subsequent gene transcription, and thus led to the detection of VDRs associated with the plasma membrane or its caveolae components [242-244]. Therefore, while the VDR is defined as a member of the nuclear receptor superfamily, which, once ligated and heterodimerised with RXR, can penetrate the deep groove of DNA and recognise VDREs to alter gene transcription of numerous target genes, it also has a number of non-DNA mediated effects. Ligation of these membrane VDRs results in the activation of second messenger systems such as phospholipase C, protein kinase C and phosphatidylinositol-3'-kinase (PI3K), thus initiating various intracellular effects such as the opening of voltage-gated calcium channels and Ras/MAPK signal transduction [244, 245]. A number of non-genomic mechanisms of the VDR have been described [246], such as stimulation of tyrosine phosphorylation cascades in muscle cells resulting in the formation of complexes between Src and the VDR which can impact contractility and myogenesis [247], inhibition of TGF-β-SMAD signal transduction via direct interaction between the 1,25$(OH)_2$D-ligated VDR and SMAD3 to suppress renal fibrosis [248], and augmentation of glucose-induced insulin release from pancreatic beta-cells via non-genomic effects of the VDR on signal transduction [249].



(Adapted from Slatopolsky et al.[250], and Parton and Simons [251])

Figure 1.3: Metabolism of 1α,25(OH)$_2$D$_3$. Vitamin D$_3$ is either obtained from dietary sources or UV synthesis, before two hydroxylations occur to produce the active metabolite 1α,25(OH)$_2$D$_3$. TLR ligation can also increase levels of CYP27B1, resulting in enhanced 1α-hydroxylation of 25(OH)D$_3$. The 1α,25(OH)$_2$D$_3$ then binds to nuclear or membrane vitamin D receptors (VDRs). Nuclear VDR ligation results in heterodimerization with retinoid X receptor (RXR) and binding to vitamin D responsive elements (VDRE) in promoter regions of responsive genes. Components of the RNA polymerase II complex are then recruited for induction of gene transcription, or transcription is repressed. Membrane caveolae-associated VDR ligation results in the activation of second messenger systems, with one effect being the initiation of Ras/MAPK signal transduction. Nuclear MAPK modulates gene expression and engages in cross-talk with the VDR-RXR-VDRE complex.

Abbreviations used: 1,25(OH)$_2$D: 1,25-dihydroxyvitamin D; 25(OH)D: hydroxyvitamin D; 7-DHC: 7-dehydrocholesterol; CYP: cytochrome p450; MAPK: mitogen-activated protein kinase; mitogen-activated protein kinase kinase; Raf: mitogen-activated protein kinase kinase kinase; Ras: mitogen-activated protein kinase kinase kinase kinase; RXR: retinoid X receptor; RNA Pol II: RNA polymerase II complex; VRD: vitamin D receptor; VDRE: vitamin D responsive element.

The detection of VDR abundance throughout the immune system [252], the observation that VDR expression is regulated by immune signalling [253, 254], and the detection of CYP27B1 expression by immune cells [254-256] has since directed vitamin D research into the area of immunology. As such, multiple observational studies and randomised control trials have looked into the effects of vitamin D in various diseases, with associations of low serum $25(OH)D_3$ levels seen with autoimmune diseases [257], cancer [258], cardiovascular disease [259] and respiratory infections [260], and *in vitro* experiments have tried to determine these effects at a cellular level. However, results have been inconsistent and the hypothesised beneficial effects of vitamin D supplementation in these important diseases are currently unclear, with a need for further randomised controlled trials [261].

1.8 Association between vitamin D status and asthma and COPD

Many studies have investigated whether there is a causal relationship between vitamin D deficiency and asthma, with the two conditions having similar risk factors (such as African American ethnicity, inner-city residence and obesity), and both seeing an increase in prevalence in recent decades [262]. Additionally, it has been observed that 60 – 70% of severe COPD patients have vitamin D deficiency [263], but it is unclear whether this deficiency contributes to COPD pathogenesis, or is just a consequence of having COPD. However, these potential relationships and the use of vitamin D in primary prevention are outside the scope of this study, with our focus being on those who already have established asthma or COPD, and how vitamin D may improve the course of these diseases by restoring function and reducing disease-related complications.

Respiratory tract infections have been shown to be the major precipitants of acute exacerbations in asthma and COPD, and it has been suggested that vitamin D may play a positive role in managing both the stable disease state and the response to exacerbations, as will be discussed later.

1.9 Vitamin D status in older adults

Vitamin D deficiency is common in the elderly in the UK, and is even greater in those living in institutions, affecting 30% of men and 33% of women in one study [264]. Randomised controlled trials have demonstrated that supplementation with vitamin D is able to prevent falls and fractures in this population [265, 266], and as such, the Department of Health advises people aged 65 and

over and not exposed to much sunlight to take a daily supplement of 10 micrograms vitamin D [267]. This prevalence of hypovitaminosis D in elderly populations can likely be attributed to limited solar exposure due to altered lifestyle factors such as outdoor activity and clothing, and also a more limited variation in diet with a lower vitamin D content [268]. Additionally, dermal production of vitamin D following exposure to UVB light decreases with age due to atrophic changes in the skin reducing the amount of 7-dehydrocholesterol and the capacity to produce pre-vitamin D_3 [269, 270].

1.10 Relationship between vitamin D status and acute respiratory tract infections

1.10.1 Observational studies

A number of observational studies have demonstrated an inverse association between low vitamin D status and increased risk of acute respiratory tract infections [271]. Cross-sectional studies have shown a 7% lower risk of acute respiratory infection with every 10nmol/l increase in 25(OH)D concentration [272], and an inverse association between serum 25(OH)D concentration and self-reported recent URTI symptoms, which was seen to be more significant in people with asthma or COPD [260]. Furthermore, in hospitalized wheezing children a low serum 25(OH)D concentration has been associated with increased risk of infection by RSV, rhinovirus, or by multiple viral causes [273].

Case-control studies have demonstrated lower serum 25(OH)D concentrations in people hospitalized with LRTIs compared to healthy controls [274-276], with a lower concentration also associated with an increased risk of admission to intensive care units [277]. Children with rickets, a disease of profound vitamin D deficiency, were also found to be more susceptible to pneumonia [278], and have an increased risk of LRTI and a prolonged hospital stay compared to healthy children [279]. Finally, children hospitalized with either acute bronchiolitis or pneumonia were found to have a lower mean vitamin D intake than healthy controls, with an intake of less than 80 international units/kg/day increasing the risk of ALRI four-fold [280].

Numerous cohort studies have investigated the relationship between vitamin D status and susceptibility to acute respiratory infection, with low serum 25(OH)D concentration associated with more days of absence from military duty due to respiratory infection [281], and increased risk of

viral ARTI in healthy adults with a longer duration of illness [282]. Serum $1,25(OH)_2D$ concentration has also been demonstrated to be inversely associated with risk of febrile respiratory illness in institutionalized elderly adults [283], and administration of either calcitriol ($1,25(OH)_2D$) or alfacalcidol (1α(OH)D, a precursor of calcitriol) in haemodialysis patients has been associated with a preventative effect on the incidence of ARTIs [284]. Finally, cord-blood levels of 25(OH)D at birth have been shown to be inversely associated with risk of respiratory infections, and specifically RSV bronchiolitis, in early childhood [285-287], and low maternal serum 25(OH)D concentrations have been associated with increased risk of LRTI, pneumonia and bronchiolitis in their offspring [288, 289].

Observational studies have also demonstrated an association between low vitamin D status and lung function and measures of asthma and COPD morbidity. In asthmatic children low serum 25(OH)D concentration resulted in increased odds of one or more severe asthma exacerbations [290], and concentrations of ≤30ng/ml were associated with higher odds of any hospitalization or emergency department visit [291]. Cross-sectional data demonstrated with each 10nmol/l increase in 25(OH)D concentration, an 8ml increase in FEV_1 and a 13ml increase in FVC (forced vital capacity) [272], and in another study mean differences of 106ml and 142ml for FEV_1 and FVC respectively when comparing the highest and lowest quintiles of serum 25(OH)D concentration [292]. Low serum 25(OH)D concentration was also shown to be associated with impaired lung function, increased airway hyperresponsiveness and reduced glucocorticoid response in adult asthma [293], and increased corticosteroid use and worsening airflow limitation in childhood asthma [294], with reduced odds of hospitalization and anti-inflammatory medication use with every log_{10} unit increase in serum 25(OH)D concentration [295].

However, while the majority of observational studies have indicated an association between low vitamin D status and increased risk of respiratory tract infections, some studies have not shown this relationship. In one case-control study there was no difference in serum 25(OH)D concentration in children hospitalized with LRTI compared to healthy controls [296], and in three cohort studies no association was demonstrated between serum 25(OH)D concentration and self-reported cases of ARI in healthy adults [297], or risk of exacerbation or rhinovirus infection in COPD patients [298, 299].

1.10.2 Intervention studies

While the previously described studies indicate an association between low vitamin D status and risk of acute respiratory tract infections, inference cannot be made until robust intervention studies have been carried out. A number of randomised clinical trials have investigated whether vitamin D supplementation can prevent the incidence of acute respiratory tract infections, with conflicting results.

In a study of healthy post-menopausal African-American women who were given 800 international units of vitamin D_3 per day for 2 years, followed by 2000 IU/day for 1 year, while there was no effect on the primary outcome of bone density loss, women in the intervention arm were reported to have had less self-reported URTI symptoms during the course of the study [300, 301]. Various trials using a range of dosing regimens have also demonstrated a reduction in the intervention arm in the number of days absent from military duty due to respiratory tract infections in healthy adults (intervention of 400 IU/day for 6 months) [302], incidence of seasonal influenza A infection in schoolchildren (1200 IU/day for 4 months) [303], risk of repeat episodes of pneumonia in young children diagnosed with non-severe or severe pneumonia (single bolus dose of 100,000 IU) [304], and the rate of parent-reported ARI in schoolchildren with vitamin D deficiency (300 IU/day for 7 weeks) [305]. In children with asthma, vitamin D supplementation reduced the number of asthma attacks in children given 1200 IU/day for 4 months [303], prevented exacerbations triggered by ARI in children given 500 IU/day + the steroid budesonide for 6 months (compared to those given placebo + budesonide) [306], and reduced the number of exacerbations, the requirement of steroids and the number of emergency visits, while improving peak expiratory flow rate in children given 60,000 IU/month for 6 months [307]. Similarly in COPD, an intervention of a monthly bolus dose of 100,000 IU for one year was shown to cause a reduction in exacerbation rate in people with severe $25(OH)D_3$ deficiency at baseline (defined as <10 ng/mL) [308].

However, not all studies have been able to demonstrate a beneficial effect of vitamin D supplementation in preventing acute respiratory tract infections. While giving elderly patients with a recent acute hip fracture high dose vitamin D_3 (2000 IU/day versus 800 IU/day) for 1 year decreased the rate of hospital readmission due to infection at any site by 90%, there was no significant difference between readmission rates specifically due to respiratory tract infections [309]. Similarly, supplementation of 2000 IU/day for 3 months had no effect on self-reported URTI incidence, URTI duration or URTI severity [310], children given 1400 IU/week or a quarterly bolus dose of 100,000 IU had no difference in pneumonia incidence [311, 312], and supplementation in

adults (1111 – 6800 IU/day or bolus dose of 100,000 – 200,000 IU/month) had no effect on incidence of influenza-like illness, or incidence or duration of URTIs [313, 314]. Finally, a recent study in 408 asthmatic adults with a serum 25(OH)D level of less than 30ng/ml who received a one-off 100,000 IU dose followed by 4000 IU/day for 28 weeks demonstrated no change in time to first exacerbation, exacerbation rate, lung function, airway hyperreactivity, asthma quality of life or asthma control compared to participants on the placebo arm [315].

The differences observed may be due to the various strengths and limitations of each trial [271]. For example, looking at ARI incidence as a secondary outcome or in post hoc analysis may result in inadequate identification of ARIs, with one study stating a surprisingly low rate of URTIs for a trial of that duration [301]. Additionally, specific pathogens were not characterised in the majority of the trials, thus not enabling any pathogen-specific effects of vitamin D supplementation to be determined. The baseline vitamin D status of trial participants may also be important, but was not measured in all studies, with, for example, important sub-group effects observed in the profoundly deficient [308]. The type of dosing regimen used may also be an important determinant, with some inadequate to produce a prolonged rise in serum 25(OH)D concentration [311], and others not allowing sufficient duration of administration and follow-up to allow prolonged repletion [310]. Additionally, bolus dosing resulting in a rapid increase followed by a slow decline in 25(OH)D levels may have a deleterious effect [316], with concentrations greater than 56ng/ml associated with impaired immunity to infection [317], and a chronic exposure to falling 25(OH)D concentration associated with an imbalance in the enzymes involved in $1,25(OH)_2D$ extra-renal synthesis and catabolism, thus reducing the concentration of this active metabolite at sites of disease [318].

Finally, none of the studies described here performed any genetic analysis of the participants, with an individual's genotype associated with the effectiveness of supplementation, as described below.

1.11 Genetic associations between vitamin D and respiratory infections

The association between vitamin D and respiratory tract infections is further supported by genetic studies which implicate various polymorphisms in the vitamin D pathway in disease susceptibility. Since the actions exerted by $1,25(OH)_2D$ can be explained by its ligation of the VDR, and the subsequent activation and binding of the VDR to vitamin D responsive elements, polymorphisms in the *VDR* gene can result in differing effects of vitamin D. The gene encoding the VDR is located on

chromosome 12, spanning approximately 75 kilobases of genomic DNA, with polymorphisms named after the restriction endonucleases used for genotyping [319]. Multiple polymorphisms have been identified, and are distributed throughout the promoter region exons, coding exons, introns and the 3'UTR. The *FokI* single nucleotide polymorphism (rs2228570) is present in the translation start site, and is the only VDR SNP which results in a VDR protein with a different structure [320, 321]. It has been associated with risk of RSV bronchiolitis, with homozygosity for the minor *f* allele resulting in a 70% higher risk of disease [322-324]. Additionally, in children hospitalised with acute lower respiratory tract infections, the *ff* VDR genotype was seen to be more prominent, further suggesting an association between the FokI VDR polymorphism and risk of acute respiratory tract infection [325]. Further characterisation of the M1 VDR genotype encoded by the *f* allele has demonstrated functional consequences for the immune response, with stronger inhibition of NFκB regulated transcription, and decreased expression of vitamin D stimulated genes due to less efficient interaction with transcription factor IIB (TFIIB) when compared to the major *F* allele M4 VDR [321, 326]. While vitamin D is normally seen to inhibit the phosphorylation and activation of STAT1 in RSV-infected cells [327], this regulation is impaired in cells expressing the M1 VDR variant, resulting in diminished control of downstream gene expression. Since bronchiolitic pathology results from an exaggerated immune response, with airways congested with mucus, infiltrated immune cells and shed epithelial cells, it is possible that this elevated STAT1 activation could contribute to exacerbated RSV disease in *ff* VDR homozygotes. As a result, vitamin D supplementation may not be beneficial for people with this VDR genotype due to the inability of $1,25(OH)_2D$ to inhibit STAT1 phosphorylation and dampen immunopathology [328].

Another VDR SNP is *TaqI* (rs731236), a synonymous codon in the VDR exon, which does not result in an altered amino acid sequence. While not an effect on an acute respiratory infection, the *TaqI* VDR polymorphism has also been demonstrated to modify the effect of vitamin D supplementation on time to sputum culture conversion in people with pulmonary tuberculosis, with participants with the *tt* genotype having an enhanced response, thus suggesting an effect on antimicrobial immunity. A response to vitamin D supplementation was not observed in the whole study population, demonstrating the potential importance of VDR genotype characterisation when assessing the immunomodulatory actions of vitamin D [329]. *In vitro,* carriage of the *t* allele has also been associated with increased $1,25(OH)_2D$-induced phagocytosis of *Mycobacterium tuberculosis*, with *BB* (*BsmI* VDR polymorphism), *AA* (*ApaI* VDR polymorphism) and *FF* (*FokI* VDR polymorphism) genotypes also demonstrated to have enhanced macrophage phagocytic activity [330, 331].

Another key component of vitamin D-induced gene transcription is the vitamin D binding protein (VDBP). Also known as Gc-globulin, it has a major role in binding, solubilising and transporting vitamin D and its metabolites, as well as contributing to macrophage activation, chemotaxis, actin scavenging, and fatty acid transport [332, 333]. The *GC* gene encoding the VDBP is highly polymorphic, with three common variants (*GC1F*, *GC1S* and *GC2*) and >120 rarer variants, resulting in differences in protein function [334]. As with VDR polymorphisms, *GC* genotype has been associated with susceptibility to tuberculosis, with the *GC2* variant VDBP strongly increasing active disease in people with vitamin D deficiency [335]. However, no association has been made with susceptibility to acute respiratory tract infections.

The effect of VDBP variant on the response of serum 25(OH)D to vitamin D supplementation has also been assessed. In adults receiving 600 or 4000 IU/day for one year, genotyping of the VDBP functional variant T436K (*GC2*) demonstrated an increased rise in mean serum 25(OH)D for the *KK* genotype (307%) compared to *TK* (151%) and *TT* (97%), although the *KK* genotype was also associated with the lowest baseline 25(OH)D concentration [336]. Therefore, the effects of vitamin D supplementation during intervention studies may be dependent on the VDBP variant present.

In addition to this, genetic studies have further displayed a possible link between vitamin D and the chronic respiratory diseases of asthma and COPD. The *GC2* VDBP has been demonstrated to be significantly associated with the risk of asthma [337], as well as six SNPs in the VDR gene, including *TaqI*, *BsmI* and *ApaI* [338]. COPD has been extensively linked to VDBP polymorphisms, with the *GC1F* genotype demonstrated to increase the risk of developing COPD and accelerate the annual decline in FEV_1 [339, 340], and *GC2* homozygosity shown to be protective [341].

Therefore, genetic studies implicating polymorphisms in key genes also demonstrate the potential role of vitamin D in the immune response to acute respiratory tract infections.

1.12 Immunological actions of vitamin D in *in vitro* models of respiratory infection

In vitro work has further demonstrated the possible association between vitamin D status and respiratory tract diseases, while also helping to characterise the underlying effects that it has on the immune system. Since the majority of immune cells express the VDR and the enzyme CYP27B1, thus

allowing the local conversion of circulating 25(OH)D to the active $1,25(OH)_2D$ and providing its receptor, the effects that vitamin D has on the immune system are diverse and extensive [241, 252, 256, 342].

1.12.1 Effects of vitamin D on the innate immune response to respiratory pathogens

The most frequently demonstrated effect of vitamin D is that on LL-37, and as such its expression is commonly used to demonstrate increased CYP27B1 expression and $1,25(OH)_2D$ activity. Cathelicidins are multifunctional antimicrobial peptides, and the sole form in humans is Human Cationic Antimicrobial Peptide of 18KDa (hCAP-18), from which the active form of LL-37 is cleaved by serine protease 3 [343]. It is expressed primarily by neutrophils and epithelial cells [344], but is also present in monocytes, NK cells, B-cells and γδ T-cells [345], and can be secreted by respiratory epithelial cells onto the airway surface to form a first line of defence against invading pathogens [346]. It has many functions, including direct facilitation of chemotaxis of immune cells [347, 348], induction of chemokine production (including CCL2, CCL4, CCL5 and CCL20) from immune and epithelial cells [349], up-regulation of chemokine receptors (including CXCR4 and CCR2) [349], suppression of neutrophil apoptosis via upregulation of BcL-XL and inhibition of caspase-3 activity [350], modification of DC differentiation [351], and immunomodulatory actions via induction of the anti-inflammatory cytokine IL-10 and suppression of the pro-inflammatory cytokines TNF-α and IL-1β [349, 352]. While LL-37 is traditionally viewed as a component of only the immune response to bacteria, with direct microbicidal activity via permeabilisation of bacterial membranes and neutralization of LPS observed [353-355], it also acts directly against viruses. Anti-viral activity of LL-37 has been demonstrated against both enveloped and non-enveloped viruses, including influenza virus and RSV [356]. It has been shown to disrupt the membrane of influenza virus [357], modify cytokine production [357], enhance TLR3 signalling [358], and have direct effects on RSV viral particles and infected epithelial cells, with diminished spread of infection and inhibited production of new virus particles [344]. Numerous studies have demonstrated the role the $1,25(OH)_2D$-ligated VDR plays in binding to a VDRE in the promoter of the cathelicidin gene to enhance hCAP-18 production [359-361], thus suggesting a potential mechanism by which vitamin D may enhance innate immunity to respiratory infections.

The proximal promoter region of DEFB4 gene also contains a VDRE, allowing $1,25(OH)_2D$ to upregulate expression of β-defensin 2 [361]. This is another antimicrobial peptide, which, similarly

to LL-37, is able to induce chemotaxis of immune cells and permeabilise bacterial membranes, whilst also inhibiting RSV infection [362, 363].

In addition to antimicrobial peptide induction, vitamin D has been demonstrated to modulate the innate immune system in a variety of other ways. Monocyte differentiation into macrophages is induced [364], with enhancement of the phagocytic and chemotactic capacity of macrophages [241, 365]. Phosphatidyinositol 3-kinase regulates anti-mycobacterial activity of $1,25(OH)_2D$ via the production of bactericidal reactive oxygen species in monocytes and macrophages [366], and inducible nitric oxide synthase (iNOS) has also been implicated, with suppression of *M.tuberculosis* growth linked to nitric oxide production [367]. However, reports have been opposing on whether vitamin D inhibits or stimulates its production in macrophages [368, 369]. The ability of $1,25(OH)_2D$ to induce monocyte autophagy has also been demonstrated. Autophagy acts as part of the immune system to remove damaged proteins and organelles, and is an important host defence mechanism against viral and intracellular bacterial infections. Vitamin D has been shown to induce autophagy by regulating multiple associated pathways, such as Bcl-2, mammalian target of rapamycin (mTOR), class III phosphatidylinositol 3-kinase complex, and cathelicidin production, thus potentially enhancing clearance of intracellular pathogens [370-372].

Pattern recognition receptors have also been demonstrated to be regulated by vitamin D. Expression of TLR2 and TLR4 is inhibited in monocytes, resulting in impaired downstream signalling and hyporesponsiveness to PAMPS. With the observation that this effect is most prominent after 72 hours, a negative feedback mechanism has been suggested, whereby excessive TLR activation is prevented at later stages of infection to dampen inflammation [241, 373]. Conversely, CD14, an accessory protein to TLR4 for LPS recognition which has also been linked to TLR2 [374], was up-regulated by $1,25(OH)_2D$ [373, 375, 376], but this effect was not sufficient to restore downstream TLR signalling [373]. Also, as opposed to its effects in monocytes, $1,25(OH)_2D$ has been demonstrated to induce up-regulation of TLR2 in keratinocytes, allowing a mechanism by which vitamin D may prevent infection of wounds [377]. Finally, the intracellular receptor NOD2 is induced by $1,25(OH)_2D$ in myeloid and epithelial cells, via two distal VDREs in the *NOD2* gene. Addition of MDP (a lysosomal breakdown product of bacterial peptidoglycan [241]) to $1,25(OH)_2D$-induced NOD2 enhanced NF-κB signalling and subsequent β-defensin 2 expression [378].

1.12.2 Effects of vitamin D on the adaptive immune response to respiratory pathogens

Vitamin D also modulates the adaptive immune response, and acts as a key intermediary between innate and adaptive immunity due to its influence on antigen presentation.

Dendritic cells are the most potent antigen-presenting cells, and as such have a direct effect on lymphocyte activation and induction of the adaptive immune response. They reside in peripheral tissues in an immature state, sampling the environment and mediating antigen uptake, until a maturation signal induces migration to local lymph nodes and subsequent T-cell activation. Addition of $1,25(OH)_2D$ has been demonstrated to inhibit DC differentiation, maturation and antigen presentation, with an associated decrease in markers such as CD1a, MHC class II, and the co-stimulatory molecules CD40, CD80 and CD86 [379-381], as well as abrogating the chemotactic response to CCL4 and CCL19 [382]. Already differentiated dendritic cells can also be redirected back towards a monocytic phenotype by the restoration of the monocytic marker CD14 [380, 383].

The antigen-presenting and T-cell stimulatory capacity of monocytes and macrophages is also impaired by $1,25(OH)_2D$, with a decrease in MHC class II, CD40, CD80 and CD86. IL-12 production is suppressed in both activated DCs and macrophages, due to the $1,25(OH)_2D$-mediated down-regulation of NF-κB activation [384], while influence on the expression of TNF-α is dependent on the differentiation state of the cells, with a reduction observed following $1,25(OH)_2D$ administration in LPS-stimulated monocytes and PBMCs [373, 385].

The main function of DCs is to initiate T-cell responses, and thus the effect of $1,25(OH)_2D$ on DCs has a major impact on T-cells. The decreased surface expression on DCs of co-stimulatory molecules and MHC class II results in a tolerogenic phenotype, with DC production of IL-12 (which is involved in driving Th1 differentiation) and IL-23 (which is involved in driving TH17 differentiation) inhibited by $1,25(OH)_2D$ [241, 383]. Even when cultured with committed T-cells, these tolerogenic DCs caused hyporesponsiveness, decreased T-cell proliferation and reduced IFN-γ secretion [379, 386]. IL-12, as well as stimulating the development of Th1 T-cells, also inhibits the development of Th2 cells, thus resulting in vitamin D shifting the balance of T-cells from a Th1 to a Th2 phenotype [387]. Concomitantly, DC production of IL-10 is increased. IL-10 is a cytokine with pleiotropic effects in immunoregulation, and levels in BAL fluid have been shown to be inversely correlated with severity/incidence of asthma [388, 389]. This IL-10 production drives development of regulatory T-cells (Tregs), and these Tregs are able to secrete more IL-10 as well as the immunomodulatory cytokine TGF-β, while release of the Treg cytokine CCL22 is also increased [241]. Th17 cells and IL-

17, have also been shown to be decreased, with calcitriol ($1,25(OH)_2D$) reducing IL-17 production in a mouse colitis model and impairing commitment to the Th17 lineage in mice with experimental autoimmune uveitis [390, 391], although these effects may not translate to the human respiratory system. Th17 cells, by releasing IL-17, initiate an inflammatory response dominated by neutrophils, with deficient levels resulting in recurrent bacterial infections. High levels of IL-17 production are associated with chronic inflammation and severe immunopathology [392], and thus vitamin D may play a role in attenuating this.

$1,25(OH)_2D$ has also been demonstrated to have direct effects on T-cells, independent of DC activity. While the role of DCs in the induction of Tregs has been described, it has also been shown that $1,25(OH)_2D$ in combination with dexamethasone can induce a Treg population in the absence of APCs [393]. The proliferation and cytokine profiles of T-cells are also directly altered by $1,25(OH)_2D$. Production of IL-2, IFN-γ, TNF-α, IL-17 and IL-21 are all inhibited [390, 394, 395], with inhibition of IFN-γ further precluding macrophage activation, thus attenuating antigen presentation and the recruitment of other T-cells [396]. This direct inhibition of Th1-priming cytokines further skews T-cell differentiation towards a Th2 phenotype. $1,25(OH)_2D$ is also able to upregulate the Th2-specific transcription factors GATA-3 and c-maf, resulting in increased production of IL-4, IL-5 and IL-10 [397]. B-cells are also affected by vitamin D, with modulation of T-cell responses altering the B-cell compartment, as well as having direct effects on B-cells themselves [241]. $1,25(OH)_2D$ is able to inhibit proliferation, plasma-cell differentiation, immunoglobulin secretion and memory B-cell generation, while inducing B-cell apoptosis [255]. As such, vitamin D supplementation has been used in the treatment of B-cell-associated autoimmune diseases such as systemic lupus erythematous [398, 399]. Finally, it has been suggested that vitamin D may affect other lymphocyte subsets, with VDR-KO mice presenting with fewer invariant natural killer (iNKT) cells [400], and $CD8^+$ T-cells from MS patients secreting less IFN-γ and TNF-α and more IL-5 and TGF-β following $1,25(OH)_2D$ treatment [401].

However, the mechanisms behind any potential beneficial role of vitamin D are unclear, with conflicting cellular studies on the effects of vitamin D on Th2 cells [402, 403], with both enhancement [397] and inhibition [404] of IL-4 synthesis demonstrated. $1,25(OH)_2D$ has also been shown to down-regulate DC-derived Ox40L, which is required for Th2 priming, thus resulting in a reduced Th2 cytokine response in $CD4^+$ T-cells from patients with allergic bronchopulmonary aspergillosis [405], thus contradicting evidence that vitamin D skews the T-cell phenotype towards a Th2 one. Additionally, the decrease in Th1 immunity which has been observed [241] would suggest a diminished immune response to pathogens, contrasting to the evidence suggesting an improved

response to respiratory tract infections after vitamin D supplementation. Finally, while studies have demonstrated direct effects of 1,25$(OH)_2$D administration on lymphocytes, others have shown that when using the inactive metabolite 25(OH)D, DCs are required to convert this precursor to the active 1,25$(OH)_2$D to exert its immunomodulatory effects [406]. This indicates that administration of different vitamin D metabolites may result in a different response. Therefore, while vitamin D clearly acts as an immunomodulatory molecule with a wide range of effects demonstrated, the precise mechanisms are currently unclear, with the conflicting results reported also adding to the uncertainty of its actions. The main immunomodulatory effects of vitamin D are summarised in Figure 1.4.

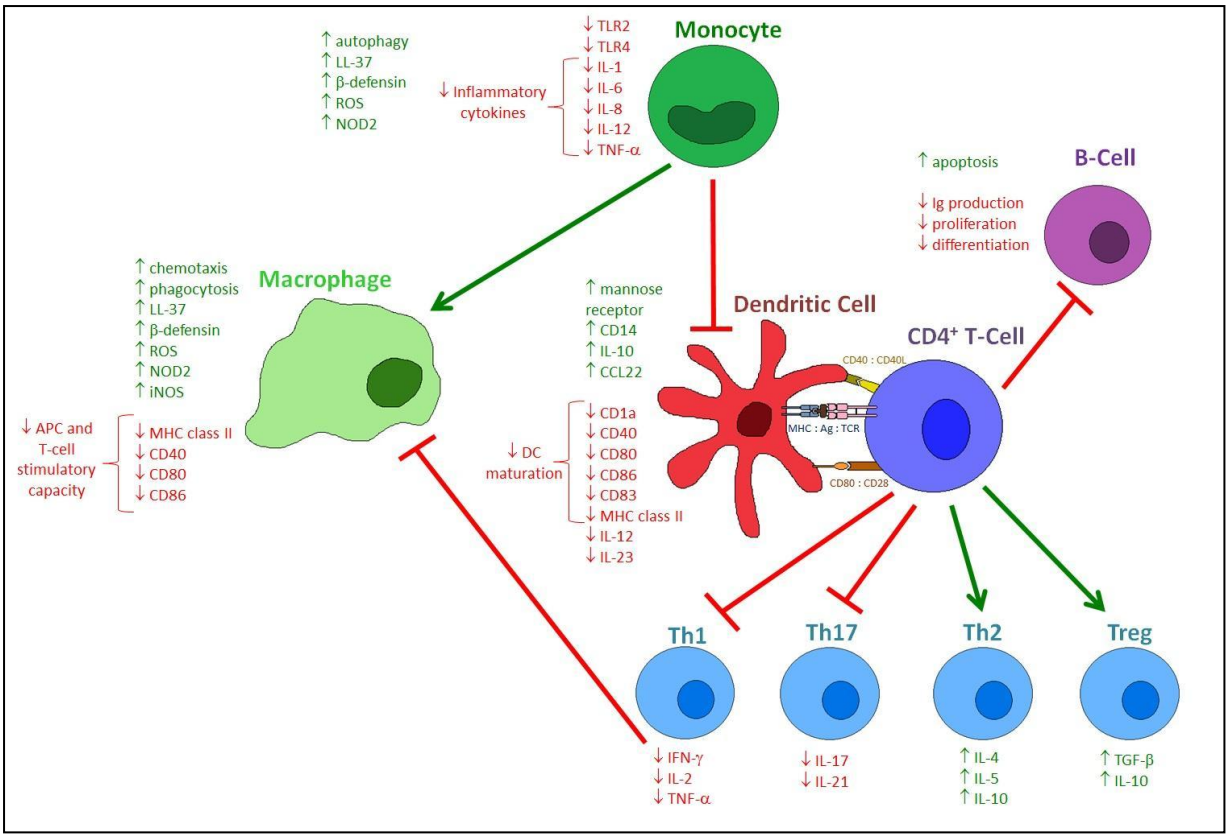


Figure 1.4: The immunomodulatory actions of 1,25(OH)$_2$D. 1,25(OH)$_2$D has diverse and extensive effects on the immune compartment. The innate immune response is affected, with monocytes producing more LL-37, β-defensin and ROS, with increased NOD2 expression and autophagy, while also producing diminished amounts of inflammatory cytokines, with decreased expression of TLR2 and TLR4. Differentiation into macrophages is increased, with macrophages having an increased capacity for phagocytosis and chemotaxis. However, their APC and T-cell stimulatory capacity is decreased. On the other hand, differentiation into DCs is inhibited, with DCs expressing decreased levels of maturation surface markers. DC production of IL-12 and IL-23 is decreased, while mannose receptor expression and production of IL-10 and CCL22 are increased. When these tolerogenic DCs interact with T-cells, development of Tregs and Th2 cells is increased, with increased production of IL-10, TGF-β, IL-4 and IL-5. The development of Th1 and Th17 cells is inhibited, with decreased production of IL-2, IFN-γ and TNF-α, and attenuation of macrophage activation. B-cells are also affected by 1,25(OH)$_2$D, demonstrating decreased immunoglobulin production, proliferation and differentiation, but increased apoptosis.

Abbreviations used: Ag: antigen; APC: antigen presenting cell; CCL: cysteine-cysteine motif ligand; CD: cluster of differentiation; DC: dendritic cell; Ig: immunoglobulin; IL: interleukin; iNOS: inducible nitric oxide synthase; MHC: major histocompatibility complex; NOD: nucleotide-binding oligomerisation domain; ROS: reactive oxygen species; TCR: T-cell receptor; TGF: transforming growth factor; TLR: Toll-like receptor; TNF: tumour necrosis factor.

1.13 Regulation of extra-renal vitamin D metabolism

Significantly, a link has been made between TLR ligation and cytokine secretion, and the expression of CYP27B1 and the VDR. The kidneys are classically the major site of 1α-hydroxylation of 25(OH)D by CYP27B1 to produce $1,25(OH)_2D$. This hydroxylation step is tightly controlled, with renal CYP27B1 regulated by the serum concentration of calcium, parathyroid hormone (PTH), phosphate, and fibroblast growth factor 23 (FGF23) [407-412]. Additionally, $1,25(OH)_2D$ inhibits CYP27B1, both directly and by inhibiting PTH and stimulating FGF23 secretion, and stimulates the catabolic 24-hydroxylase enzyme (CYP24A1), which generates $24,25(OH)_2D$ and $1,24,25(OH)_3D$ from 25(OH)D and $1,25(OH)_2D$ respectively, thus providing negative feedback pathways [413, 414]. However, extra-renal 1α-hydroxylation in cells such as monocytes and macrophages is unaffected by these calcium homeostatic agents [415-417], except for FGF23 which has been demonstrated to inhibit CYP27B1 expression in monocytes [418]. Additionally, negative feedback is defective due to expression of a splice variant form of CYP24A1 which is catalytically inactive, thus potentially resulting in accumulation of $1,25(OH)_2D$ [419, 420]. Instead, regulation is primarily by immune inputs, therefore rendering the immune system responsive to circulating levels of 25(OH)D and allowing the sustained and potentially beneficial production of $1,25(OH)_2D$ during an immune response [420].

Vitamin D metabolism in macrophages is linked to pathogen recognition, thus making it an integral part of the innate immune response [421]. Ligation of the TLR2/1 heterodimer in macrophages has been demonstrated to up-regulate CYP27B1 and the VDR in microarray studies. This was associated with increased downstream production of LL-37 in the presence of 25(OH)D replete human serum [422]. The TLR8 ligands CL097 and ssRNA40 have also both been demonstrated to induce a dose-dependent increase in CYP27B1 mRNA and protein expression in macrophages [423]. Similarly, ligation of TLR4 by LPS up-regulated CYP27B1 expression in monocytes [424, 425], again with increased LL-37 production with replete serum from *in vivo* vitamin D-supplemented patients [426], and with the same response observed in dendritic cells [427]. Additionally, ligation of TLR4 by monophosphoryl lipid A (MPLA), a synthetic LPS derivative with vaccine adjuvant properties which utilizes the TRIF signalling pathway, has been demonstrated to induce expression of CYP27B1 in dendritic cells, resulting in regulation of DC migration and activation of T-cells [428]. Also in dendritic cells, TLR3 ligation by polyI:C resulted in increased CYP27B1 expression, again altering the migratory properties of DCs to allow their localisation into non-draining secondary lymphoid organs to present antigen peptides to $CD4^+$ T-cells [254, 429]. Similarly, in human tracheobronchial epithelial (hTBE) cells, polyI:C stimulation and addition of live RSV were demonstrated to increase CYP27B1 expression, enhance 25(OH)D conversion to $1,25(OH)_2D$, and amplify cathelicidin mRNA

[430]. With the expression of TLRs in multiple cell types, and the ability to respond to a variety of pathogens, it is also possible that other TLRs or alternate PRRs may promote extra-renal expression of CYP27B1, allowing locally generated $1,25(OH)_2D$ to have even more extensive effects on the immune response.

The exact mechanism by which TLR ligation enhances CYP27B1 production has not been fully defined. Upregulation of CYP27B1 expression following ligation of TLR4 by LPS in human monocytes and the mouse RAW264.7 macrophage cell line has been shown to require the JAK-STAT, NF-κB and p38 MAPK pathways. Each signalling pathway is essential, with inhibitors to block MAPK, NF-κB and JAK resulting in inhibition of LPS-induced CYP27B1 production, demonstrating an intricate cross-talk between the pathways. Phosphorylation of the transcription factor C/EBPβ by p38 MAPK was also shown to be essential, with direct binding of C/EBPβ to recognition sites in the CYP27B1 promoter region necessary in enabling LPS-stimulated upregulation [425]. Additionally, IL-15 has been implicated as an intermediary in promoting localized activity of CYP27B1 and synthesis of $1,25(OH)_2D$. TLR 2/1 ligation by a triacylated lipopeptide of the *M.tuberculosis* 19 kDa antigen in human monocytes induced IL-15 secretion, which was required for the upregulation of CYP27B1 and the VDR, and subsequent downstream production of LL-37. Furthermore, IL-15 induces the differentiation of monocytes into macrophages with enhanced phagocytic activity against *M.tuberculosis*. Therefore, IL-15 produced in response to TLR2/1 ligation is able to amplify the innate immune response by inducing macrophage differentiation and upregulation of CYP27B1 and the VDR, thus increasing $1,25(OH)_2D$ synthesis and subsequent activation of the antimicrobial pathway and LL-37 production [431]. With the observation that patients with granulomatous diseases such as sarcoidosis frequently present with over-production of $1,25(OH)_2D$ and elevated expression of IL-15 [432, 433], it is also possible that IL-15-mediated CYP27B1 induction provides the link between vitamin D as a regulator of the normal innate immune response and the dysregulated $1,25(OH)_2D$ production associated with such inflammatory diseases [434].

As well as IL-15, other cytokines have been implicated in the regulation of CYP27B1 and vitamin D metabolism. Lung epithelial cells express CYP27B1 and are able to convert 25(OH)D to $1,25(OH)_2D$ locally [430], with IL-13 demonstrated to increase CYP27B1 and enhance the ability of 25(OH)D to increase production of LL-37 [435]. IFNγ induces CYP27B1 expression in differentiated THP1 macrophages on its own [436], and acting synergistically with LPS stimulation in human monocytes [425], while also up-regulating TLR2/1 induction of CYP27B1 and VDR in monocytes [437]. In contrast, IL-4 was shown to induce CYP24A1-dependent catabolism of 25(OH)D to the inactive $24,25(OH)_2D$ both on its own and in combination with the TLR2/1 ligand [437]. While IL-17 has been

demonstrated to have no effect on vitamin D metabolism [437], TNF-α, IL-1 and IL-2 were all able to increase monocyte CYP27B1, with an associated increase in $1,25(OH)_2D$ production [416, 438].
The pathways involved in cytokine-regulated transcription of CYP27B1 are likely the same ones as utilized by TLR ligation-induced regulation, as described above [436]. Additionally, the phospholipase A2 (PLA2)-arachidonic acid pathway has been implicated in influencing macrophage 1α-hydroxylation. Specifically, signal transduction through the 5-lipoxygenase pathway with generation of leukotriene C4 was critical to an increase in synthesis of $1,25(OH)_2D$ [439]. Chloroquine, a compound with actions in the PLA2-arachidonic acid pathway, was demonstrated to completely inhibit synthesis of $1,25(OH)_2D$ in macrophages, thus explaining its effectiveness in patients with sarcoidosis to reduce serum $1,25(OH)_2D$ and hypercalcaemia [440, 441].

Finally, and of potential importance to asthmatics who use glucocorticoids to reduce their chronic airway inflammation, dexamethasone has been shown to inhibit 25(OH)D 1 α-hydroxylation in pulmonary alveolar macrophages and monocytes [416, 438, 442]

Therefore, extra-renal $1,25(OH)_2D$ synthesis has been shown to be regulated by TLR ligation and cytokine secretion, utilizing an intricate cross-talk between various signalling pathways. As such, in the presence of sufficient circulating levels of 25(OH)D, infection by respiratory pathogens resulting in recognition by TLRs and cytokine production is able to increase levels of $1,25(OH)_2D$, hypothetically altering the immune response to better respond to these pathogens, as described in the previous two sections of this report.

1.14 Summary

In summary, respiratory infections are a major burden in terms of economics, morbidity and mortality, especially in those with chronic respiratory conditions such as asthma and COPD, and vulnerable populations such as the elderly. In such people, the immune response is altered, resulting in an ineffective immune response to respiratory pathogens, with inadequate antimicrobial activity and unregulated inflammatory responses potentially causing immunopathology, thus leading to an increased chance of hospitalization and death. Reviews of the literature linking vitamin D deficiency to increased incidence of acute respiratory tract infections suggest that supplementing with vitamin D_3, thus providing the 25(OH)D substrate for extra-renal 1α-hydroxylation to the active metabolite $1,25(OH)_2D$, may prevent such infections, as well as improving lung function in people

with asthma or COPD. However, intervention studies have so far been inconclusive, and the mechanisms behind any observed benefits are unclear, with conflicting *in vitro* results pertaining to the effects of vitamin D supplementation on the Th1/Th2 balance. Thus larger and better-designed randomised controlled trials are required, as well as further elucidation of the *in vitro* effects of vitamin D on cell types and function. When the immunomodulatory effects of vitamin D are further established, this molecule may provide a cheap and effective preventative agent for those at increased risk of acute respiratory infection. Individuals have been shown to be able to tolerate doses as high as 10,000 IU, which corresponds to 250μg per day [443], and the general public are unfazed by the consumption of supplements, with £220 million spent on vitamin and mineral tablets in 2006 in the UK and 43% admitting to taking some in the past 12 months [444].

1.15 Aims of the project

The overarching aim of this PhD project was to test the hypothesis that vitamin D metabolites, given *in vivo* in the form of supplementation in a randomised controlled trial, or used during *in vitro* cell culture, will augment the antimicrobial immune responses to respiratory pathogens and suppress immunopathological inflammation, thus potentially translating to beneficial effects in the host in the form of prevention of acute respiratory tract infections and exacerbations.

The specific aims were as follows:

1. To develop a whole blood assay to characterise the effects of *in vivo* vitamin D supplementation on *ex vivo* immune responses to TLR ligands and respiratory pathogens in peripheral blood
2. To characterise the effects of *in vitro* 25(OH)D and 1,25$(OH)_2$D on epithelial receptor expression, cytokine secretion and resistance to rhinovirus infection in alveolar A549 cells
3. To characterise the effects of *in vivo* vitamin D supplementation on the cellular profiles of the lower airways by analysis of induced sputum samples
4. To characterise the effects of *in vivo* vitamin D supplementation on the cellular profile of peripheral whole blood
5. To characterise the effects of *in vivo* vitamin D supplementation on *ex vivo* immune responses to TLR ligands and respiratory pathogens in peripheral blood
6. To analyse the differences between sputum cellular profiles in asthma vs. COPD patients

7. To analyse the differences between peripheral blood cellular profiles and the *ex vivo* immune responses to TLR ligands and respiratory pathogens in asthma vs. COPD patients vs. elderly adults living in sheltered accommodation with neither condition.

2. Materials and Methods

2.1 Clinical trial participants

The blood and sputum samples used in this study were from participants of three randomised, double-blind, placebo-controlled trials of vitamin D supplementation, in patients with asthma (ViDiAs) or COPD (ViDiCO) or in people living or working in sheltered accommodation who had neither condition (ViDiFlu) (Table 2.1).

Table 2.1: Summary of the three clinical trials

	ViDiAs	**ViDiCO**	**ViDiFlu**
Full Title	Randomised, multi-centre, double-blind, placebo-controlled trial of vitamin D supplementation in adult and adolescent patients with asthma	Randomised, multi-centre, double-blind, placebo-controlled trial of vitamin D supplementation in patients with chronic obstructive pulmonary disease	Cluster-randomised, double-blind, placebo-controlled trial of vitamin D supplementation for the prevention of influenza and other respiratory infections in sheltered accommodation
Ref Number (ClinicalTrials.gov)	NCT00978315	NCT00977873	NCT01069874
Sponsor	Barts and the London NHS Trust	Barts and the London NHS Trust	Barts and the London NHS Trust
Funding Body	National Institute of Health Research	National Institute of Health Research	National Institute of Health Research
Ethics Approval	East London and The City Research Ethics Committee ref 09/H0703/67	East London and The City Research Ethics Committee ref 09/H0703/76	East London and The City Research Ethics Committee ref 09/H0703/112
EudraCT (EU Drug Regulating Authorities Clinical Trials) number	2009-010083-42	2009-010084-16	2009-010085-35
Primary Objective	To determine whether a 2-monthly oral dose of 3mg vitamin D influences time to severe exacerbation and time to upper respiratory tract infection in patients with asthma	To determine whether a 2-monthly oral dose of 3mg vitamin D influences time to moderate/severe exacerbation and time to upper respiratory tract infection in patients with COPD	To determine whether vitamin D supplementation prevents respiratory infections in sheltered accommodation schemes, residential care homes, and nursing home residents and attendees of day centres and groups for older adults

Power calculations for each trial were based on the required sample size to enable detection of a clinically significant difference in the co-primary outcomes of time from randomisation to severe asthma exacerbation, moderate/severe COPD exacerbation or respiratory tract infection between intervention and control arms. For ViDiAs, with estimates of a median time to exacerbation of 120 days [445-447], a total of 200 participants (100 in each arm) was needed to be recruited in order to detect a 60 day difference in median time to exacerbation between intervention and control groups with 80% power using a 2-sided test at the 5% significance level, assuming an accrual period of 2.5 years and a follow-up period of one year for each participant [448]. A difference of this magnitude represents a hazard ratio of 0.67, which would be clinically significant. To compensate for patient drop-out, this number was increased by 25%, giving a total sample size of 250. Similarly for ViDiCO, with estimates of a median time to exacerbation of 60 days [449], a total of 192 participants (96 in each arm) was needed to be recruited in order to detect a 30 day difference in median time to exacerbation between intervention and control groups with 80% power using a 2-sided test at the 5% significance level, assuming an accrual period of 3.25 years and a follow-up period of one year for each participant [448]. A difference of this magnitude represents a hazard ratio of 0.67, which would be clinically significant, and to compensate for patient drop-out, this number was increased by 25%, giving a total sample size of 240. For ViDiFlu, the proportion of the population experiencing at least one respiratory tract infection per year has been reported to be between 68% and 92% [13, 188, 450]. Employing the Xie and Waksman formula for sample size estimation in clinical trials with clustered survival times as the primary endpoint [451], and assuming an average of 5 residents and attendees per cluster, with intra-cluster coefficient of 0.05, and equal numbers of units allocated to intervention and control arms of the study, a total of 68 units was estimated to be needed to be randomised to demonstrate a 10% reduction in the proportion of residents and attendees experiencing at least one respiratory tract infection in one year from 80% to 72%, with 80% power at the 5% significance level. To allow for loss of eight clusters to follow-up, this number was increased to a total of 116 clusters to be recruited.

Study design, recruitment of participants and study visits were performed by members of the vitamin D research team. In total, 250 people were recruited and randomised to the ViDiAs trial, 240 to ViDiCO and 240 to ViDiFlu. The majority of participants for ViDiAs and ViDiCO were recruited by approaching London-based primary care providers, who identified potentially eligible individuals and asked them if they would like to be contacted by a member of the study teams. Additionally, posters advertising the studies were displayed in appropriate public places such as hospital out-patient departments and GP surgeries, and emails were circulated within Queen Mary, University of London. Any individual expressing an interested was provided with a patient information sheet

(PIS), before screening was arranged. For ViDiFlu, London-based units, defined as sheltered accommodation schemes, residential care homes, nursing homes, day centres or groups for older adults, were approached to participate in the trial. A representative for the management of each unit was required to sign an agreement to participate, and units were excluded if they offered care exclusively for clients with dementia, learning disabilities, mental health needs, alcohol dependency or drug dependency. In total, 108 units were eligible and agreed to participate. Managers of each participating unit were provided with letters, and asked to forward these to potentially eligible staff, carers, attendees and residents. These letters provided an introduction to the study and an invitation to attend a presentation about the trial within the unit, or to meet with a researcher on a one-to-one basis. Researchers then visited participating units and met with individuals who had expressed an interest in the study, clearly explaining the trial and answering any questions. Patient information sheets were provided, and screening was then arranged.

2.1.1 Investigational medicinal product (IMP)

Active IMP for all three trials was Vigantol® Oil (Merck Serono, Darmstadt, Germany) – an oily solution containing 0.5mg cholecalciferol (vitamin D_3) per millilitre. The placebo was Miglyol® 812 oil (Caesar and Loretz, Hilden, Germany) – an organoleptically identical mixture of palm oil and coconut oil widely used in pharmaceutical practice. Miglyol® 812 oil is the excipient for cholecalciferol in Vigantol® Oil, and is thus identical to Vigantol® Oil in every respect except for the absence of cholecalciferol. Nova Laboratories Ltd (Wigston, UK) produced a computer-generated randomisation sequence and bottled study medication, and treatment allocation was concealed from trial participants and study staff.

2.1.2 ViDiAs trial: participant characteristics

Patients were deemed eligible if they were between 16 and 80 years of age, had a medical diagnosis of asthma treated at BTS (British Thoracic Society) Step 2 (taking inhaled corticosteroids daily [452]) or above, and had documented either a ≥12% increase in forced expiratory volume in one second (FEV_1) after inhalation of 400μg of salbutamol, a ≥20% diurnal variability peak in expiratory flow (PEFR), or methacholine PC20 (concentration of methacholine causing a 20% fall in FEV_1) < 8g/L. In addition to this, patients agreed to the required visits, were contactable by telephone, were able to

give written informed consent to participate and, if a woman of child-bearing potential, agreed to use a reliable form of contraception for the duration of the study.

Patients were excluded if they had a diagnosis of COPD, known sarcoidosis, hyperparathyroidism, nephrolithiasis, active tuberculosis, vitamin D intolerance, liver failure, renal failure or any malignancy not in remission for at least 3 years. Taking dietary supplements containing >10µg per day of vitamin D, a baseline corrected serum calcium >2.65mmol/L, baseline serum creatinine > 125µmol/L and a smoking history > 15 pack years (calculated by the number of packs of cigarettes smoked per day divided by 20, and multiplied by the number of years the person has smoked for) were additional exclusion criteria. Finally, participants were excluded if they were breastfeeding, pregnant or planning a pregnancy, undergoing treatment with any other IMP or device up to 4 months before randomisation onto this trial, or taking a cardiac glycoside (e.g. Digoxin), carbamazepine, phenobarbital, phenytoin, primidone or benzothiadiazine derivatives at a dose higher than recommended in the British National Formulary (BNF) or in combination with a calcium supplement.

Successfully recruited and randomised patients were given 6 x 2-monthly doses of 6ml of active IMP (containing 3mg of Vitamin D_3, equating to 120,000 IU) or placebo over the period of one year.

2.1.3 ViDiCO trial: participant characteristics

Patients were deemed eligible if they were over 40 years old, had a medical diagnosis of COPD, emphysema or bronchitis, had a post-bronchodilator ratio of forced expiratory volume in one second to forced vital capacity (FEV_1/FVC) < 70%, or post-bronchodilator FEV_1/slow VC < 70%, and had a smoking history ≥ 15 pack years. In addition to this, patients agreed to the required visits, were contactable by telephone, were able to give written informed consent to participate and, if a woman of child-bearing potential, agreed to use a reliable form of contraception for the duration of the study.

Patients were excluded if they had a current diagnosis of asthma, known clinically significant bronchiectasis, sarcoidosis, hyperparathyroidism, nephrolithiasis, active tuberculosis, vitamin D intolerance, liver failure, renal failure or any malignancy not in remission for at least 3 years. Taking dietary supplements containing >10µg per day of vitamin D, a baseline corrected serum calcium

>2.65mmol/L, baseline serum creatinine > 125µmol/L and the requirement of long-term oxygen therapy for ≥ 12 hours per day were additional exclusion criteria. Finally, participants were excluded if they were breastfeeding, pregnant or planning a pregnancy, undergoing treatment with any other IMP or device up to 4 months before randomisation onto this trial, or taking a cardiac glycoside (e.g. Digoxin), carbamazepine, phenobarbital, phenytoin, primidone or benzothiadiazine derivatives at a dose higher than recommended in the British National Formulary (BNF) or in combination with a calcium supplement.

Successfully recruited and randomised patients were given 6 x 2-monthly doses of 6ml of active IMP (containing 3mg of Vitamin D_3, equating to 120,000 IU) or placebo over the period of one year.

2.1.4 ViDiFlu trial: participant characteristics

Participants were deemed eligible if they were a permanent resident, attendee, staff member or carer at a participating unit, and were aged 16 or over. In addition to this, patients agreed to the required visits, were able to give written informed consent to participate and, if a woman of child-bearing potential, agreed to use a reliable form of contraception for the duration of the study.

Patients were excluded if they had a current diagnosis of asthma or COPD, chronic respiratory infection or chronic cough, any condition requiring treatment with a vitamin D dose of > 10µg per day, known sarcoidosis, hyperparathyroidism, nephrolithiasis, active tuberculosis, vitamin D intolerance, liver failure, renal failure or any malignancy not in remission other than cutaneous basal/squamous cell carcinoma. Taking dietary supplements containing >10µg per day of vitamin D, a baseline corrected serum calcium >2.65mmol/L, baseline serum creatinine > 125µmol/L and inability to complete the symptom diary were additional exclusion criteria. Finally, participants were excluded if they were breastfeeding, pregnant or planning a pregnancy, undergoing treatment with any other IMP or device up to 4 months before randomisation onto this trial, or taking a cardiac glycoside (e.g. Digoxin), carbamazepine, phenobarbital, phenytoin, primidone, long-term immunosuppressant therapy, or benzothiadiazine derivatives at a dose higher than recommended in the British National Formulary (BNF) or in combination with a calcium supplement.

Successfully recruited and randomised participants had various dosing regimens depending on their status as resident or staff/carer (Table 2.2), as it was deemed unethical to withhold vitamin D

supplementation in individuals aged ≥ 65 with limited sunlight exposure. As such, staff and carers were randomised to receive 6 x 2-monthly doses of 6ml of active IMP (containing 3mg of Vitamin D_3, denoted PR1) or placebo over the period of one year (denoted PL1), while residents were randomised to receive low-dose vitamin D supplementation (a daily dose of 20µl of active IMP for 1 year, denoted PR3, as well as 6 x 2-monthly doses of 4.8ml of placebo, denoted PL2) or high-dose vitamin D supplementation (a daily dose of 20µl of active IMP for 1 year, denoted PR3, as well as 6 x 2-monthly doses of 4.8ml active IMP containing 2.4mg of Vitamin D_3, denoted PR2), thus allowing a comparison between high-dose vitamin D_3 and placebo in staff and carers, and between high-dose vitamin D_3 and low-dose vitamin D_3 in residents and attendees.

Table 2.2: ViDiFlu trial dosing regimens

Participant, arm	IMP regimen received	Vitamin D_3 received	Total vitamin D_3 dose in 1 year	Equivalent daily dose of vitamin D_3
Resident and attendee,control arm	PR3 + PL2	10 micrograms daily	3.65 milligrams	10 micrograms
Resident and attendee, intervention arm	PR2 + PR3	10 micrograms daily + 6 bolus doses of 2.4 milligrams	18.05 milligrams	50 micrograms
Staff member and carer, control arm	PL1	Nil	Nil	Nil
Staff member and carer, intervention arm	PR1	6 bolus doses of 3 milligrams	18 milligrams	50 micrograms

** PR1, PR2 and PR3 are Vigantol ® regimens*

*** PL1 and PL2 are Miglyol ® regimens*

2.1.5 Primary and Secondary Endpoints

The co-primary endpoints for ViDiAs and ViDiCO were time from randomisation to first upper respiratory tract infection (URTI), and time from randomisation to first severe asthma exacerbation or first moderate/severe COPD exacerbation, respectively. A severe asthma exacerbation was defined as a deterioration in asthma resulting in the need for treatment with oral corticosteroids, hospital admission, emergency treatment or a decrease in the morning peak expiratory flow rate (PEFR) to more than 25% below the baseline value on 2 or more consecutive days. COPD exacerbations were defined as the occurrence of ≥ 2 major symptoms or 1 major symptom and ≥ 1 minor symptom for at least 2 consecutive days, with major symptoms comprising an increase in

dyspnoea, sputum volume or sputum purulence, and minor symptoms consisting of an increase in nasal congestion or discharge, wheeze, sore throat or cough. Any exacerbation fulfilling these requirements plus involving treatment with systemic steroids or antibiotics was classified as moderate, while severe exacerbations were defined as those which fulfilled the requirements above, involved treatment with systemic steroids or antibiotics, and additionally required hospital admission or a visit to a hospital emergency department. URTIs were defined as influenza-like illness (presence of cough, feeling of fever/chilliness, and muscle pain) [453], or using Jackson's criteria for a cold [454]. Participants were asked to score each of the 8 Jackson symptoms (sneezing, sore throat, headache, subjective sensation of fever or chilliness, malaise, nasal discharge, nasal obstruction, cough) and one additional symptom (muscle pain) from 0 (no symptoms) to 3 (symptoms severe enough to interfere with activity or sleep) in a provided diary on a daily basis. A cold was recorded when the total Jackson score was ≥ 14 with a subjective impression of having a cold, the total Jackson score was ≥ 14 with increased nasal discharge for at least 3 days, or the total Jackson score was < 14 with a subjective impression of having a cold plus an increase in nasal discharge for ≥ 3 days.

For ViDiFlu, the primary outcome measure was time from randomisation to first respiratory tract infection (upper or lower). Respiratory tract infections were defined as an episode of illness meeting the stated criteria of either URTI, lower respiratory tract infection (LRTI), or both. URTIs were defined as described above, while LRTIs were defined using the Macfarlane criteria [450]. When completing their daily diary entries, participants were asked whether or not they had a cough, cold or flu symptoms, with those answering 'yes' asked to score the 5 Macfarlane symptoms (cough, sputum production, dyspnoea, wheeze, chest discomfort/pain) from 0 (no symptoms) to 3 (symptoms severe enough to interfere with activity or sleep). LRTIs were defined as the presence of a cough scoring at least one point over that recorded during the run-on period, plus ≥ 1 other lower respiratory tract symptom scoring at least one point over that recorded during the run-in period.

Secondary endpoints encompassed measures of respiratory morbidity, health service use, adverse events, medication use, physiological outcomes, microbiological outcomes, immunological outcomes, biochemical outcomes, genetic parameters, quality of life, and economic outcomes. The focus of this thesis is on the immunological outcomes, with methodology described in greater detail below. However, since some of the other trial outcomes are presented in later results chapters in order to offer a comparison to the immunological findings, a summary of the secondary endpoints measured for each of the three trials is provided in Table 2.3. The majority of these outcomes were determined from the diaries which participants were asked to complete on a daily basis, indicating

respiratory symptom scores and PEFR (peak expiratory flow rate) readings (ViDiAs only). Participants were also asked to record any adverse events, medication use and costs associated with this, time off work, and any hospital or GP visits. A lifestyle questionnaire was completed by each participant during their screening visit, with asthma control test (ACT, ViDiAs only), Mini-rhinoconjunctivitis quality of life questionnaire (Mini-RQLQ, ViDiAs only), St. George's Respiratory Questionnaire (SGRQ, ViDiAs and ViDiCO) and EQ-5D (all 3 trials) questionnaires completed at screen, and 2, 6 and 12 months post-randomisation. For ViDiAs participants, exhaled nitric oxide was measured using a handheld Niox Mino device to assess airway inflammation, and FEV_1 (forced expiratory volume) was measured using a handheld spirometer In addition to FEV_1, spirometry was also used in ViDiCO participants to record FVC (forced vital capacity) at screening visits, and 2, 6 and 12 months post-randomisation. Blood samples were also collected at screening visits and 2, 6 and 12 months post-randomisation to allow quantification of concentrations of vitamin D metabolites and measures of calcium homeostasis. Finally, nose and throat swabs were taken from a subset of participants from each trial to allow polymerase chain reaction (PCR) detection of respiratory pathogens to act as validation for the definitions of respiratory tract infections based on symptom scores alone.

Table 2.3: Summary of the secondary endpoints for all three clinical trials

Secondary Endpoints		Trials outcomes were measured in
Respiratory Morbidity	Rate of severe exacerbation per participant-year	ViDiAs; ViDiCO
	Rate of URTI per participant-year	ViDiAs; ViDiCO
	Rate of URTI/LRTI per participant-year	ViDiFlu
	Proportion of participants experiencing ≥ 1 severe exacerbation during the trial	ViDiAs; ViDiCO
	Proportion of participants experiencing ≥ 1 URTI during the trial	ViDiAs; ViDiCO
	Proportion of participants experiencing ≥ 1 URTI/LRTI during the trial	ViDiFlu
	Proportion of severe exacerbations associated with URTI	ViDiAs; ViDiCO
	Proportion of exacerbations associated with increased sputum purulence	ViDiCO
	Proportion of exacerbations requiring antibiotics or corticosteroids	ViDiCO
	Duration of URTIs/exacerbations	ViDiAs; ViDiCO
	Median recovery time for episodes of URTI/LRTI	ViDiFlu
	Proportion of nights with awakenings due to asthma	ViDiAs
	Asthma control test score	ViDiAs
	Asthma symptom score	ViDiAs
Health Service Use	Unscheduled GP consultation with exacerbation or URTI	ViDiAs; ViDiCO
	Unscheduled health service use for respiratory tract infection	ViDiFlu
	A&E attendance with exacerbation or URTI	ViDiAs; ViDiCO
	A&E attendance for respiratory tract infection	ViDiFlu
	Unscheduled hospital admission with exacerbation or URTI	ViDiAs; ViDiCO
	Unscheduled hospital admission for respiratory tract infection	ViDiFlu
	Duration of hospital admissions for exacerbation or URTI	ViDiAs; ViDiCO
	Duration of hospital admissions for respiratory tract infection	ViDiFlu
Adverse Events	Proportion of participants reporting severe adverse events	ViDiAs; ViDiCO; ViDiFlu
	Proportion of participants withdrawing from the trial	ViDiAs; ViDiCO; ViDiFlu
	Mortality due to exacerbation or respiratory infection	ViDiAs; ViDiCO; ViDiFlu
	All-cause mortality	ViDiAs; ViDiCO; ViDiFlu
Medication Use	Use of oral corticosteroids	ViDiAs; ViDiCO
	Use of antibiotics	ViDiAs; ViDiCO; ViDiFlu
	Use of over-the-counter medication for URTI symptoms	ViDiAs; ViDiCO; ViDiFlu
	Use of inhaled relief medication or inhaled corticosteroids	ViDiAs; ViDiCO
Physiological Outcomes	FEV_1 (% of predicted value)	ViDiAs; ViDiCO
	FVC (% of predicted value)	ViDiCO
	Morning PEFR	ViDiAs
	Exhaled nitric oxide concentration	ViDiAs
Microbiological Outcomes	Proportion of nasal/throat swabs where respiratory pathogens were detected	ViDiAs; ViDiCO; ViDiFlu
	Determination of respiratory pathogens in induced sputum	ViDiAs; ViDiCO
Biochemical Outcomes	Concentrations of vitamin D metabolites in serum and plasma	ViDiAs; ViDiCO; ViDiFlu
	Concentrations of measures of calcium homeostasis in serum and plasma	ViDiAs; ViDiCO; ViDiFlu
Genetic Parameters	Polymorphisms in genes influencing vitamin D transport, metabolism and signalling	ViDiAs; ViDiCO; ViDiFlu
Quality of Life	SGRQ	ViDiAs; ViDiCO
	mini-RQLQ	ViDiAs
	EQ-5D	ViDiAs; ViDiCO; ViDiFlu
Economic Outcomes	Costs incurred by participants due to exacerbation or URTI	ViDiAs; ViDiCO
	Costs incurred by participants due to respiratory infection	ViDiFlu
	Costs of medical care provided for exacerbation or URTI	ViDiAs; ViDiCO
	Costs of medical care provided for participants	ViDiFlu
	Days of absence from work due to exacerbation or URTI	ViDiAs; ViDiCO
	Days of absence from work due to respiratory infection	ViDiFlu

2.1.6 Sputum induction

A subset of participants in the ViDiAs (n = 50) and ViDiCO (n = 50) trials were invited to give induced sputum samples in addition to other study samples at visits 2 (before IMP administration), 3 (2 months post randomisation) and 5 (12 months post randomisation). Further to the normal inclusion and exclusion criteria, to be eligible for sputum induction ViDiAs participants must have had treatment at BTS step 2 or 3, FEV_1> 50% of predicted value and no tobacco product use within the previous 6 months, while ViDiCO participants must have had a post-bronchodilator FEV_1 ≥ 40% of predicted value and have been an ex-smoker for ≥ 6 months.

Sputum inductions were carried out and samples collected according to standard protocols as described elsewhere [455]. After ensuring that the participant had not experienced an exacerbation or URTI within the last 28 days, pre- and post-bronchodilator FEV_1 values were obtained, with a post-bronchodilator FEV_1 of >1 litre necessary to continue. From these values safety thresholds were calculated, with post-bronchodilator FEV_1 required to be ≥50% predicted for ViDiAs, and ≥40% predicted for ViDiCO. Induction with 4.5% nebulised saline at a rate of 1ml/minute was then carried out for 5 minutes, with expectoration following this. Nebulisation was carried out a maximum of 3 times to obtain an adequate sample, with FEV_1 determined following each nebulisation to ensure that there had not been a ≥ 20% fall in FEV_1 from the baseline post-bronchodilator value. Collected sputum samples were kept on ice until processing.

2.1.7 Blood samples

Participants from all three trials had blood samples taken at screen, 2 months post randomisation and 12 months post randomisation by a member of the study team who was a trained phlebotomist. There was a limit to the volume of blood permitted to be taken from each participant at each study visit, with 6ml allocated for immunological tests. This 6ml of blood was collected into a sodium heparin vacutainer (BD, Franklin Lakes, USA) and kept at 4^oC until stimulation as part of a whole blood assay to investigate effects of vitamin D supplementation on the release of inflammatory mediators.

Blood was also collected into serum separating tubes (SST) and underwent centrifugation to yield serum samples. Serum concentrations of $25(OH)D_2$ and $25(OH)D_3$ were measured by Marion Rowe

(Department of Clinical Biochemistry, Homerton Hospital, London) by isotope-dilution liquid chromatography–tandem mass spectrometry [456], and summed to give values for total 25(OH)D concentration. Sensitivity for this assay was 10nmol/l. Coulter counts were carried out on whole blood samples to enumerate leukocyte populations by the Haematology Laboratory at the Royal London Hospital.

2.2 Stimulation of whole blood with TLR ligands and pathogens

Whole blood collected from study participants was stimulated with TLR ligands and pathogens. 180µl of blood was incubated with 20µl of stimulant or D-PBS in a humidified incubator at 37^{o}C and 5% CO_2 for 24 hours. Following this, plasma was aspirated and stored at -80^{o}C until further analysis by multiplex ELISA.

2.2.1 TLR ligand preparation

TLR ligands (InvivoGen, San Diego, USA) were dissolved according to product inserts to provide stock solutions, before being further diluted using Dulbecco's phosphate buffered saline (D-PBS) (Sigma-Aldrich, St Louis, USA) to produce the required working concentration (Table 2.4). Sterile 96-well polystyrene microplates (Corning Incorporated, Corning, USA) were prepared by adding 20µl of D-PBS or TLR ligand to the appropriate wells, before being stored at -80^{o}C until use.

Table 2.4: TLR ligands used in this study

Antigen	Dissolve In	Stock Conc.	Working Conc.	Final Conc.
LPS	1ml sterile water	5mg/ml	1µg/ml	0.1µg/ml
Pam2CSK4	1ml endotoxin free water	100µg/ml	0.1µg/ml	0.01µg/ml
Pam3CSK4	1ml endotoxin free water	1mg/ml	10µg/ml	1µg/ml
CpG-ODN 2216	2ml endotoxin free water	100µg/ml	100µg/ml	10µg/ml
Poly I:C	2.5ml endotoxin free water	10mg/ml	1mg/ml	100µg/ml
R848	500µl endotoxin free water	1mg/ml	10µg/ml	1µg/ml

2.2.2 Pathogen preparation

Pathogens were kindly provided by Professor Johnston's lab at the National Heart and Lung Institute, Imperial College London.

For both RV-16 and RV-1B, viral stocks were generated following standard procedures [457]. HeLa Ohio cell monolayers (MRC Common Cold Unit, Salisbury, UK) were infected and, when cytopathic effect (CPE) was fully developed, freeze/thaw cycles were carried out to lyse the cells, before centrifugation to pellet the debris. The viruses were concentrated using filtration columns, before aliquots were frozen and placed in -80^{o}C storage. Titration of the frozen aliquots was performed by exposing confluent monolayers of HeLa cells to serial dilutions of the viral stock. Cells were cultured at 37^{o}C in 5% CO_2 with plates inspected daily for cytopathic effect before fixation and staining with 5% formaldehyde, 5% ethanol and 0.1% crystal violet in PBS. The endpoint titre, defined as the highest dilution at which CPE was observed in half or more of the wells, was read and expressed as the inverse log of this dilution, giving a final $TCID_{50}$/ml of 9.82 x 10^7 for RV-16, and 8.76 x 10^7 for RV-1B.

Respiratory syncytial virus (RSV) (strain A2) was grown on Hep2 cells and confluent cell monolayers were incubated for 4 hours at 37^{o}C, 5% CO_2. 2% FCS medium was added, and after 48 hours (approximately 70% cytopathic affect) the virus was harvested. Following a 5 minute sonication using an ice bath, samples were centrifuged to remove cell debris, and the viruses were snap frozen using liquid nitrogen, before being transferred to -80^{o}C storage. Titration was carried out on Hep2 cells by fixing with methanol and staining for RSV, before brown plaques were counted, giving a titre of 4 x 10^6 PFU/ml (plaque forming units).

S.pneumoniae (strain D39) was grown on Todd Hewitt+yeast broth, and once at an OD of 0.4 read at 600nm, was added to 10% glycerol and aliquoted. Titration was carried out on solid media (Columbia horse blood agar) by serial dilution, and the numbers of colonies were counted, giving a titre of 2.3 x 10^8 CFU/ml (colony forming units).

H.influenzae (Strain ATCC 49247) was grown in Brain Heart Infusion broth + haem supplement. Once at an OD of 0.4 read at 600nm, 10% glycerol was added and it was aliquoted. Titration was carried out on solid media using serial dilutions, and the numbers of colonies were counted, giving a titre of 8.13 x 10^8 CFU/ml.

Aliquots of the pathogens were thawed daily and added to the prepared 96-well microplates to avoid repeated freeze-thaw cycles. The viruses RV-16, RV-1B and RSV were added neat, with 20μl and 180μl of blood per well, resulting in a final concentration in the blood of 1/10. The bacteria *H.influenzae* and *S.pneumoniae* were diluted to 1/100 in D-PBS before 20μl was added to each well, resulting in a final concentration in the blood of 1/1000. Therefore, the final titres of each pathogen when diluted 1/10 in the blood were: RV-16 9.82 x 10^6 $TCID_{50}$/ml, RV-1B 8.76 x 10^6, RSV 4 x 10^5 PFU/ml, *S.pneumoniae* 2.3 x 10^5 CFU/ml and *H.influenzae* 8.13 x 10^5 CFU/ml.

2.3 Processing induced sputum samples

2.3.1 Cytospin preparation from induced sputum samples

Sputum samples were processed according to methods described elsewhere [455], and all procedures were carried out on ice or at 4^{o}C. Sputum plugs from induced sputum samples were selected using forceps and condensed into one mass of approximately 100mg – 500mg. A 1% stock solution of DL-Dithiothreitol (DTT) (Sigma-Aldrich) was initially prepared using dH_2O, with a subsequent dilution using D-PBS to produce a 0.1% working solution. A volume of 4ml of 0.1% DTT per 1g of selected sputum was added to the sample, and placed on a bench rocker for 15 minutes. Following this, an equal volume of D-PBS was added and the contents filtered through 48μm nylon gauze (SEFAR LTD, Bury, UK). After centrifugation at 790g for 10 minutes, the supernatant was aspirated and stored at -80^{o}C for subsequent analysis by multiplex ELISA, and the pellet resuspended in D-PBS to give a concentration of 0.5 x 10^6 cells/ml. The resulting solution was used to produce cytospin slides using a Shandon Cytospin Cytocentrifuge (Thermo Scientific, Waltham, USA), with 3 slides produced from every sample. Slides were stained using a Hemacolor Rapid Staining Kit (Merck, Whitehouse Station, USA) and a coverslip added, before a differential cell count was carried out.

2.3.2 Differential cell counts of induced sputum samples

Slides were studied under a microscope and the number of eosinophils, neutrophils, columnar epithelial cells, lymphocytes and macrophages were counted using a tally counter until the total reached 400 cells. Counts were done in this way for 3 cytospin slides for each sputum sample and

averages were taken. Percentages of each cell type were recorded, and absolute numbers were calculated using the total number of leukocytes in the sample as determined using a haemocytometer preceding centrifugation during sputum processing.

2.4 Flow cytometry

Flow cytometry was used to enumerate leukocyte populations in induced sputum and peripheral whole blood. Monoclonal mouse anti-human antibodies were acquired from BD (Franklin Lakes, USA) or Miltenyi Biotec (Cologne, Germany), as detailed in Table 2.5. Combinations of monoclonal mouse anti-human antibodies were used in equal volumes to create master mixes (Table 2.6), with mouse IgG1 isotype controls for FITC, PE, APC and Alexa Fluor 647 (BD, Franklin Lakes, USA) used to facilitate determination of DC and Treg populations.

Table 2.5 Monoclonal mouse anti-human antibodies used in this study

Antibody - Conjugate	**Target for this Study**	**Manufacturer (catalogue number)**
CD3 - FITC *	T-Cells	BD (345763)
CD3 - PerCP	T-Cells	BD (345766)
CD4 - APC	Helper T-Cells	BD (555349)
CD4 - PE	Helper T-Cells	BD (345769)
CD8 - PE	Cytotoxic T-Cells	BD (555367)
CD11c - PE	Myeloid DCs	BD (555392)
CD14 - FITC *	Monocytes	BD (345784)
CD16 - FITC *	Granulocytes	BD (555406)
CD19 - FITC *	B-Cells	BD (555412)
CD20 - FITC *	B-Cells	BD (555622)
CD25 - FITC	Regulatory T-Cells	BD (555431)
CD56 - FITC *	Natural Killer Cells	BD (345811)
CD127 - Alexa Fluor 647	Naive T-Cells	BD (558598)
BDCA-4 - APC	Plasmacytoid DCs	Miltenyi Biotec (130-090-900)
HLA-DR - PerCP	Dendritic Cells	BD (347402)
FITC isotype control	n/a	BD (555748)
Alexa Fluor 647 isotype control	n/a	BD (557714)
APC isotype control	n/a	BD (555751)
PE isotype control	n/a	BD (555749)

**Antibodies used in Lineage Cocktail*

Table 2.6 Antibody master mixes used for flow cytometry

Master Mix	**FITC**	**PE**	**PerCP**	**APC**	**Alexa 647**
Lymphocyte	CD19	CD8	CD3	CD4	n/a
Treg	CD25	CD4	CD3	n/a	CD127
DC	Lin Cocktail*	CD11c	HLA-DR	BDCA-4	n/a
Treg Isotype	Isotype	CD4	CD3	n/a	Isotype
DC Isotype	Lin Cocktail*	Isotype	HLA-DR	Isotype	n/a

** Lineage Cocktail as detailed in Table 2.5*

2.4.1 Flow cytometric analysis of peripheral whole blood

In addition to stimulation with pathogens and TLR ligands in the whole blood assay, a subset of whole blood samples underwent further analysis by flow cytometry.

3µl of the required antibody master mixes (Table 2.6) were incubated with 125µl of whole blood at 4^{o}C for 45 minutes in the dark. Following this, 2ml of 1x FACs lysing solution (BD, Franklin Lakes, USA), was added and the solution left to stand until reaching transparency, or for a maximum of 5 minutes, before centrifugation at 200g for 5 minutes. The supernatant was discarded and the pellet resuspended in 1ml of FACSFlow Sheath Fluid (BD, Franklin Lakes, USA), before being centrifuged again. This resuspension and centrifugation was repeated once more, before the pellet was finally resuspended in 500µl of FACSFlow. Samples were left on ice until analysis using a BD FACS Canto II machine and FACSDiva software.

2.4.2 Flow cytometric analysis of induced sputum

In addition to cytospin production and differential cell counts, a subset of sputum samples underwent further analysis by flow cytometry. Sputum was processed as above until the point of filtration. Upon determination of cell numbers, if adequate cells (> 2 million) were present in the sample the solution was split, resulting in one cell suspension for cytospin production (described above) and one cell suspension (containing > 1 million cells) for flow cytometric analysis.

The separated cell suspension was centrifuged at 790g for 10 minutes at 4^{o}C, before the pellet was re-suspended in 700µl of 4% FCS in FACSFlow Sheath Fluid. 3µl of the required antibody master mixes (Table 2.6) were incubated with 100ul of cell suspension at 4^{o}C for 30 minutes in the dark. Following this, 1ml of 4% FCS in FACSFlow was added to each tube, before undergoing centrifugation at 200g for 5 minutes and the supernatant was discarded. The pellet was resuspended in 1ml of 4% FCS in FACSFlow, centrifuged again, and the resulting pellet resuspended in 250µl of 1% PFA in FACSFlow. Samples were left on ice until analysis using a BD FACS Calibur machine and CellQuest Pro software.

2.4.3 Foxp3 staining

Forkhead box protein 3 (Foxp3) is a transcription factor specifically expressed in Tregs which is required in maintaining their regulatory function [458, 459]. A subset of sputum and blood samples underwent FoxP3 staining to validate the results generated using CD25 and CD127 as markers of Tregs using an Anti-Human Foxp3 APC Staining Set (eBioscience, San Diego, USA).

Two 9µl solutions containing equal volumes of the antibodies CD25-FITC, CD4-PE and CD3-PerCP (BD) were each incubated at 4^{o}C for 45 minutes in the dark with 200µl peripheral whole blood, or 100µl of an induced sputum solution containing at least 1 million cells. Blood samples underwent an additional step at this point of being mixed with 2ml of 1x FACs lysing solution (BD, Franklin Lakes, USA) until reaching transparency or for a maximum of 5 minutes, before centrifugation at 200g for 5 minutes. After discarding the supernatant, the pellet was resuspended in 1ml FACSFlow, while the sputum sample solution had 1ml of 4% FCS in FACS Flow added to it. Both blood and sputum samples were centrifuged at 200g for 5 minutes, the supernatant discarded and the pellet resuspended in 1ml FACSFlow or 4% FCS in FACSFlow respectively. This centrifugation was repeated and the pellets resuspended in 1ml of 1x Fixative solution, before being incubated in the dark at room temperature for 45 minutes. Following this, 1ml of 1x permeabilisation buffer was added and the samples centrifuged at 200g for 5 minutes. The supernatant was discarded, the pellet resuspended in 1ml of 1 x permeabilisation buffer and the centrifugation repeated, before 5µl of FoxP3 or Rat IgG Isotype was added to the appropriate tubes. The samples were incubated in the dark at room temperature for 45 minutes. Following this, 1ml of 1x permeabilisation buffer was added and the samples centrifuged at 200g for 5 minutes. The supernatant was discarded, the pellet resuspended in 500µl of FACSFlow and the centrifugation repeated. The resulting pellet was resuspended in 500µl of FACSFlow, and the samples were left on ice until analysis using a BD FACS Calibur machine and CellQuest Pro software (sputum) or BD FACS Canto II machine and FACSDiva software (blood).

2.4.4 Gating technique to determine lymphocyte populations

Lymphocytes were gated on based on their forward and side scatter properties (Figure 2.1). B-cells were distinguished as the $CD3^{-}CD19^{+}$ population, while T-cells were selected as being $CD3^{+}CD19^{-}$. The $CD3^{+}$ population was divided into $CD4^{+}$ and $CD8^{+}$ T-cells to enable enumeration of the two main phenotypes of helper and cytotoxic T-cells.

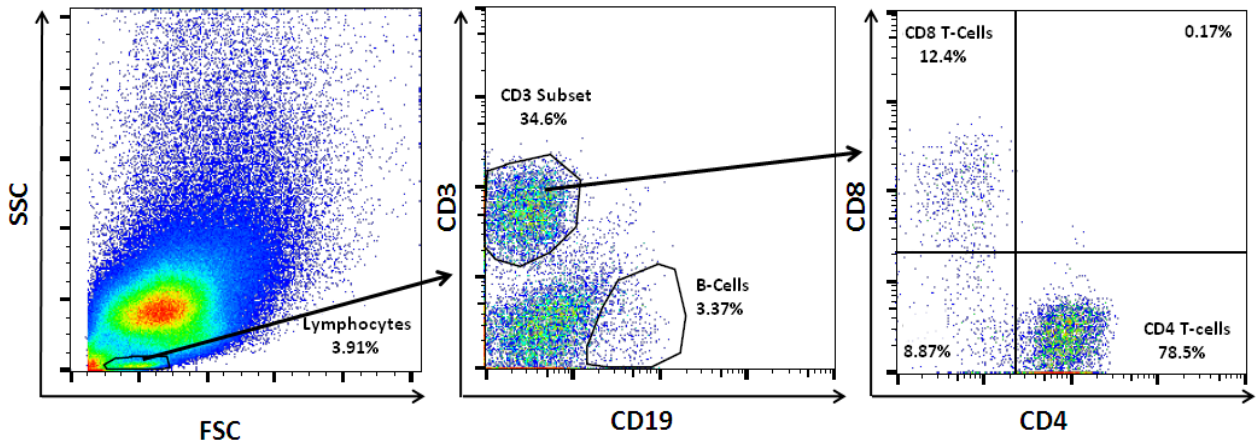


Figure 2.1: Gating technique for lymphocytes subtypes. Lymphocytes were gated on based on their forward and side scatter properties. $CD19^{+}CD3^{-}$ cells were characterised as B-cells, and $CD3^{+}$ subsets were selected and differentiated as $CD4^{+}$ or $CD8^{+}$ T-cells.

2.4.5 Gating technique to determine dendritic cell populations

Dendritic cells were selected by gating for lymphocytes based on their forward and side scatter properties. From this population, cells that were Lineage Cocktail negative ($CD3^{-}$, $CD14^{-}$, $CD16^{-}$, $CD19^{-}$, $CD20^{-}$, $CD56^{-}$) and HLA-DR^{+} were selected as being dendritic cells (Figure 2.2). From this population, myeloid DCs were selected as being $CD11c^{+}$BDCA-4^{-} and plasmacytoid DCs as being $CD11c^{-}$BDCA-4^{+}. Isotype controls were used to help determine what constituted the $CD11c^{+}$BDCA-4^{-} population.

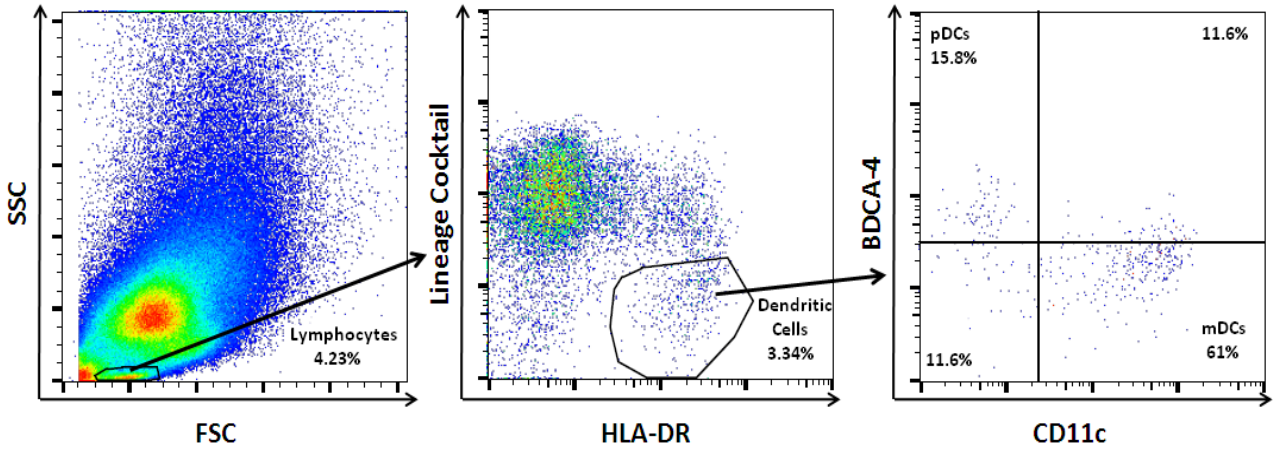


Figure 2.2: Gating technique for dendritic cells. Lymphocytes were gated on based on their forward and side scatter properties, and dendritic cells were selected as being HLA-DR$^+$ Lineage Cocktail$^-$. From this population, mDCs were characterised as being CD11c$^+$BDCA-4$^-$, and pDCs as being CD11c$^-$BDCA-4$^+$.

2.4.6 Gating technique to determine regulatory T-cell populations

To enumerate regulatory T-cells, lymphocytes were gated on based on their forward and side scatter properties. The CD3$^+$CD4$^+$ population was selected and used to determine CD25$^+$CD127$^-$ T-regs (Figure 2.3). Isotype controls were used to help determine what constituted the CD25$^+$CD127$^-$ population.

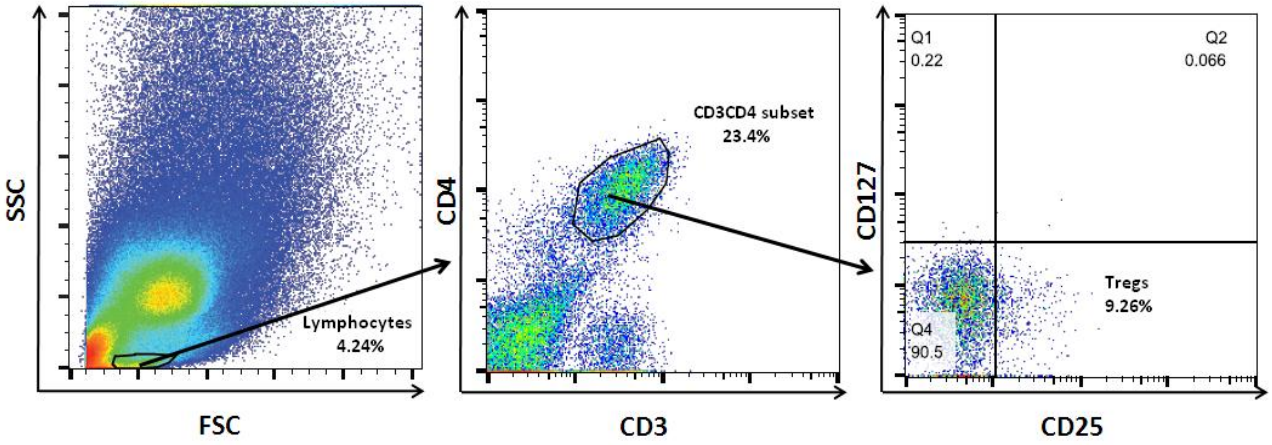


Figure 2.3: Gating technique for regulatory T-cells. Lymphocytes were gated on based on their forward and side scatter properties. CD3$^+$CD4$^+$ cells were selected and Tregs were characterised as being CD25$^+$CD127$^-$.

In a subset of samples FoxP3 staining was used to validate the results generated using CD25 and CD127 as markers of Tregs, using an Anti-Human Foxp3 APC Staining Set. Following gating of the lymphocyte population based on forward and side scatter properties and selection of the $CD3^+CD4^+$ population as before, regulatory T-cells were distinguished as being $CD4^+CD25^+FoxP3^+$ (Figure 2.4). Isotype controls were used to help determine what constituted the $CD25^+$ population.

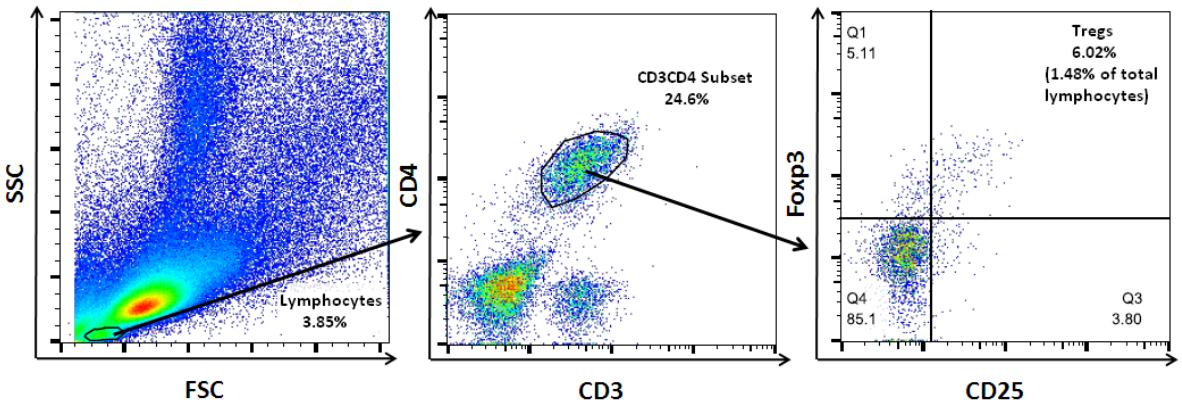


Figure 2.4: Gating technique for regulatory T-cells using the transcription factor FoxP3. Lymphocytes were gated on based on their forward and side scatter properties. $CD3^+CD4^+$ cells were selected and Tregs were characterised as being $CD25^+FoxP3^+$.

2.5 TNF-α and IFN-αA2 ELISA

During development of the whole blood assay, aspirated plasma samples from whole blood stimulated with TLR ligands or pathogens were analysed by ELISA to assay concentrations of TNF-α or IFN-αA2. Meso Scale Discovery ® (MSD) ultra-sensitive cytokine assay kits (Rockville, Maryland, USA) were used for all experiments involved in the development of the whole blood assay except the final bacterial dose response, for which a Quantikine ® TNF-α immunoassay (R&D Systems, Minneapolis, Minnesota, USA) was used.

2.5.1 Meso Scale Discovery ® (MSD) ultra-sensitive assay

Single-spot plates were used for both the IFN-αA2 and TNF-α assays, following the manufacturer's instructions. All reagents and samples were thawed and brought to room temperature. 25µl of

Diluent 2 was added to each well, before incubation on a plate rocker (300 – 1000rpm) at room temperature for 30 minutes. Calibrator solutions were prepared, with an initial 1:100 dilution of the stock solution using Diluent 2, before a serial 1:4 dilution. 25µl of sample or calibrator solutions were added to the appropriate wells, before incubation on a plate rocker (300 – 1000rpm) at room temperature for 2 hours. The plate was washed three times with PBS-T (phosphate buffered saline with 0.05% Tween), before addition of 25µl of 1X Detection Antibody Solution to each well. Following a 2 hour incubation on the plate rocker (300 – 1000rpm) at room temperature, the plate was washed 3 times with PBS-T, and 150µl of 2X Read Buffer was added to each well. Plates were analysed using a SECTOR Imager 6000 and MSD Discovery Workbench software.

2.5.2 Quantikine ® TNF-α immunoassay

A TNF-α ELISA was carried out following manufacturer's instructions. All samples and reagents were thawed and brought to room temperature, before 50µl Assay Diluent RD1F was added to all wells. Plasma samples were diluted 1:5 using Calibrator Diluent RD6-35, and standards were prepared using an initial 1:10 dilution and a subsequent 1:2 serial dilution. 200µl of standards or samples were added to the appropriate wells, before incubation for 2 hours at room temperature. The plate was washed 4 times using Wash Buffer, before addition of 200µl TNF-α Conjugate to each well. Plates were incubated for 2 hours at room temperature, before being washed 4 times using Wash Buffer. 200µl Substrate Solution was added to each well, and following a 20 minute incubation at room temperature in the dark, 50µl Stop Solution was added to each well. Plates were read immediately on a Bio-Rad 680 microplate reader (Bio-Rad, Hercules, California, USA) at 450nm and 570nm for wavelength correction.

2.6 Multiplex ELISA

Induced sputum supernatants acquired during cytospin preparation, aspirated plasma from whole blood stimulation with TLR ligands and pathogens, and supernatants from *in vitro* cell culture underwent multiplex ELISA analysis to enable quantification of the concentration of a panel of 30 inflammatory mediators (IL-1β, IL-2, IL-4, IL-5, Il-6, IL-7, IL-8 (CXCL8), IL-10, IL-12, IL-13, IL-15, IL-17, IL-1RA, IL-2R, IFN-α, IFN-γ, TNF-α, MCP-1 (CCL2), MIP-1α (CCL3), MIP-1β (CCL4), RANTES (CCL5),

eotaxin (CCL11), MIG (CXCL9), IP-10 (CXCL10), EGF, FGF-basic, HGF, VEGF, G-CSF, GM-CSF). The Invitrogen Human Cytokine Magnetic 30-plex Panel was used (Invitrogen, Camarillo, CA, USA), following manufacturer's instructions. 25µl of the antibody bead solution was added to each well of a 96-well plate, before washing two times with 200µl wash solution and the addition of 50µl of incubation buffer. Standards were prepared using a serial 1:2 dilution, with 100µl standard or 50µl assay diluents + 50µl sample added to the appropriate wells. Following a 2 hour incubation at room temperature on an orbital shaker, the plate was washed twice with 200µl wash solution, and 100µl of 1x biotinylated detector antibody was added to each well. After a 1 hour incubation at room temperature on an orbital shaker, the plate was washed twice with 200µl wash solution, 100µl of 1x streptavidin-RPE was added to each well, and the plate was incubated for a further 30 minutes. Three washes with 200µl wash solution were carried out, before 125µl wash solution was added to each well to re-suspend the beads. Plates were analysed using the Magpix ® platform (powered by Luminex xmap Technology) and the Luminex xponent ® software.

2.7 Genetic analysis of SNPs associated with vitamin D status or disease risk

All genetic analyses were performed by David Jolliffe (Blizard Institute, Queen Mary University of London). A systemic literature review was carried out which identified 54 single nucleotide polymorphisms (SNPs) in 11 genes in the vitamin D pathway, and one SNP in the class I MHC-restricted T-cell-associated molecule (CRTAM) gene which has been reported to modify the influence of vitamin D status on asthma exacerbation risk [460], which were typed using Taqman allelic discrimination assays. For those SNPs in high linkage disequilibrium ($r^2 \geq 0.8$) according to the HapMap database (release #27), six tag SNPs were selected as proxies using a previously-developed algorithm [461] and were typed (Table 2.7). DNA was extracted from peripheral whole blood using the salting-out method [462] on the Miomek FX robot (Beckam Coulter), quantified using the Nanodrop spectrophotometer and normalized to 5ng/µl. 10ng DNA was used as a template for 2µl Taqman assays (Applied Biosystems, Foster City, California, USA) performed on the ABI 7900HT platform in 384-well format before analysis with Autocaller software. Pre-developed assays were used to type all SNPs except three, which had customized assays:
rs2740574 in CYP3A4 (forward primer sequence CCAGGCATAGGTAAAGATCTGTAGGT, reverse primer sequence CTCAAGTGGAGCCATTGGCATA, reporter sequences ACAAGGGCAAGAGAG and ACAAGGGCAGGAGAG); rs3740165 in CUBN (forward primer sequence

GCAATGAGATTAAATCTTCAGGAAACACA, reverse primer sequence CTGGAGGTATAGGAAGCAGTGAAG, reporter sequences CCGCCATATGGCCTG and CGCCATACGGCCTG); rs7861779 in RXRA (forward primer sequence TGGCCCATGCACGAGTAG, reverse primer sequence ACCGAGACAGGCCAAACTC, reporter sequences CAGCAGAGGTGGCCGA and CAGCAGAGATGGCCGA). Alleles at all loci conformed to the Hardy-Weinberg equilibrium. Typing for two SNP (rs6127118 and rs11574010) failed.

Table 2.7: Single nucleotide polymorphisms (SNPs) identified as putative modifiers of the effects of vitamin D supplementation

Gene	Chromosomal Region	Target SNP	Functional Consequence	Tag SNP [1]	r^2
CYP24A1	20q13	rs2762934	utr variant 3 prime	-	-
		rs6127118	intron variant	-	-
		rs2248137	intron variant	-	-
		rs2762939	intron variant	-	-
CYP27B1	20q13.1-q13.3	rs4646536	intron variant	-	-
		rs10877012	downstream variant 500B,upstream variant 2KB	rs4646536	1.00
		rs703842	upstream variant 2KB,utr variant 3 prime	rs4646536	1.00
		rs4646537	intron variant	-	-
CYP2R1	11p15.2	rs2060793	upstream variant 2KB	-	-
		rs10741657	upstream variant 2KB	rs2060793	1.00
		rs1993116	intron variant,upstream variant 2KB	rs2060793	1.00
		rs7116978	intron variant	rs2060793	0.92
		rs10500804	intron variant,upstream variant 2KB	-	-
		rs12794714	nc transcript variant,synonymous codon,upstream variant 2KB	rs10500804	1.00
		rs10766197	?	-	-
CYP3A4	7q21.3-q22.1	rs2740574	upstream variant 2KB	-	-
CYP27A1	2q35	rs17470271	intron variant	-	-
VDR	12q13.11	rs1544410	intron variant	-	-
		rs731236	synonymous codon	-	-
		rs4516035	upstream variant 2KB	-	-
		rs4334089	intron variant	-	-
		rs10783219	intron variant	-	-
		rs7976091	?	-	-
		rs11574010	upstream variant 2KB	-	-
		rs2853559	intron variant	-	-
		rs2238136	intron variant	-	-
		rs7975232	intron variant	-	-
		rs2228570	missense	-	-
		rs7970314	?	-	-
		rs11568820	?	-	-
DBP	4q12-q13	rs4588	intron variant,missense	-	-
		rs2282679	intron variant	rs4588	1.00
		rs3755967	intron variant	rs4588	1.00
		rs17467825	?	rs4588	1.00
		rs1155563	intron variant	rs4588	0.83
		rs2298850	intron variant	rs4588	0.95
		rs7041	intron variant,missense	-	-
		rs222035	intron variant	rs7041	0.92
		rs842999	intron variant	rs7041	0.96
		rs2298849	intron variant	-	-
		rs16846876	?	-	-
		rs12512631	?	-	-
		rs2070741	intron variant	-	-
DHCR7	11q13.4	rs12785878	intron variant	-	-
		rs4944957	intron variant	rs12785878	1
		rs4945008	?	rs12785878	0.95
		rs3794060	intron variant	rs12785878	1
		rs7944926	intron variant	rs12785878	1
		rs12800438	intron variant	rs12785878	1
		rs3829251	intron variant	-	-
CUBN	10p13	rs3740165	synonymous codon	-	-
RXRA	9q34	rs9409929	?	-	-
		rs7861779	intron variant	-	-
LRP2	2q31.1	rs3755166	upstream variant 2KB	-	-
CRTAM	11q22	rs2272094	missense	-	-

[1] Six tag SNP were selected as proxies for target SNP in high linkage disequilibrium ($r^2 \geq 0.8$) in the HapMap database (release #27) and typed

Abbreviations used: CYP: cytochrome p450 family of enzymes; VDR: vitamin D receptor; DBP: vitamin D binding protein; DHCR7: 7-dehydrocholesterol reductase; CUBN: intrinsic factor-cobalamin receptor / cubilin; RXRA: retinoid x receptor alpha; LRP2: low density lipoprotein receptor-related protein 2 / megalin; CRTAM: class I MHC-restricted T-cell-associated molecule; utr: untranslated region

2.8 A549 cell culture and stimulation

A549 cells (obtained from Sigma-Aldrich) were cultured in complete Dulbecco's Modified Eagle Medium (DMEM), containing 10% FCS, 1% penicillin/streptomycin and 1% L-glutamine (all obtained from Lonza, Basel, Switzerland). Upon reaching confluence, cells were trypsinized and resuspended in complete DMEM giving a concentration of approximately 200,000 cells/ml. 1ml was added to each well of a 24-well plate, and cells were incubated for 24 hours at 37^{o}C with 5% CO_2. Subsequently, supernatants were aspirated and cells were incubated for 48 hours with 25(OH)D or 1,25$(OH)_2$D at final concentrations of 10^{-7}M, or vehicle (0.1% ethanol). Following this, supernatants were aspirated, and 200µl of incomplete media, RV-16 (MOI 1), filtered virus, or UV-inactivated virus were added. After incubation at room temperature on an orbital shaker for 1 hour, supernatants were aspirated, and replaced with 200µl of incomplete DMEM, before a further 5 or 23 hour incubation at 37^{o}C with 5% CO_2. Supernatants were aspirated and either used immediately in the toxicology assay, or stored at -80^{o}C for subsequent analysis, and 350µl Buffer RLT (Qiagen, Valencia, USA) was used for cell lysis, with the lysates stored at -80^{o}C for subsequent RNA extraction.
A549 cells used in these experiments were passaged no more than 20 times.

2.8.1 Vitamin D metabolite preparation

1α,25-Dihydroxyvitamin D3 and 25-Hydroxycholecalciferol (Sigma-Aldrich) were dissolved in anhydrous ethanol to stock concentrations of 10^{-4}M. They were stored at -80^{o}C under a layer of argon to prevent oxidization. The final concentrations of 10^{-7}M were obtained following dilution in incomplete DMEM containing 2% FCS.

2.8.2 Stimulant preparation

RV-16 stocks were kindly provided by Professor Johnston's lab at the National Heart and Lung Institute, Imperial College London, and prepared as described previously. Viral controls were obtained by filtering the virus using Amicon Ultra centrifugal filters with a 30kDa molecular weight cut-off pore size (Sigma-Aldrich), or by UV-inactivation for 30 minutes at 120,000µJ/cm^2 using a UV crosslinker.

2.8.3 Toxicology assay

Cytotoxicity was measured using a lactate dehydrogenase (LDH)-based *in vitro* toxicology assay kit (Sigma-Aldrich). 50µl supernatants were aspirated following cell culture of A549 cells (as described above) and a 100µl mixture of equal volumes of LDH assay substrate solution, LDH assay dye solution and 1 x LDH assay cofactor preparation was added. Following incubation in the dark at room temperature for 20 minutes, the reaction was terminated by the addition of 20µl 1N hydrochloric acid. Absorbance was measured spectrophotometrically at a background wavelength of 650nm, which was subtracted from the measurements obtained at a wavelength of 450nm.

2.8.4 Cytopathic effect (CPE) assay

A549 cells were co-cultured with vitamin D metabolites or vehicle before infection for 6 or 24 hours with RV-16, and supernatants were aspirated and stored at -80^{o}C, as described above. Supernatants were thawed and serial 1 in 10 dilutions were prepared using complete DMEM. Five dilutions were prepared for each sample in total, (including a neat sample), and 50µl of each were plated in quadruplicate in a 96-well plate. A 150µl volume of HeLa Ohio cells (1 x 10^5 cells/ml) was added to each well, before incubation at 37^{o}C with 5% CO_2 for 4 days. Cytopathic effect was observed by light microscopy, and TCID/50 values were calculated using the Spearman-Kaerber method [463, 464].

2.9 RNA extraction and RT-PCR

RNA extraction was carried out using an RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. RNA was immediately reverse transcribed using SuperScript VILO Mastermix (Invitrogen, Life Technologies), with each reaction volume containing 9µl of RNA following the manufacturer's instructions, and stored at -80^{o}C.

In a 20µl reaction volume, 1µl of cDNA was used for quantitative RT-PCR, using TaqMan gene expression master mix (Applied Biosystems, Life Technologies) and pre-developed TaqMan gene expression assays for ICAM-1, CYP27B1, CYP24A1, IκBα, IFN-β, IFN-α2, HBD2, CAMP and PafR (Table 2.8). Gene expression assays had a FAM reporter dye at the 5′ end of the TaqMan MGB probe and a non-fluorescent quencher at the 3′ end of the probe. Each assay was a 20x mix, with primers

present at a concentration of 18µM and probes at a concentration of 5µM. A gene expression assay for viral mRNA was custom-made (Table 2.9), with final concentrations of 50nM forward primer, 300nM reverse primer and 100nM probe used as described elsewhere [465]. All samples were run in triplicate. A 7500 Real Time PCR System (Applied Biosystems) and 7500 software v2.0.6 were used with thermal cycling conditions set according to the manufacturer's instructions. Each reaction was normalised to the GAPDH content, and the ΔΔCT method was used to give the fold induction over unstimulated samples.

Table 2.8 Assay ID for TaqMan Gene Expression Assays Used

Gene	**Assay ID (Life Technologies)**
GAPDH	Hs02758991_g1
ICAM-1	Hs00164932_m1
CYP27B1	Hs01096154_m1
CYP24A1	Hs00167999_m1
IκBα	Hs00355671_g1
IFN-β	Hs01077958_s1
IFN-α2	Hs00265051_s1
HBD2	Hs00823638_m1
CAMP	Hs00189038_m1
PafR	Hs00265399_s1

Table 2.9 RV-16 Primer and Probe Sequences (5' – 3')

Gene	**Forward Primer**	**Reverse Primer**	**Probe**
RV-16	GTGAAGAGCCSCRTGTGCT	GCTSCAGGGTTAAGGTTAGCC	TGAGTCCTCCGGCCCCTGAATG

2.10 Statistical analysis

All data were checked visually for normal distribution. Analyses of *in vitro* A549 work, vitamin D status, and patient characteristics were carried out using GraphPad Prism version 6.04 (GraphPad Software Inc, La Jolla, USA), and normally distributed data are presented as means + SEM, with Student's T-tests or one-way ANOVA used to determine any statistically significant differences between data-sets, while non-normally distributed data are presented as median + IQR, with Mann-Whitney or Kruskal-Wallis tests used to determine any statistically significant differences between data-sets. Contingency tables were also used to compare participant characteristics and were analysed using a chi-squared test. Significance was inferred for $p < 0.05$ throughout. Method validation was carried out using Pearson's R Correlation Test and Bland-Altman analysis, also using GraphPad Prism.

Clinical samples were analysed using the statistical programme Qlucore Omics Explorer 2.3 (Lund, Sweden). Analyte concentrations were automatically generated from the MFI values recorded following Magpix analysis, and values lower than the sensitivity thresholds stated on the technical data sheets were assigned a value of 0 pg/ml. For the whole blood assay results, the unstimulated control was subtracted from each stimulus. A small constant for each analyte was added where necessary, sufficient to ensure all values were positive. Log2 transformation was carried out on all data to achieve normal distribution and minimise variance, and K nearest neighbour (KNN) imputation was used for missing values. For prospective analyses, interaction variables were generated for each data point for time-point and allocation (i.e. p1 for a baseline placebo sample, p2 for a 12 month post-randomisation placebo sample, d1 for a baseline intervention sample, d2 for a 12 month post-randomisation intervention sample). A multi-group comparison was carried out on the log transformed data using Qlucore Omics Explorer on these interaction variables, which equates to a repeated measures ANOVA test, with this model including adjustment for time-point, allocation and study. An important assumption of repeated measures ANOVA is sphericity, whereby the variance of the differences between all combinations of groups is equal. A violation in sphericity, where the variances are not equal, can increase type I error. Data were log transformed to minimise variance, and Qlucore also automatically measures the extent to which sphericity is violated, with p-values calculated taking this violation into account using the Greenhouse-Geisser method [466].

Cross-sectional analysis of baseline data was also carried out on log2 transformed data using Qlucore Omics Explorer. Analysis was modelled using a two-group comparison between each study (i.e. asthma, COPD, or people with neither condition living or working in sheltered accommodation),

which is the equivalent to Student's T-test. The model included adjustment for age, sex, baseline 25(OH)D concentration, ethnicity, smoking status, vaccination record and inhaled corticosteroid use.

For both prospective and cross-sectional analysis, data are presented as raw median values, i.e. before log transformation, with IQRs only stated when a statistical significant difference was evident. The Benjamini-Hochberg procedure for multiple testing correction was applied to analysis to give a measure of the false discovery rate at 10% [467]. Significance was inferred for $p < 0.05$ throughout when associated with false discovery rate (q) < 0.1.

3. Development of a whole blood assay to investigate effects of vitamin D on the immune response to TLR ligands and respiratory pathogens

3.1 Introduction

As detailed in Chapter 1.10, vitamin D deficiency has previously been associated with an increased risk of acute respiratory tract infections, with supplementation demonstrating a protective effect in some intervention studies [468]. A variety of immunological actions have also been described from *in vitro* models, although the effects of vitamin D on cytokine and chemokine production have not been definitively characterised. Since the focus of this study is the immunomodulatory actions of vitamin D in the protection against respiratory tract infections, an assay was developed to assess the effect of *in vivo* vitamin D supplementation on the release of inflammatory mediators in peripheral blood following *ex vivo* stimulation with TLR ligands and respiratory pathogens.

For these preliminary experiments, aimed around developing the assay for further use, TLR ligand concentrations were selected based on the InvivoGen product inserts and the literature available [469-474], and pathogens were diluted down from the prepared stock solutions provided by the Johnston laboratory (as described in Chapter 2.2.2). The TLR ligands used are listed in Table 3.1. The only cytokines measured were TNF-α in response to bacterial stimuli, and IFN-αA2 in response to viral stimuli. TNF-α is a pleiotropic pro-inflammatory cytokine and pyrogen, which is vital in the innate immune response, especially to bacteria. Its functions include stimulation of acute phase protein synthesis, increasing vascular permeability to enhance leukocyte migration from the blood, activation of endothelial cells to trigger clotting and containment of infection, stimulation of DC maturation, and generation of reactive oxygen species [77, 475]. Additionally, it has been demonstrated to upregulate CYP27B1 mRNA expression in microglia and astrocytes [476], suggesting a potential role in the local generation of active $1,25(OH)_2D$ to exert its immunomodulatory effects. LPS, *H.influenzae* and *S.pneumoniae* are all potent stimulators of TNF-α production [477-479], with TNF-α antagonism from agents such as Infliximab associated with an increased risk of upper respiratory tract infections [480]. TNF-α is also thought to be of particular importance in asthma and COPD, with high levels of TNF-α linked to asthmatic complications [475], and an elevated concentration in whole blood associated with COPD disease severity and cachexia development [481]. Type I IFNs are vital in innate protection against viral infections, with features of the anti-viral state including resistance to viral replication, induction of apoptotic cell death in infected cells,

increased MHC class I expression, activation of DCs and macrophages, and stimulation of NK cells [43]. Of the main types of anti-viral IFN, IFN-α is the most strongly induced in whole blood cultures and PBMCs, with IFN-λ the principal interferon produced in bronchial epithelial cells, and IFN-β production induced in all cell types [482, 483]. The volume of whole blood used in the assay was based on restrictions of the amount of blood that could be taken from trial participants, whilst also ensuring a sufficient yield of plasma supernatant for analysis by multiplex ELISA.

Therefore, an assay was developed using whole blood to investigate the effect of *in vivo* vitamin D supplementation on TNF-α or IFN-αA2 secretion following *ex vivo* stimulation with TLR ligands and respiratory pathogens. The optimum duration of co-culture of whole blood with stimuli, and the optimum concentration of each stimulus was determined, in order to optimise this assay and allow the characterisation of the effects of vitamin D on immune responses from future assays.

Table 3.1: TLR ligands used and the PAMPs recognised

TLR	Location	PAMP Recognised	Relevant Pathogens	Ligands (InvivoGen)
1/2	Plasma membrane	Triacyl lipopeptides	*Mycoplasma pneumoniae* *Haemophilus influenzae*	Pam3CSK4 (Pam3)
2/6	Plasma membrane	Zymosan Lipoteichoic acid Diacyl lipopeptides	*Streptococcus* spp *Mycoplasma pneumoniae* *Haemophilus influenzae*	Pam2CSK4 (Pam2)
3	Endosomal membrane	ds RNA ss RNA	Rhinovirus Influenza A	PolyI:C
4	Plasma membrane	LPS Mannan Envelope proteins Glycoinositolphospholipids	RSV *Haemophilus influenzae* *Chlamydiophila pneumoniae*	LPS
7/8	Endosomal membrane	ss RNA	Rhinovirus RSV Influenza A Influenza B Enterovirus Metapneumovirus Coronavirus	R848
9	Endosomal membrane	ds DNA CpG-DNA motifs Hemozoin	Adenovirus	CpG ODN

3.2 Results

3.2.1 Time course to determine the optimum duration of co-culture of whole blood with TLR ligands

Initial concentrations of TLR ligands used for the time course were at the higher end of the suggested ranges in the literature and InvivoGen product inserts in order to ensure a response was seen [469-474]. Peripheral whole blood from 6 donors (2 healthy, 2 with asthma and 2 with COPD) was incubated with TLR ligands for 0, 3, 6 or 24 hours, as detailed in Chapter 2.2. The plasma was aspirated and stored at -80^{o}C before analysis by ELISA for either TNF-α or IFN-αA2, with the results presented in Figure 3.1. TNF-α production following stimulation with LPS, Pam2CSK4 and Pam3CSK4 was highest at the 6 hour time-point. Incubation of whole blood with bacterial TLR ligands for 3 hours or 24 hours, while lower than that achieved with 6 hour stimulation, resulted in a robust TNF-α response, with levels of the cytokine diminished at the 0 hour time-point. IFN-αA2 production following stimulation with CpG ODN and PolyI:C was highest at 24 hours, with concentrations undetectable at 0, 3 and 6 hours. Stimulation of whole blood with R848 resulted in robust IFN-αA2 production at all time-points, with the highest concentrations observed with co-incubation of 6 or 24 hours. As such, the 24 hour time-point was selected for use in further experiments, on the basis that this was the only time-point at which both TNF-α and IFN-αA2 responses to stimuli were detectable.

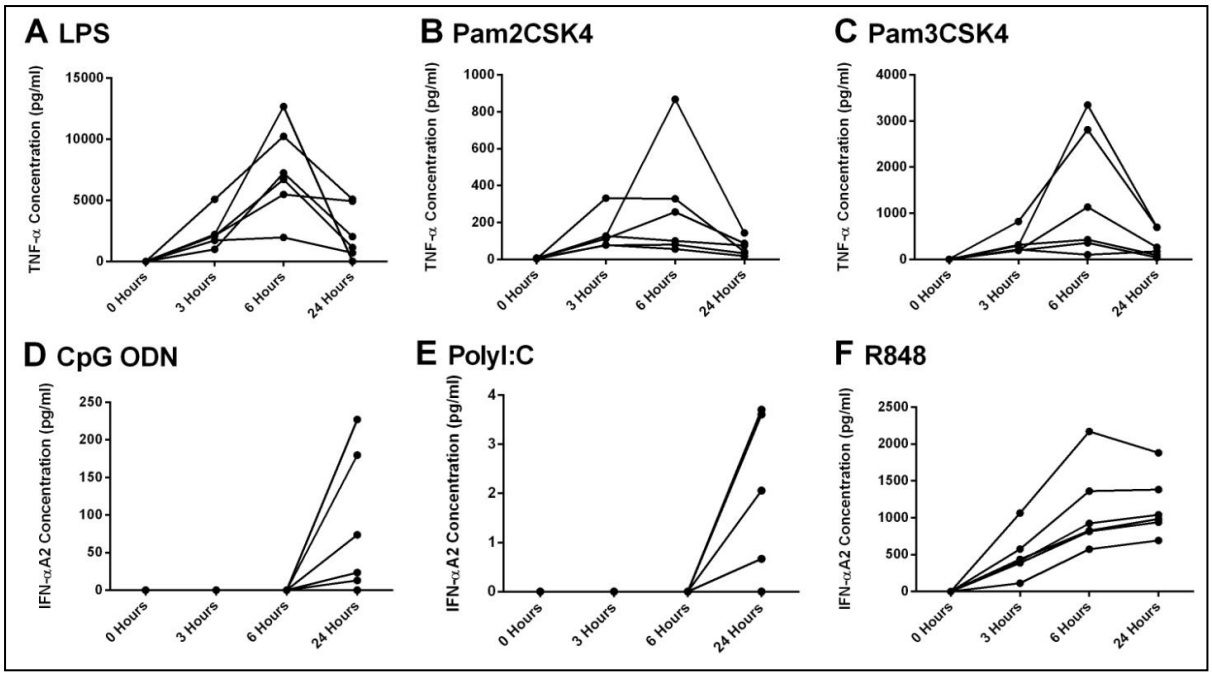


Figure 3.1: Time course data for cytokine secretion in whole blood assay stimulated with TLR ligands. Data are from 6 donors (2 healthy, 2 with asthma, 2 with COPD) with lines joining the data-points for each donor. Blood was stimulated with the TLR ligands LPS (A), Pam2CSK4 (B), Pam3CSK4 (C), CpG ODN (D), PolyI:C (E) or R848 (F) for 0, 3, 6 or 24 hours, and TNF-α (A – C) or IFN-αA2 (D – F) concentrations were assayed. The concentrations of TLR ligands used were: LPS 1µg/ml, Pam2CSK4 1µg/ml, Pam3CSK4 10µg/ml, CpG 10µg/ml, PolyI:C 100µg/ml, R848 10µg/ml.

3.2.2 Dose response to determine the optimum concentration of TLR ligands to use for co-culture with whole blood

Peripheral whole blood from 3 healthy donors was incubated with TLR ligands at the concentration used for the time-course experiment above, and at dilutions ten times and one hundred times lower to determine the optimal concentration to use for co-culture with whole blood. Following incubation for 24 hours, plasma was aspirated and stored at -80^{o}C before analysis by ELISA for either TNF-α for supernatants of whole blood stimulated with bacterial TLR ligands, or IFN-αA2 supernatants of whole blood stimulated with viral TLR ligands, with the results presented in Figure 3.2.

The concentration of each stimulus to be employed in the assay was selected on the basis that it stimulated a cytokine response roughly 50% of the maximal response, so that any decrease or increase caused by vitamin D supplementation could be observed, and so that a significant response compared to PBS could be seen. As such, the concentrations selected for use in further assays were

LPS at 0.1µg/ml, Pam2CSK4 at 0.01µg/ml, Pam3CSK4 at 1µg/ml, CpG ODN at 10µg/ml, PolyI:C at 100µg/ml and R848 at 1µg/ml.

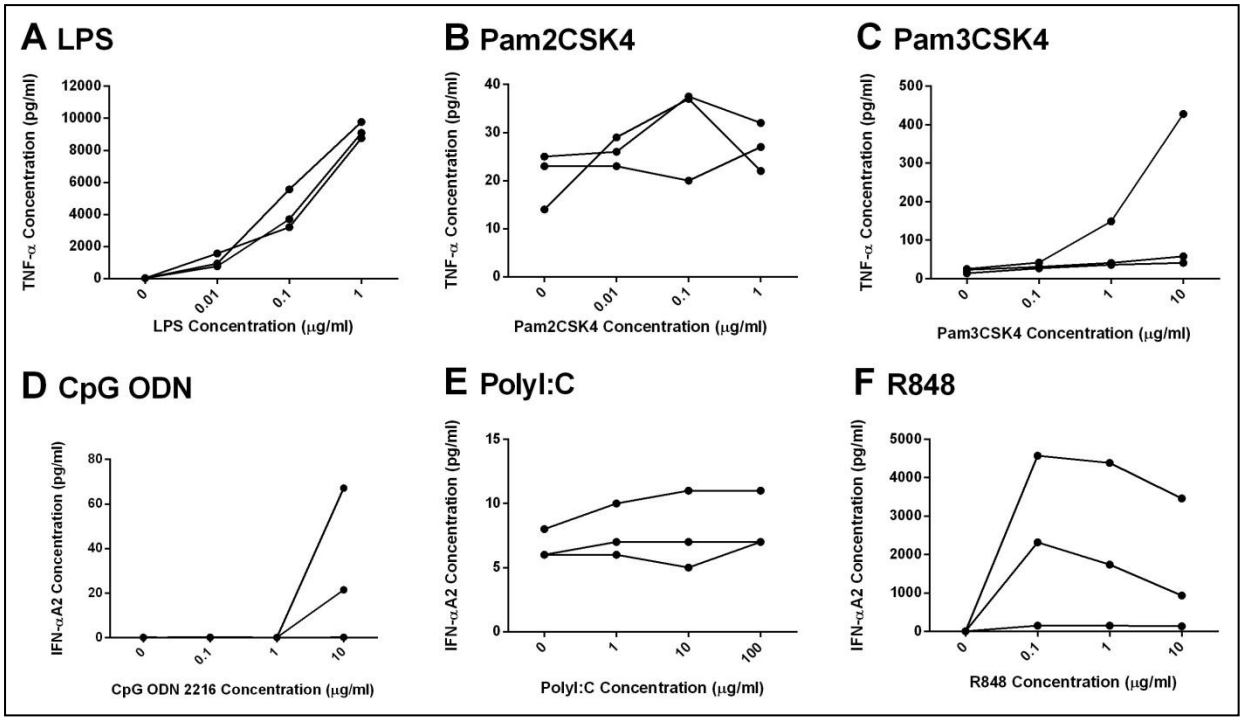


Figure 3.2: Dose response data for cytokine secretion in whole blood stimulated with TLR ligands. Data are from 3 healthy donors with lines joining the data-points for each donor. Blood was stimulated for 24hours with PBS or the TLR ligands LPS (A), Pam2CSK4 (B), Pam3CSK4 (C), CpG ODN (D), PolyI:C (E) or R848 (F), and TNF-α (A – C) or IFN-αA2 (D – F) concentrations were detected. The highest concentrations of TLR ligands used were: LPS 1µg/ml, Pam2CSK4 1µg/ml, Pam3CSK4 10µg/ml, CpG 10µg/ml, PolyI:C 100µg/ml, R848 10µg/ml, with additional dilutions of 1:10 and 1:100.

3.2.3 Dose response to determine the optimum titre of viral pathogens to use for co-culture with whole blood

Whole viruses were added to provide a more physiologically accurate stimulus of blood in order to determine the immune response induced by the ligation of multiple PRRs. Rhinovirus and RSV were chosen due to their common precipitation of exacerbations in both asthma and COPD [146, 484-487]. Two rhinovirus serotypes were used, one major (RV-16) and one minor (RV-1B), with the main difference being that major serotypes utilise ICAM-1 for cellular entry [488], while minor serotypes exploit low-density-lipoprotein receptors [489].

Peripheral whole blood from 6 healthy donors was incubated with virus for 24 hours. Virus preparations were obtained from the Johnston laboratory (as detailed in Chapter 2.2.2) and were used undiluted (RV-16 at 9.82 x 10^7 TCID50/ml, RV-1B at 8.76 x 10^7 TCID50/ml and RSV at 4 x 10^6 PFU/ml), and at dilutions of 1:2 and 1:5, with PBS used for a negative control. The plasma was aspirated and stored at -80^{o}C before analysis by ELISA for IFN-αA2 production, with results presented in Figure 3.3. The titre of RV-16 selected for use in further assays was 9.82 x 10^7 TCID50/ml, on the basis that this titre stimulated a cytokine response roughly 50% of the maximal response, so that any decrease or increase caused by vitamin D supplementation could be observed, and so that a significant response compared to the PBS negative control could be seen. As it was desirable to be able to compare responses to the major and minor serotypes of rhinovirus between individuals, titres similar to each other were chosen for RV-16 and RV-1B, with a titre of 8.76 x 10^7 TCID50/ml selected for RV-1B. The IFN-αA2 response to RV-1B and RSV at titres of 8.76 x 10^7 TCID50/ml and 4 x 10^6 PFU/ml respectively did not appear to be saturated, suggesting that any increase in cytokine production caused by vitamin D would still be able to be detected using this assay. Additionally, the highest titres of RV-1B and RSV were able to induce detectable IFN-αA2 production even in the donor with a diminished cytokine response to viral stimuli. As such, the titre of RSV selected for use in further assays was 4 x 10^6 PFU/ml.

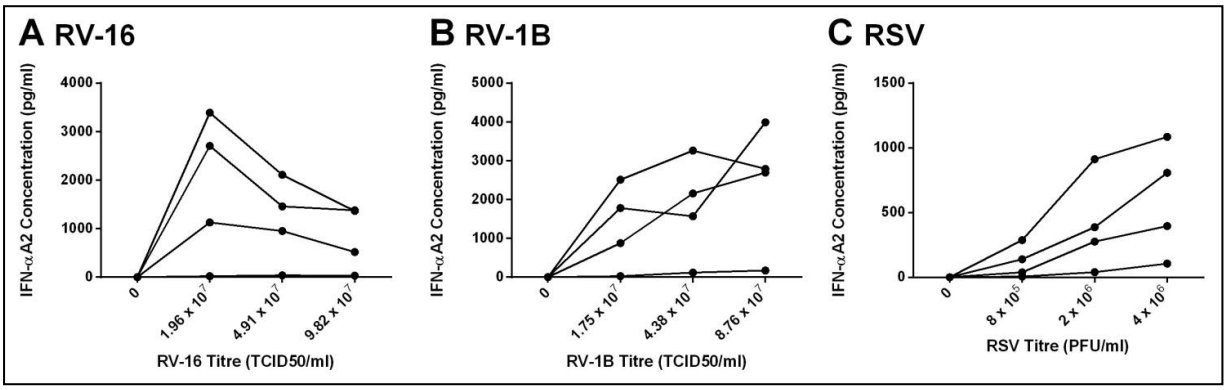


Figure 3.3: Dose response data for IFN-αA2 secretion in whole blood following stimulation with viral pathogens. Data are from 4 healthy donors with lines joining the data-points for each donor. Blood was stimulated for 24hours with PBS or the viruses RV-16 (A), RV-1B (B) or RSV (C), and IFN-αA2 concentrations were detected. The highest titres of viruses used were: RV-16 9.82x10^7 TCID/50/ml, RV-1B 8.76x10^7 TCID50/ml, RSV 4x10^6 PFU/ml, with additional dilutions of 1:2 and 1:5.

3.2.4 Dose response to determine the optimum titre of bacterial pathogens to use for co-culture with whole blood

In addition to viruses, bacteria were also added to provide a more physiologically accurate stimulus of blood in order to determine the immune response induced by the ligation of multiple PRRs. The bacteria *H.influenzae* and *S.pneumoniae* were chosen for the whole blood assay, as these are the most commonly implicated microbes in COPD and asthma exacerbations, and are also common causative agents of pneumonia in residents of sheltered accommodation schemes [490-492].

Peripheral whole blood from 4 healthy donors was incubated with bacterial pathogens for 24 hours. Bacteria preparations were obtained from the Johnston laboratory (as detailed in Chapter 2.2.2) and were used undiluted (*H.influenzae* at 8.13 x 10^8 CFU/ml and *S.pneumoniae* at 2.3 x 10^8 CFU/ml), and at dilutions of 1:2 and 1:5, with PBS used for a negative control. The plasma was aspirated and stored at -80^{o}C before analysis by ELISA for TNF-α production, with results presented in Figure 3.4. *H.influenzae* stimulation at these dilutions resulted in a saturated TNF-α response, with concentrations higher than the limit of detection of 10,000pg/ml. *S.pneumoniae* stimulation at these dilutions also resulted in a saturated TNF-α response for one donor, with concentrations higher than the limit of detection of 10,000pg/ml, while the remaining 3 donors produced responses at the higher end of detection as well.

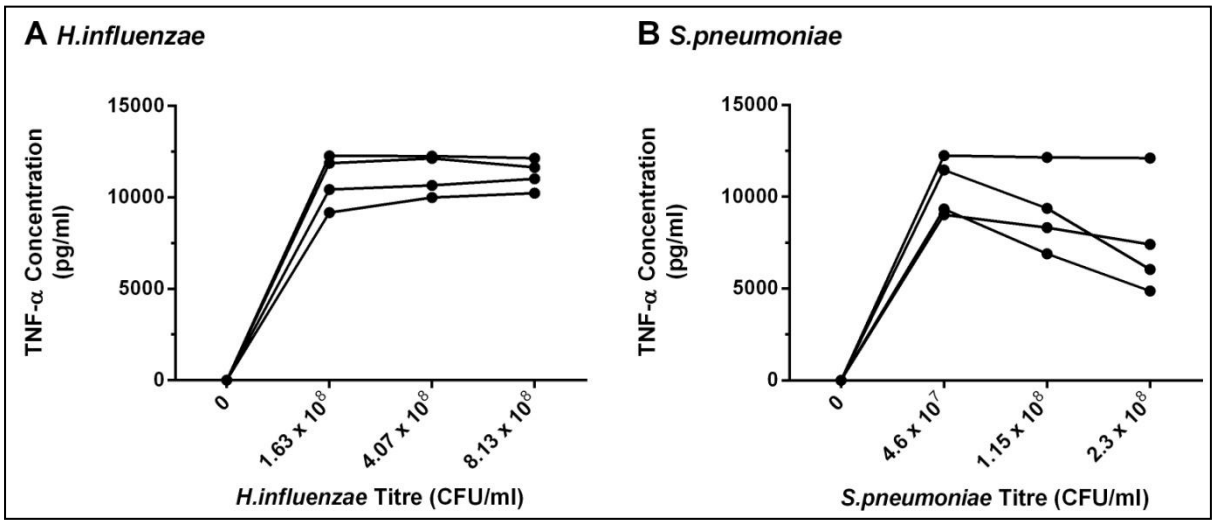


Figure 3.4: Dose response data for TNF-α secretion in whole blood stimulated with bacterial pathogens. Data are from 4 healthy donors with lines joining the data-points for each donor. Blood was stimulated for 24hours with PBS or the bacteria H.influenzae (A) or S.pneumoniae (B) and TNF-α concentrations were detected. The highest titres of bacteria used were 8.13 x 10^8 for H.influenzae and 2.3 x 10^8 for S.pneumoniae, with additional dilutions of 1:2 and 1:5.

It was preferable to use titres of stimuli that resulted in cytokine responses falling within the detectable range, so as to avoid having to dilute supernatants at the ELISA stage. This way, it would enable the detection of multiple inflammatory mediators from one supernatant at a single dilution using multiplex ELISA. As such, the dose response was repeated, using a highest titre of 8.13 x 10^6 CFU/ml for *H.influenzae* and 2.3 x 10^6 CFU/ml for *S.pneumoniae* (1:100 dilutions from the original stocks), and additional dilutions of 1:1000 and 1:10000 from the stock preparations received from the Johnston laboratory. Peripheral blood from 3 healthy donors was incubated with the pathogens for 24 hours, before plasma aspiration and TNF-α analysis (Figure 3.5). TNF-α production in response to stimulation with *H.influenzae* was now in the detectable range, with concentrations of the cytokine decreasing with the declining titre of pathogen. Stimulation with decreasing titres of *S.pneumoniae* also resulted in a progressively lower TNF-α response for 2 donors, with the third donor showing a steady concentration of the cytokine, although all responses were within the limit of detection. Again, the titre of each stimulus to be employed in the assay was selected on the basis that it stimulated a cytokine response roughly 50% of the maximal response, so that any decrease or increase caused by vitamin D supplementation could be observed, and so that a significant response compared to the PBS negative control could be seen. As such, the titre of *H.influenzae* selected for use in further assays was 8.13 x 10^6 CFU/ml, and for *S.pneumoniae* was 2.3 x 10^6 CFU/ml.

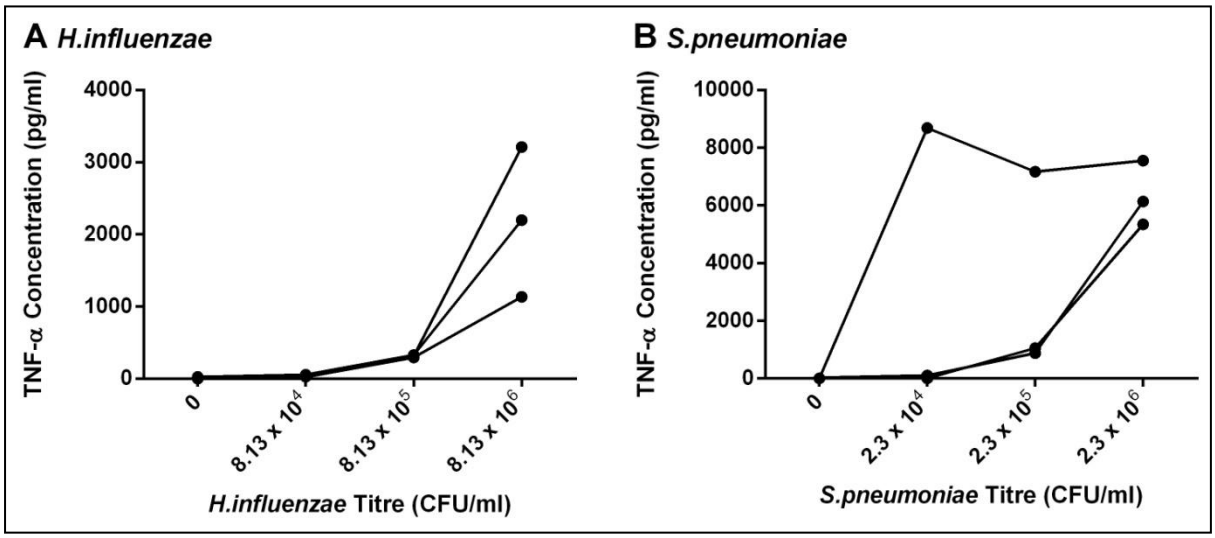


Figure 3.5: Dose response data for TNF-α secretion in whole blood stimulated with bacterial pathogens. Data are from 3 healthy donors with lines joining the data-points for each donor. Blood was stimulated for 24hours with PBS or the bacteria H.influenzae (A) or S.pneumoniae (B) and TNF-α concentrations were detected. The highest titres of bacteria used were 8.13 x 10^6 for H.influenzae and 2.3 x 10^6 for S.pneumoniae, with additional dilutions of 1:10 and 1:100.

3.3 Discussion

Here I have shown how a whole blood assay was developed to enable the evaluation of how *in vivo* vitamin D supplementation in trial participants affects the *ex vivo* cytokine response to TLR ligands and key pathogens. The assay was optimised by time course and dose response experiments to give the best chance of detecting any effect of *in vivo* vitamin D supplementation on antigen-stimulated immune responses.

The co-culture of whole blood with various stimuli offers several advantages as a model by which to investigate the immunomodulatory actions of vitamin D. Other investigators carrying out similar studies have used peripheral blood mononuclear cells (PBMCs) or isolated monocytes, co-cultured with TLR ligands for 24 hours [426, 493, 494]. PBMC populations include lymphocytes, monocytes, macrophages and dendritic cells, but not polymorphonuclear cells such as eosinophils and neutrophils. Neutrophils have a multitude of roles in the immune response, such as phagocytosis, cytokine and chemokine production to co-ordinate the responses of other immune cells, microbicidal activity, and formation of neutrophil extracellular traps [495, 496]. While neutrophils may have limited function in anti-viral responses [47], they are vital in the defence against respiratory bacteria, with depletion resulting in impaired clearance of bacteria such as *S.pneumoniae* and *Legionella pneumophila*, and repletion improving defence and murine survival in response to bacterial infection [497-499]. Eosinophils, while normally associated with allergy, also have an important role in the immune response to respiratory pathogens. They are able to degranulate, releasing cytotoxic ribonucleases such as eosinophil cationic protein (ECP) and eosinophil-derived neurotoxin (EDN), which have antiviral properties against RSV [500], as well as being able to produce NO in response to TLR ligands, with MyD88-dependent accelerated clearance of RSV in hypereosinophilic mice [501]. Additionally, eosinophils are important in the immune response to bacteria, demonstrating bactericidal properties of ECP and the oxidative burst, plus the ability to directly phagocytose bacteria [502-505], with adoptive transfer of eosinophils improving bacterial clearance of *Pseudomonas aeruginosa* and congenital eosinophil deficiency impairing clearance in mice [506]. Therefore, a model whereby these cells are not present does not represent the normal cellular milieu, and thus the immune response to stimuli may be altered As such, it was decided to use whole blood for the development of this assay, as it is advantageous over PBMCs with all immune cell types present.

The use of whole blood also offers an advantage over PBMCs as 25(OH)D received from supplementation in the clinical trial would be present in the whole blood, as the vitamin D binding

protein and 25(OH)D itself are present at physiological concentrations in plasma. Studies using PBMCs require media supplementation, and thus any vitamin D present becomes diluted, and furthermore many cellular assays in the literature have received additional supplementation with vitamin D *in vitro* [494, 507]. The model employed here allows for the effect of physiological concentrations of 25(OH)D to be assessed, whilst still being directly associated with the *in vivo* supplementation of trial participants. Another benefit of this assay is the determination of concentrations of stimuli to be used to ensure a detectable cytokine response without the need for dilution of the plasma. This enables multiple samples to be assayed at once with ease, and means that numerous inflammatory mediators can be measured in single wells using a multiplex ELISA platform.

The decision was made to focus on the innate immune response, as innate antimicrobial responses are important in affording protection against respiratory infections, and vitamin D has been demonstrated to have a key role in altering this response. TLR ligands were selected to stimulate an innate immune response, especially considering the potential importance of the up-regulation of CYP27B1 following TLR ligation allowing conversion of inactive 25(OH)D to the active metabolite $1,25(OH)_2D$ (as described in Chapter 1.13). The TLR ligands were chosen based on their relevance to pathogens precipitating exacerbations in asthma and COPD, and those commonly implicated in respiratory tract infections in the elderly (Table 3.1). The Class A CpG 2216 was chosen over Classes B and C due to its distinct induction of type-I IFN production [508], which is of particular interest in this study due to its importance in the defence against respiratory viruses [509]. Whole pathogens were also used in order to provide more physiologically accurate stimulation of the blood, as these can stimulate signalling through multiple PRRs simultaneously. Thus, the use of stimuli in the form of both TLR ligands and pathogens gives the opportunity to assess the effect of vitamin D on the physiologically relevant immune response to whole pathogens, as well as enabling the dissection of the immune response to determine the individual TLRs ligated and how vitamin D affects these responses.

There are also some limitations to this assay. Firstly, the methods used in the development of the assay may not have allowed for the optimum conditions to be determined. The time course to determine the optimum length of time for co-culture of whole blood with stimuli was only carried out with TLR ligands, and the optimum time needed for co-culture with whole pathogens may have been different. Different blood donors were also used for the various preliminary experiments due to availability, with some being healthy, and others having asthma or COPD. As a result, any difference between optimum times for co-culture and concentrations of stimuli for different disease

states would have been missed. For example, a dysregulated anti-viral immune response in asthma has been demonstrated, with macrophages producing less IFN-λ, bronchial epithelial cells having an impaired IFN-β response, PBMCs secreting less IFN-α, and pDCs less able to synthesise IFN-α following viral challenge [172, 510-512]. The TNF-α response to LPS stimulation in whole blood and isolated monocytes has also been demonstrated to be elevated in severe COPD and COPD with associated weight-loss [513, 514]. Therefore, concentrations of stimuli may have been needed to be altered to induce a detectable immune response in these populations. Additionally, only TNF-α and IFN-αA2 were assayed in preliminary experiments, with other inflammatory mediators potentially responding differently to the stimuli and requiring higher or lower concentrations of these stimuli for detectable responses. However, while a separate time-course was not carried out for the pathogens, a robust immune response was detected following incubation for 24 hours, thus allowing any effects of vitamin D supplementation to be observable, despite this potentially not being the optimum conditions. Equally, while blood donors had differing immune responses following stimulation, this was observed in the assays performed with only healthy donors as well as those with a mixture of healthy, asthmatic and COPD patient donors, thus negating any potential issues over the health status of donors in the development of this assay.

Therefore, an assay was successfully developed for use with whole blood samples from clinical trial participants to assess the effects of *in vivo* vitamin D supplementation on the *ex vivo* innate immune response to TLR ligands and whole pathogens.

4. Investigation into the effects of vitamin D metabolites on resistance to viral infection, secretion of inflammatory mediators and receptor expression in a respiratory epithelial cell line

4.1 Introduction

The respiratory epithelium is the first line of defence against inhaled foreign organisms and pathogens. It acts as a physical barrier, with cell-cell junctions blocking viral access to receptors in the basolateral membrane thus preventing entry and dissemination into the submucosa, and goblet cells producing mucus, which acts as a barrier impermeable to most pathogens, with ciliated epithelial cells allowing clearance of pathogens via the mucociliary escalator [515]. In addition to this, airway epithelial cells express the full complement of TLRs, as well as RIG-I, MDA5, NOD1 and NOD2, thus allowing recognition of pathogens and regulation of the innate and adaptive immune response. They are able to produce a range of cytokines and chemokines, such as CXCL8, CXCL1 and CXCL5 to recruit neutrophils, IL-5, GM-CSF, eotaxin and RANTES to recruit eosinophils, IL-1β, MIP-1α, MCP-1 and TNF-α to recruit monocytes/macrophages, and IFN-α, IFN-β and MIP-1α to enhance NK cell recruitment and activity. The adaptive immune response can also be mediated by respiratory epithelial cells, via the production of MIP-3α to enhance conventional DC (cDC) migration to the epithelium, IL-15 and type I IFNs to drive local cDC differentiation and maturation, RANTES, MIG and IP-10 to induce migration of Th1 cells, and IL-1β to stimulate Th2 cell migration to the mucosa. Finally, the respiratory epithelium can secrete molecules with direct anti-viral properties, such as type I and type III IFNs which initiate the antiviral state, lactoferrin which blocks viral cellular receptors, HBD2 which suppress viral replication, nitric oxide which inhibits viral replication and possess pro-apoptotic properties, and LL-37 which has numerous anti-microbial actions [515, 516].

It has been demonstrated that the respiratory epithelium is able to convert 25(OH)D to the active form of $1,25(OH)_2D$, and that viral infection increases this local activation of 25(OH)D [430]. Therefore, a microenvironment with high levels of active $1,25(OH)_2D$ is created, allowing increased expression of vitamin D-regulated genes. Due to the importance of the respiratory epithelium as the first line of defence to invading pathogens, the localised immunomodulatory actions of vitamin D may play a critical role in protection against acute respiratory tract infections.

Therefore, a series of *in vitro* experiments was conducted to determine the effect of vitamin D metabolites on the immune response to rhinovirus in a respiratory epithelial cell line, by looking at both mRNA expression of key molecules, and concentrations of a panel of cytokines and chemokines in culture supernatants. Rhinovirus was chosen as the pathogen in this study since it is the most common aetiologic agent of the common cold [517], which is the most frequent acute illness in the industrialised world, associated with 2-3 episodes per year in adults, and 5-7 per year in children [518, 519]. Rhinoviruses are also a frequent cause of exacerbations in both asthma and COPD patients [148, 484, 520-522], who are of particular interest in this study due to their increased susceptibility to acute respiratory tract infections. Since 90% of rhinovirus serotypes are in the major group, utilizing ICAM-1 for cellular entry, and the majority are also classified as the A species, RV-16, a major group A species rhinovirus serotype was chosen for experimental work [523].

4.2 Results

4.2.1 Co-culture of A549 cells with vitamin D metabolites results in transient resistance to RV-16 infection

As detailed in Chapter 2.8, A549 cells were co-cultured with vitamin D metabolites or vehicle (0.1% ethanol) for 48 hours, before incubation for 6 or 24 hours with RV-16 or controls of filtered and UV-inactivated virus. Cells were lysed and, following RNA extraction and reverse transcription, RT-PCR was used to quantify viral mRNA (Figure 4.1). Three technical replicates were used for each sample, from which an average was taken, and the experiment was repeated 3 times, providing three biological replicates. Due to only having n = 3 biological replicates, statistical tests could not be performed on these data. RV-16 mRNA was increased approximately 2000-fold at both 6 and 24 hours post-infection in cells co-cultured with vehicle compared to the UV-inactivated RV controls. In cells co-cultured with 25(OH)D or $1,25(OH)_2D$ preceding a 6 hour incubation with RV-16, viral mRNA was significantly reduced by 70% and 37% respectively compared to the ethanol control. Presence of vitamin D metabolites did not however significantly reduce viral mRNA expression following a 24 hour incubation with RV-16.

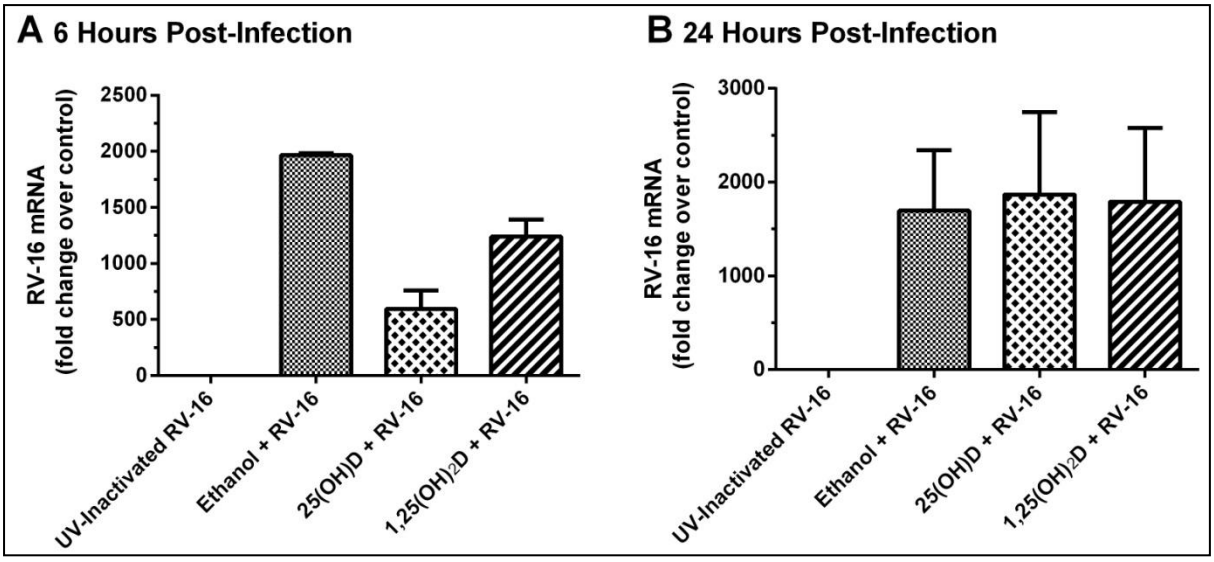


Figure 4.1: Effect of co-culture with vitamin D metabolites on RV-16 mRNA expression. A549 cells were co-cultured with vitamin D metabolites or vehicle (0.1% ethanol) for 48 hours, before incubation with RV-16 or control (UV-inactivated RV-16) for 6 (A) or 24 (B) hours. RV-16 mRNA was quantified by RT-PCR and analysed using the ΔΔCT method, compared to the UV-inactivated control. Each RT-PCR reaction was carried out in triplicate and a mean value was taken of the technical replicates. Data are from 3 repeats of the same experiment, represented as mean + SEM. P-values could not be calculated due to the small sample size of n = 3.

4.2.2 Co-culture of A549 cells with vitamin D metabolites results in attenuation of RV-induced ICAM-1 expression

ICAM-1 is the main receptor for major serotypes of rhinovirus, such as RV-16 [524, 525]. As such, it was desirable to test whether the effects of vitamin D metabolites on viral mRNA were associated with an effect on ICAM-1 expression. As described in chapter 2.8, A549 cells were cultured, and cells lysed following an overnight incubation. Culture was extended for some cells, which underwent co-culture with vitamin D metabolites or vehicle (0.1% ethanol) for 48 hours before lysis. A final set of cells were cultured further following addition of vitamin D metabolites or vehicle, with RV-16 incubation for 6 or 24 hours before cell lysis. RNA was extracted from the cell lysates and reverse transcription carried out, before RT-PCR was used to quantify ICAM-1 mRNA (Figure 4.2). Three technical replicates were used for each sample, from which an average was taken, and the experiment was repeated 3 times, providing three biological replicates. Due to only having n = 3 biological replicates, statistical tests could not be performed on these data. Co-culture with vitamin D metabolites had no effect on constitutive expression of ICAM-1 (Figure 4.2 A). Incubation with RV-

16 for 6 hours increased ICAM-1 expression 50-fold, with the 24 hour incubation increasing ICAM-1 to a lesser extent (3.5-fold). Following co-culture with vitamin D metabolites, RV-16-induced ICAM-1 expression was attenuated. Co-culture with 25(OH)D resulted in a 70% reduction in ICAM-1 expression 6 hours post-infection, and a 30% reduction 24 hours post-infection, while co-culture with $1,25(OH)_2D$ resulted in a 37% decrease 24 hours post-infection, and a 30% reduction 6 hours post-infection.

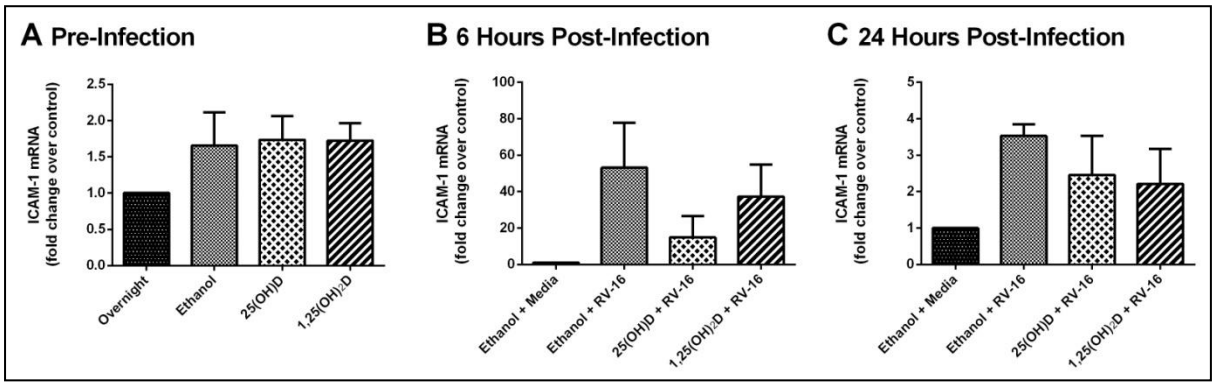


Figure 4.2: Effect of co-culture with vitamin D metabolites and RV infection on ICAM-1 mRNA expression. A549 cells were cultured overnight, before co-culture with vitamin D metabolites or vehicle (0.1% ethanol) for 48 hours, pre-infection (A). Some cells underwent further culture and were incubated with RV-16 for 6 (B) or 24 (C) hours. ICAM-1 mRNA was quantified by RT-PCR and analysed using the ΔΔCT method. Each RT-PCR reaction was carried out in triplicate and a mean value was taken of these technical replicates. Data are from 3 repeats of the same experiment, represented as mean + SEM. P-values could not be calculated due to the small sample size of n = 3.

4.2.3 Co-culture off A549 cells with vitamin D metabolites results in increased IκBα expression

ICAM-1 expression has been demonstrated to be regulated by the transcription factor NF-κB [111, 457, 526]. NF-κB is inactive in the cytoplasm due to the direct binding of inhibitors such as IκBα to the p65 subunit of the NF-κB heterodimer. Upon stimulation of the cell, the IκB kinase complex (IKK) is activated, resulting in phosphorylation of IκBα, and subsequent ubiquitination and degradation by the proteasome. Therefore, NF-κB is free to translocate to the nucleus, bind DNA, and activate transcription of numerous target genes [527]. It has been demonstrated elsewhere that $1,25(OH)_2D$ is able to increase the expression of the inhibitor IκBα in airway epithelial cells in a dose-dependent manner [327]. As such, it was decided to assess the effect of vitamin D metabolites on expression of IκBα, to determine whether attenuation of RV-induced ICAM-1 was associated with an increase in

IκBα. A549 cells were co-cultured with vitamin D metabolites or vehicle (0.1% ethanol) for 48 hours, before incubation for 6 or 24 hours with RV-16. Cells were lysed and, following RNA extraction and reverse transcription, RT-PCR was used to quantify IκBα mRNA (Figure 4.3). Three technical replicates were used for each sample, from which an average was taken, and the experiment was repeated 3 times, providing three biological replicates. Co-culture of A549 cells with 25(OH)D marginally increased constitutive expression of IκBα by 1.3-fold, while co-culture with $1,25(OH)_2D$ also increased constitutive expression of IκBα 1.5-fold. Incubation with RV-16 for 6 or 24 hours had no effect on IκBα mRNA expression. Co-culture of A549 cells with vitamin D metabolites combined with a 6 hour incubation with RV-16 increased the expression of IκBα 3-fold compared to cells cultured with vehicle and RV-16. Co-culture of A549 cells with $1,25(OH)_2D$ combined with a 24 hour incubation with RV-16 increased IκBα expression 1.7-fold, but no difference was seen with 25(OH)D co-culture compared to cells cultured with vehicle and RV-16.

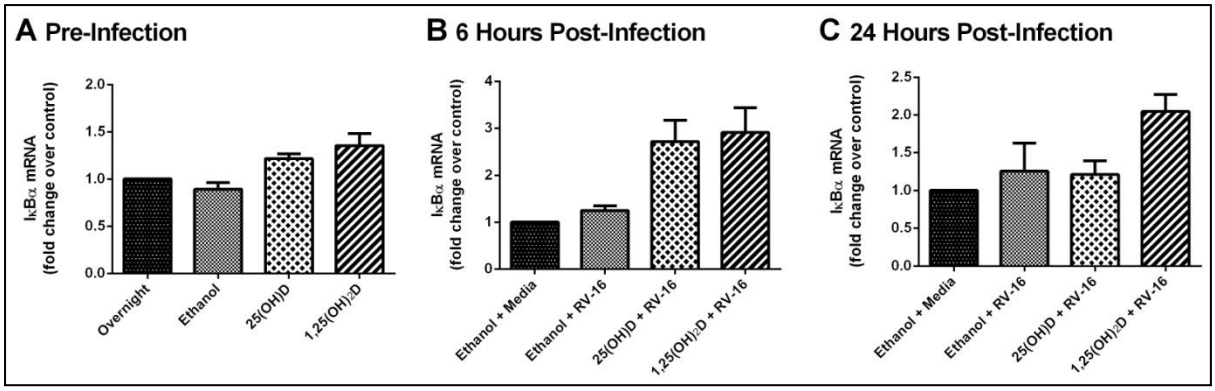


Figure 4.3: Effect of co-culture with vitamin D metabolites and RV infection on IκBα mRNA expression. A549 cells were cultured overnight, before co-culture with vitamin D metabolites or control for 48 hours, pre-infection (A). Following this, cells were incubated with RV-16 for 6 (B) or 24 (C) hours. IκBα mRNA was quantified by RT-PCR and analysed using the ΔΔCT method. Each RT-PCR reaction was carried out in triplicate and a mean value was taken of the technical replicates. Data are from 3 repeats of the same experiment, represented as mean + SEM. P-values could not be calculated due to the small sample size of n = 3.

4.2.4 Co-culture off A549 cells with vitamin D metabolites results in increased cathelicidin mRNA expression

Vitamin D is associated with increased expression of antimicrobial peptides (AMPs), such as cathelicidin and human beta defensins [528]. The *CAMP* (cathelicidin antimicrobial peptide) gene encodes the pro-peptide hCAP18, which contains an N-terminal cathelin domain and a C-terminal

peptide which, once cleaved, has antimicrobial activity and is known as LL-37 [343, 529]. A vitamin D responsive element (VDRE) has been identified in the promoter region of the *CAMP* gene [361], and vitamin D has been demonstrated to have a critical role in the regulation of *CAMP* [359, 530], while also increasing the protein hCAP18 [531, 532]. As a result, the effect of vitamin D on expression of *CAMP* was assessed, to determine whether vitamin D-mediated resistance to RV-16 infection was associated with an increase in cathelicidin. A549 cells were co-cultured with vitamin D metabolites or vehicle (0.1% ethanol) for 48 hours before incubation for 6 or 24 hours with RV-16. Cells were lysed and, following RNA extraction and reverse transcription, RT-PCR was used to quantify *CAMP* mRNA (Figure 4.4). Three technical replicates were used for each sample, from which an average was taken, and the experiment was repeated 3 times to provide three biological replicates. Due to only having n =3 biological replicates, statistical tests could not be performed on these data. Co-culture of A549 cells with vitamin D metabolites resulted in increased constitutive expression of *CAMP*, with 25(OH)D resulting in a 4-fold increase, and $1,25(OH)_2D$ in a 5.5-fold increase compared to vehicle. Incubation with RV-16 resulted in decreased *CAMP* expression, by 50% 6 hours post-infection and 40% 24 hours post-infection. Co-culture with 25(OH)D preceding incubation with RV-16 for 6 hours resulted in a modest 2-fold increase in *CAMP* expression compared to co-culture with ethanol, while there was no difference following infection for 24 hours. Co-culture with $1,25(OH)_2D$ increased *CAMP* expression compared to the ethanol + RV-16 control, with a 3-fold increase both 6 hours and 24 hours post-infection.

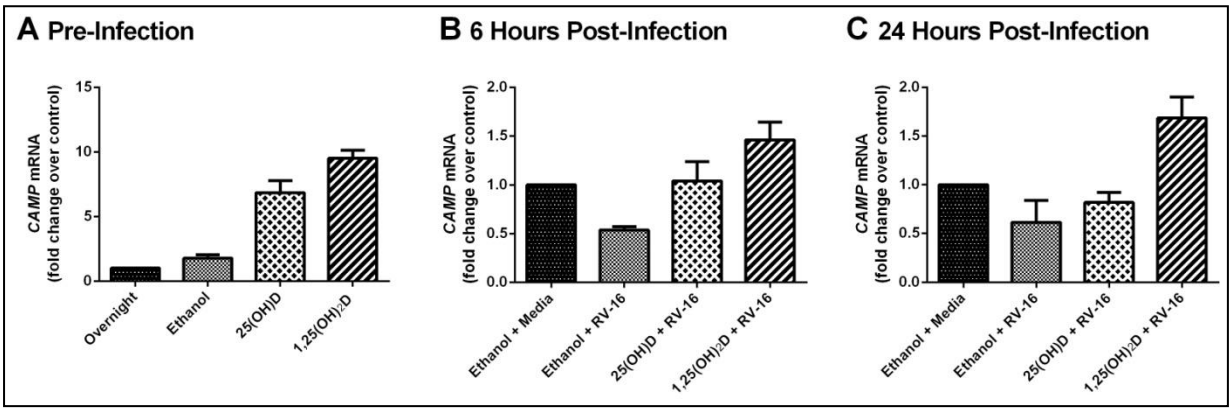


Figure 4.4: Effect of co-culture with vitamin D metabolites and RV infection on CAMP mRNA expression. A549 cells were cultured overnight, before co-culture with vitamin D metabolites or control for 48 hours, pre-infection (A). Following this, cells were incubated with RV-16 for 6 (B) or 24 (C) hours. CAMP mRNA was quantified by RT-PCR and analysed using the ΔΔCT method. Each RT-PCR reaction was carried out in triplicate and a mean value was taken from these technical replicates. Data are from 3 repeats of the same experiment and are expressed as mean + SEM. P-values could not be calculated due to the small sample size of n = 3.

4.2.5 Co-culture off A549 cells with vitamin D metabolites results in increased IFN-α2 mRNA expression

Type I IFNs are vital in the immune response to viral infection, with a broad range of effects such as inducing apoptosis of infected cells, enhancing antigen presentation by increasing expression of MHC Class I, and enhancing cytolytic activity of NK cells [533]. Surprisingly, given the reported protective effect of vitamin D against viral infections, other studies have demonstrated that culture of monocytes or human tracheobronchial epithelial cells with $1,25(OH)_2D$ suppresses type I IFN expression following antigenic stimulation [327, 382, 534]. As such, the effect of vitamin D metabolites on expression of IFN-α and IFN-β was assessed, to determine whether vitamin D-mediated resistance to RV-16 infection was associated with an increase in type I IFNs. A549 cells were co-cultured with vitamin D metabolites or vehicle (0.1% ethanol) for 48 hours before incubation for 6 or 24 hours with RV-16 or controls. Cells were lysed and, following RNA extraction and reverse transcription, RT-PCR was used to quantify IFN-α and IFN-β mRNA (Figure 4.5). Three technical replicates were used for each sample, from which an average was taken, and the experiment was repeated 3 times to provide 3 biological replicates. Due to only having n = 3 biological replicates, statistical tests could not be performed on these data. Constitutive expression of IFN-β was decreased by 25(OH)D by 27% and increased 1.5-fold by $1,25(OH)_2D$ compared to vehicle, and similarly constitutive IFN-α expression was decreased by 25(OH)D by 30%, and increased 2-fold by $1,25(OH)_2D$. Incubation with RV-16 resulted in decreased expression of IFN-α both 6 hours and 24 hours post-infection, by 51% for both. Co-culture with 25(OH)D was able to attenuate RV-induced reduction of IFN-α expression 6 hours post-infection with a 2-fold increase, while $1,25(OH)_2D$ had no effect. At 24 hours post-infection, co-culture with vitamin D metabolites decreased IFN-α expression by 60% for 25(OH)D and 40% for $1,25(OH)_2D$ compared to vehicle. Incubation with RV-16 also resulted in a 73% reduction in IFN-β expression 24 hours post-infection, although this effect was not observed 6 hours post-infection. Co-culture with vitamin D metabolites reduced IFN-β expression 6 hours post-infection by 80% compared to vehicle. 25(OH)D marginally increased IFN-β expression by 1.7-fold at 24 hours post-infection, while $1,25(OH)_2D$ increased expression 3-fold compared to vehicle.

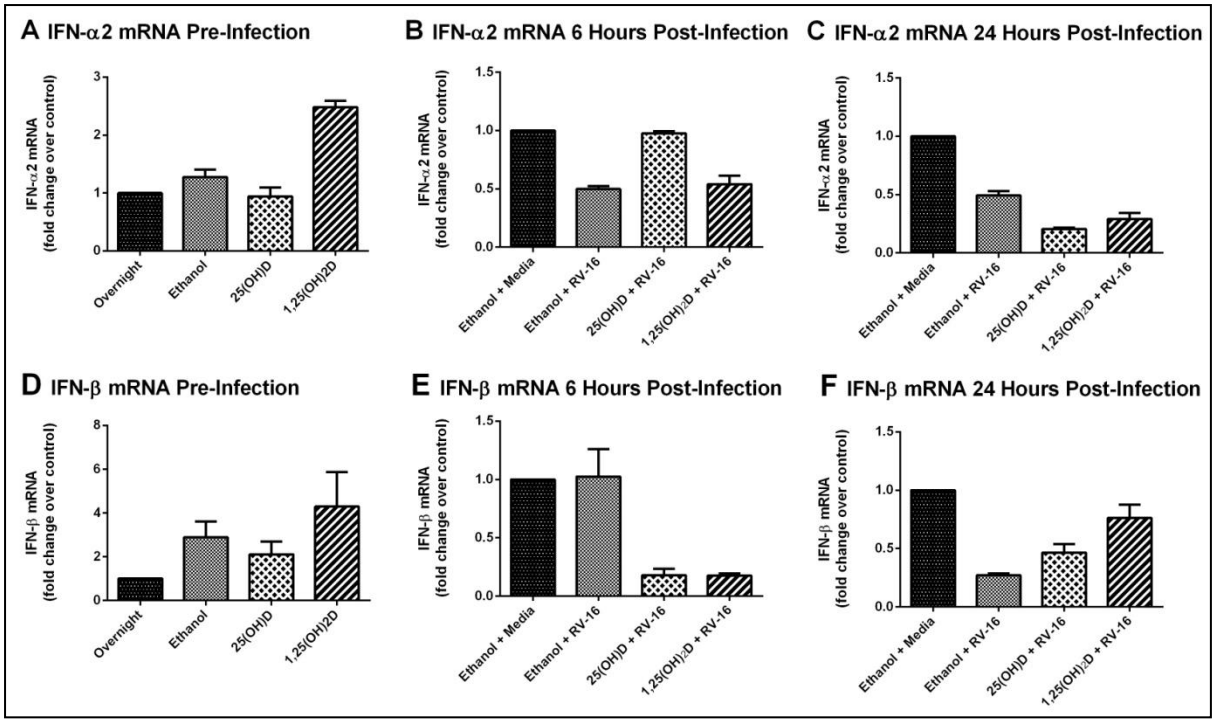


Figure 4.5: Effect of co-culture with vitamin D metabolites and RV infection on type I IFN mRNA expression. A549 cells were cultured overnight, before co-culture with vitamin D metabolites or control for 48 hours, pre-infection (A and D). Following this, cells were incubated with RV-16 for 6 (B and E) or 24 (C and F) hours. IFN-α2 (A to C) and IFN-β (D to F) mRNA was quantified by RT-PCR and analysed using the ΔΔCT method. Each RT-PCR reaction was carried out in triplicate and a mean value was taken of these technical replicates. Data are from 3 repeats of the same experiment, represented as mean + SEM. P-values could not be calculated due to the small sample size of n = 3.

4.2.6 Co-culture off A549 cells with vitamin D metabolites results in decreased PafR mRNA expression

Viral infections are commonly seen to predispose patients to an increased incidence and severity of secondary bacterial infections, with the majority of bacterial sepsis cases reportedly following acute viral infections, and the majority of deaths during the 1918 influenza pandemic attributable to secondary bacterial infections [100, 535-537]. Platelet activating factor receptor (PAF-r) has been shown to be a receptor for *S.pneumoniae* and *H.influenzae*, the two most common bacterial respiratory pathogens, and its expression has been shown to be increased by RSV, RV-14, influenza and coronavirus infections [88, 101, 102]. While vitamin D has been demonstrated to have many anti-bacterial functions [538], such as stimulating the production of antimicrobial peptides, we

hypothesised that vitamin D may further act in the inhibition of secondary bacterial infections by attenuating viral induced receptor expression. As such, A549 cells were co-cultured with vitamin D metabolites or vehicle (0.1% ethanol) for 48 hours before incubation for 6 or 24 hours with RV-16 or controls. Cells were lysed and, following RNA extraction and reverse transcription, RT-PCR was used to quantify PafR mRNA (Figure 4.6). Three technical replicates were used for each sample, from which an average was taken, and the experiment was repeated 3 times to provide three biological replicates. Due to only having n = 3 biological replicates, statistical tests could not be performed on these data. Co-culture of A549 cells with vitamin D metabolites did not affect constitutive expression of PafR. Incubation with RV-16 for 6 hours resulted in a 2.5-fold increase in PafR mRNA, with co-culture with both vitamin D metabolites resulting in an 80% reduction compared to vehicle. Conversely, incubation with RV-16 for 24 hours resulted in a 53% decrease in PafR expression, with co-culture with vitamin D metabolites having no further effect.

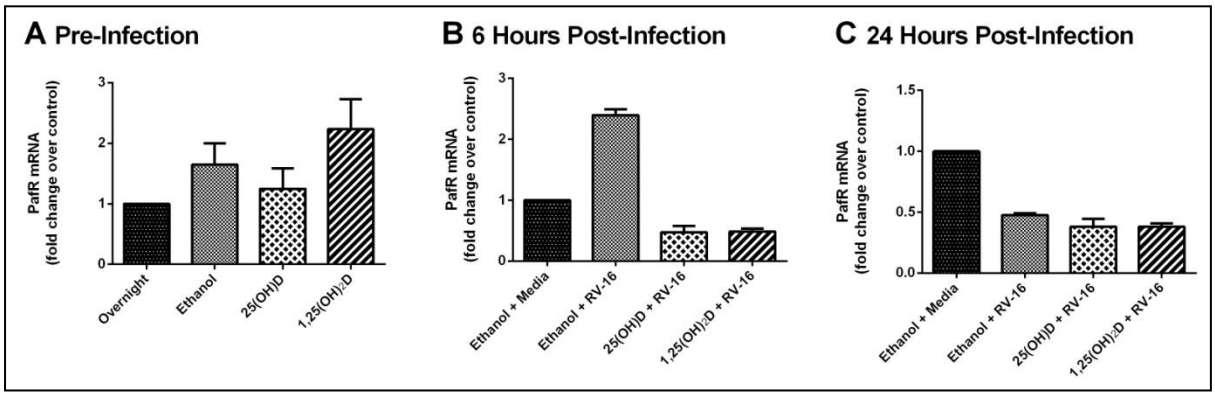


Figure 4.6: Effect of co-culture with vitamin D metabolites and RV-16 infection on PafR mRNA expression. A549 cells were cultured overnight, before co-culture with vitamin D metabolites or control for 48 hours, pre-infection (A). Following this, cells were incubated with RV-16 for 6 (B) or 24 (C) hours. PafR mRNA was quantified by RT-PCR and analysed using the ΔΔCT method. Each RT-PCR reaction was carried out in triplicate and a mean value was taken of these technical replicates. Data are from 3 repeats of the same experiment, represented as mean + SEM. P-values could not be calculated due to the small sample size of n = 3.

4.2.7 Cytokine and chemokine response to RV-16 infection of A549 cells co-cultured with vitamin D metabolites

The respiratory epithelium is able to secrete cytokines and chemokines to mount an immune response following pathogenic stimulation [539-542]. It has been demonstrated that respiratory epithelial cells can convert 25(OH)D to 1,25$(OH)_2$D [430], and this active form can alter the

production of local inflammatory mediators [327]. Therefore, it was decided to employ a multiplex approach to determine the effect of vitamin D metabolites on the cytokines, chemokines and growth factors secreted following RV-16 infection. A549 cells were co-cultured with vitamin D metabolites or vehicle (0.1% ethanol) for 48 hours before incubation for 6 or 24 hours with RV-16 or controls. Supernatants were aspirated and analysed by multiplex ELISA and significant results are presented in Figures 4.7 (for 6 hours post-infection) and 4.8 (for 24 hours post-infection). Incubation with RV-16 resulted in increased concentrations of a large number of inflammatory mediators in the supernatants of cultured A549 cells. RV-16 incubation for 6 hours increased concentrations of IL-6 ($p = 0.0006$), IL-8 ($p = 0.007$), IL-12 ($p = 0.001$), IL-2R ($p = 0.009$), IFN-α ($p < 0.0001$), IFN-γ ($p < 0.0001$), RANTES ($p = 0.005$), eotaxin ($p = 0.01$), MIP-1α ($p = 0.01$), MIP-1β ($p = 0.007$), MCP-1 ($p = 0.002$), EGF ($p = 0.002$), HGF ($p < 0.0001$) and VEGF ($p < 0.0001$). Incubation with RV-16 for 24 hours also increased concentrations of IL-6 ($p = 0.0001$), IL-8 ($p = 0.0005$), IFN-α ($p = 0.002$), RANTES ($p = 0.0005$), MIP-1β ($p < 0.0001$), MCP-1 ($p = 0.01$), EGF ($p = 0.008$) and HGF ($p = 0.008$). Infection of A549 cells with RV-16 did not affect supernatant concentrations of IL-1β, IL-2, IL-4, IL-5, IL-7, IL-10, IL-13, IL-15, IL-17, IL-1RA, TNF-α, MIG, IP-10, FGF-Basic, G-CSF or GM-CSF either 6 or 24 hours post-infection. Co-culture with $1,25(OH)_2D$ for 48 hours before a 6 hour incubation with RV-16 resulted in decreased concentrations of IL-2R ($p = 0.02$), IFN-γ ($p = 0.01$), RANTES ($p = 0.009$), MIP-1β ($p = 0.04$), EGF ($p = 0.04$), HGF ($p = 0.02$) and VEGF ($p = 0.005$) compared to cells co-cultured with vehicle alone preceding viral infection. However, the magnitude of the decrease was very small in all cases. These effects were not observed following a 24 hour incubation with RV-16, with co-culture of A549 cells with $1,25(OH)_2D$ resulting in an increased concentration of IL-6 ($p = 0.008$), although again by a very small magnitude (1.2-fold), while both 25(OH)D and $1,25(OH)_2D$ caused an increased concentration of FGF-Basic in the supernatant of cultured A549 cells (2-fold, $p = 0.04$ for both).

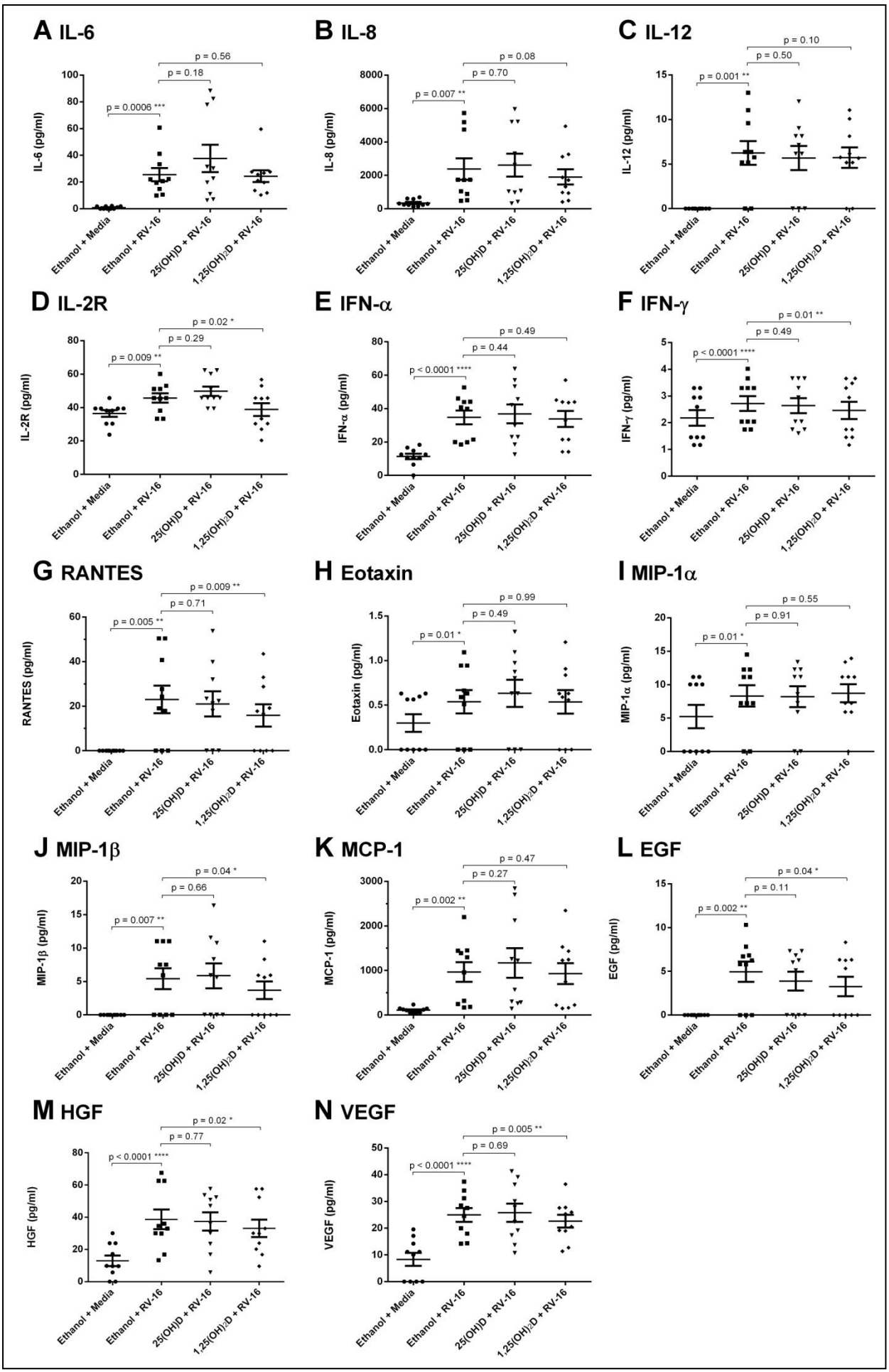

A IL-6
IL-6 (pg/ml)
p = 0.0006 ***
p = 0.18
p = 0.56
B IL-8
IL-8 (pg/ml)
p = 0.007 **
p = 0.70
p = 0.08
C IL-12
IL-12 (pg/ml)
p = 0.001 **
p = 0.50
p = 0.10
D IL-2R
IL-2R (pg/ml)
p = 0.009 **
p = 0.29
p = 0.02 *
E IFN-α
IFN-α (pg/ml)
p < 0.0001 ****
p = 0.44
p = 0.49
F IFN-γ
IFN-γ (pg/ml)
p < 0.0001 ****
p = 0.49
p = 0.01 **
G RANTES
RANTES (pg/ml)
p = 0.005 **
p = 0.71
p = 0.009 **
H Eotaxin
Eotaxin (pg/ml)
p = 0.01 *
p = 0.49
p = 0.99
I MIP-1α
MIP-1α (pg/ml)
p = 0.01 *
p = 0.91
p = 0.55
J MIP-1β
MIP-1β (pg/ml)
p = 0.007 **
p = 0.66
p = 0.04 *
K MCP-1
MCP-1 (pg/ml)
p = 0.002 **
p = 0.27
p = 0.47
L EGF
EGF (pg/ml)
p = 0.002 **
p = 0.11
p = 0.04 *
M HGF
HGF (pg/ml)
p < 0.0001 ****
p = 0.77
p = 0.02 *
N VEGF
VEGF (pg/ml)
p < 0.0001 ****
p = 0.69
p = 0.005 **
Ethanol + Media
Ethanol + RV-16
25(OH)D + RV-16
1,25(OH)2D + RV-16

Figure 4.7: Effect of co-culture with vitamin D metabolites and RV-16 infection for 6 hours on inflammatory mediator concentration in supernatants. A549 cells were cultured overnight, before co-culture with vitamin D metabolites or control for 48 hours. Following this, cells were incubated with RV-16 for 6 hours before supernatants were collected and multiplex ELISA was carried out. Supernatants were collected from 3 repeats of the same experiment, and n=10 samples were analysed for each variable. Data are expressed as scatter plots with mean + SEM, and p-values were calculated using a paired T-test.

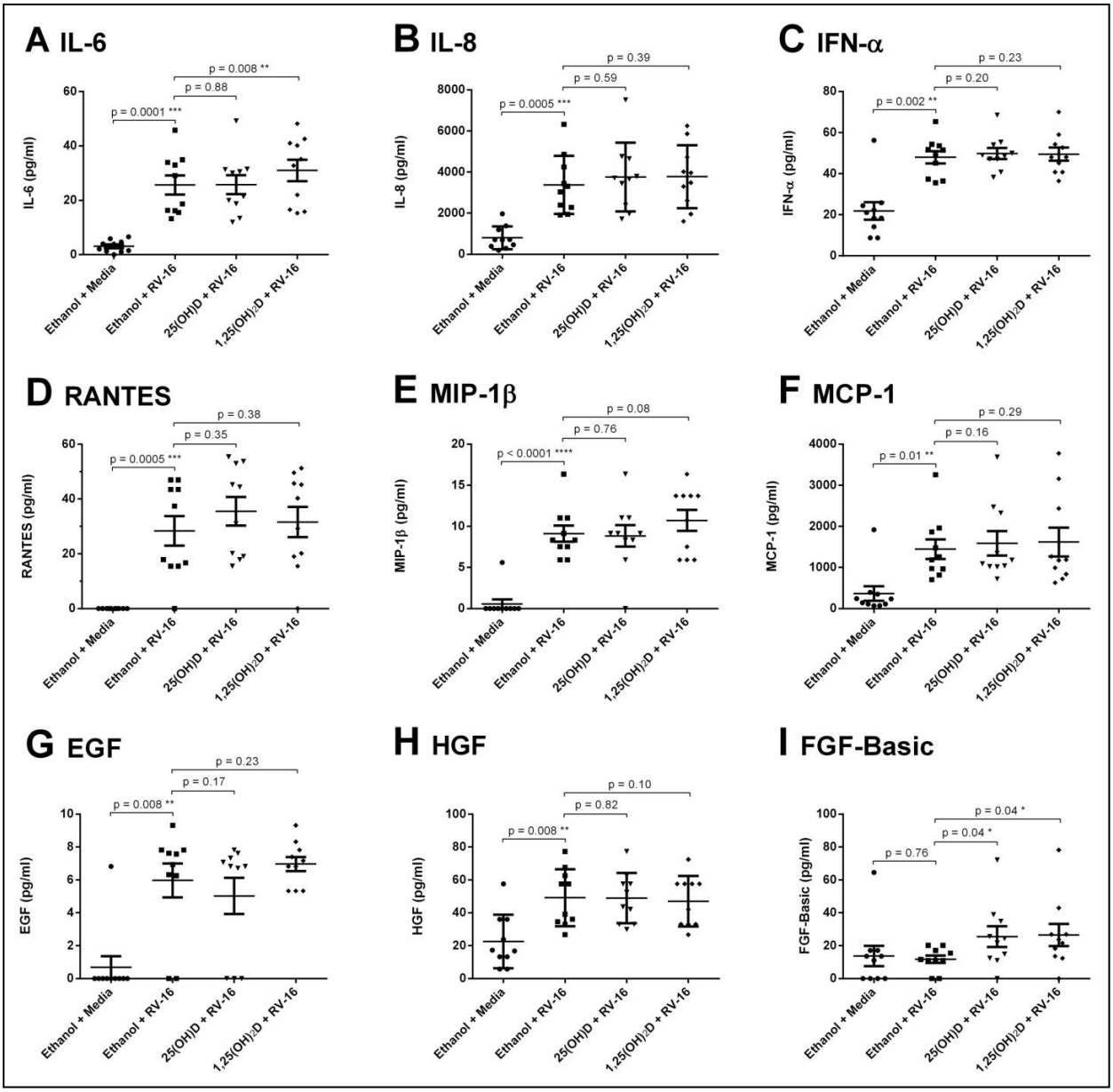


Figure 4.8: Effect of co-culture with vitamin D metabolites and RV-16 infection for 24 hours on inflammatory mediator concentration in supernatants. A549 cells were cultured overnight, before co-culture with vitamin D metabolites or control for 48 hours. Following this, cells were incubated with RV-16 for 24 hours before supernatants were collected and multiplex ELISA was carried out. Supernatants were collected from 3 repeats of the same experiment, and n=10 samples were analysed for each variable. Data are expressed as scatter plots with mean + SEM, and p-values were calculated using a paired T-test. Concentrations below the lower limit of detection for each analyte, as specified by the technical data sheet, were recorded as 0.

4.2.8 Co-culture of A549 cells with vitamin D metabolites increases A549 viability 24 hours post RV-16 infection

Control experiments were carried out to check that the observed transient effect of vitamin D metabolites on viral mRNA expression was not due to vitamin D directly effecting A549 viability. The lactate dehydrogenase (LDH) assay can be used to assess cytotoxicity *in vitro*. It is based on the reduction of nicotinamide adenine dinucleotide (NAD^+) to NADH by LDH, which is utilized in the stoichiometric conversion of a tetrazolium dye, with the resulting coloured compound measured spectrophotometrically. LDH is a cytosolic enzyme present in many different cell types. When the plasma membrane is damaged, for example following infection, LDH is released into the culture media, allowing the reduction of NAD^+, and the detection of the resulting coloured compound. A549 cells were co-cultured with vitamin D metabolites or vehicle (0.1% ethanol) for 48 hours before incubation for 6 or 24 hours with RV-16 or controls (filtered virus or UV-inactivated virus). Supernatants were aspirated and analysed by a lactate dehydrogenase-based toxicology assay, as described in Chapter 2.8.3, to determine the effects of vitamin D metabolites on A549 cell viability (Figure 4.9). Three technical replicates were used for each sample, from which an average was taken, and the experiment was repeated 3 times to provide three biological replicates. Due to only having n = 3 biological replicates, statistical tests could not be performed on these data. Infection of A549 cells with RV-16 for both 6 and 24 hours resulted in increased cellular cytotoxicity, regardless of pre-incubation with vehicle by between 2 and 3.5-fold. Vitamin D metabolites did not affect RV-induced cytotoxity 6 hours post infection compared to cells co-cultured with vehicle. However, at 24 hours post-infection, vitamin D metabolites reduced cytotoxicity compared to A549 cells co-cultured with vehicle, with a 28% reduction in cytotoxicity of 25(OH)D-treated RV-infected cells vs. vehicle-treated RV-infected cells, and a 20% reduction in cytotoxicity of $1,25(OH)_2D$-treated RV-infected cells vs. vehicle-treated RV-infected cells.

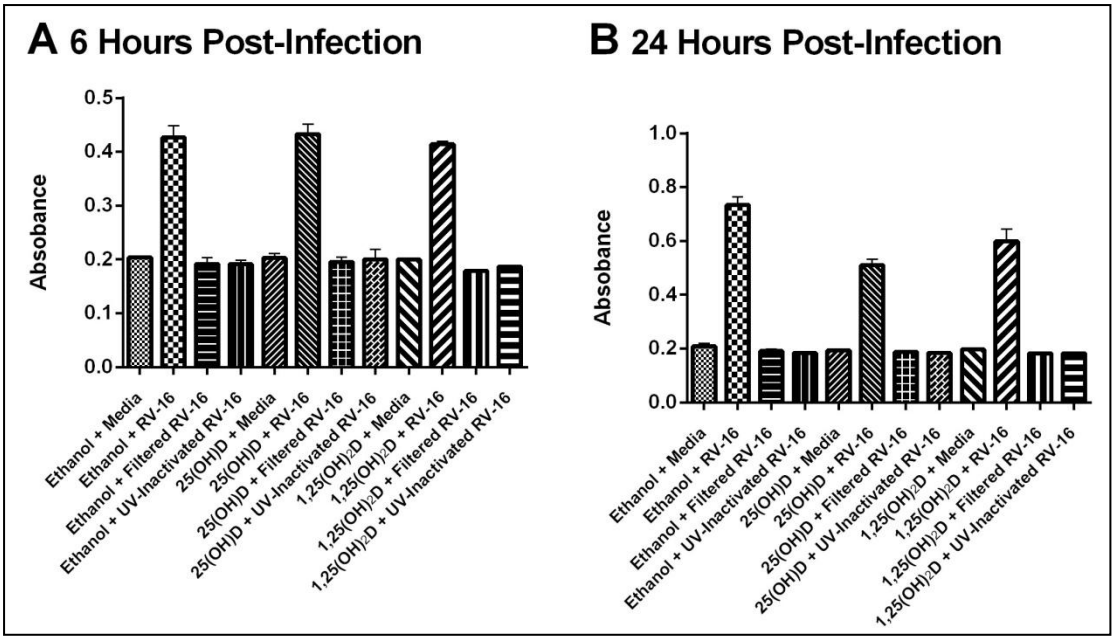


Figure 4.9: Effect of co-culture with vitamin D metabolites and RV-16 infection on A549 cytotoxicity. A549 cells were cultured overnight, before co-culture with vitamin D metabolites or vehicle control for 48 hours. Following this, cells were incubated with RV-16 or controls of filtered virus and UV-inactivated virus for 6 (A) or 24 (B) hours, and supernatants were aspirated. NAD^+ reduction by LDH was measured using a lactate dehydrogenase-based toxicology assay, with the reduced compound NADH enabling the stoichiometric conversion of a tetrazolium dye to produce a coloured compound to be measured spectrophotometrically and expressed as an absorbance value. Each reaction was carried out in triplicate and a mean value was taken of these technical replicates. Data are from 3 repeats of the same experiment, represented as mean + SEM. P-values could not be calculated due to the small sample size of n = 3.

4.2.9 Co-culture of A549 cells with vitamin D metabolites does not affect viral titre present in culture supernatant

An additional control experiment was carried out to assess whether the transient effect of vitamin D metabolites on viral mRNA expression was associated with a decreased release of the live virus into culture supernatant. Cytopathic effect assays are used to assess the damage done to host cells during viral invasion, thus allowing quantification of viral titre. This approach was utilised to determine whether co-culture with vitamin D metabolites affected viral release from A549 cells following infection. A549 cells were co-cultured with vitamin D metabolites or vehicle (0.1% ethanol) for 48 hours before incubation for 6 or 24 hours with RV-16. Supernatants were aspirated and stored, before being used in a cytopathic effect assay, as described in Chapter 2.8.4. Viral titres

(TCID/50) were calculated using the Spearman-Kaerber method [463, 464]. Co-culture with vitamin D metabolites did not significantly affect viral titre at either 6 or 24 hours post-infection, despite an observed trend towards an increase in TCID/50 in cells cultured with $1,25(OH)_2D$ compared to cells cultured with vehicle (Figure 4.10).

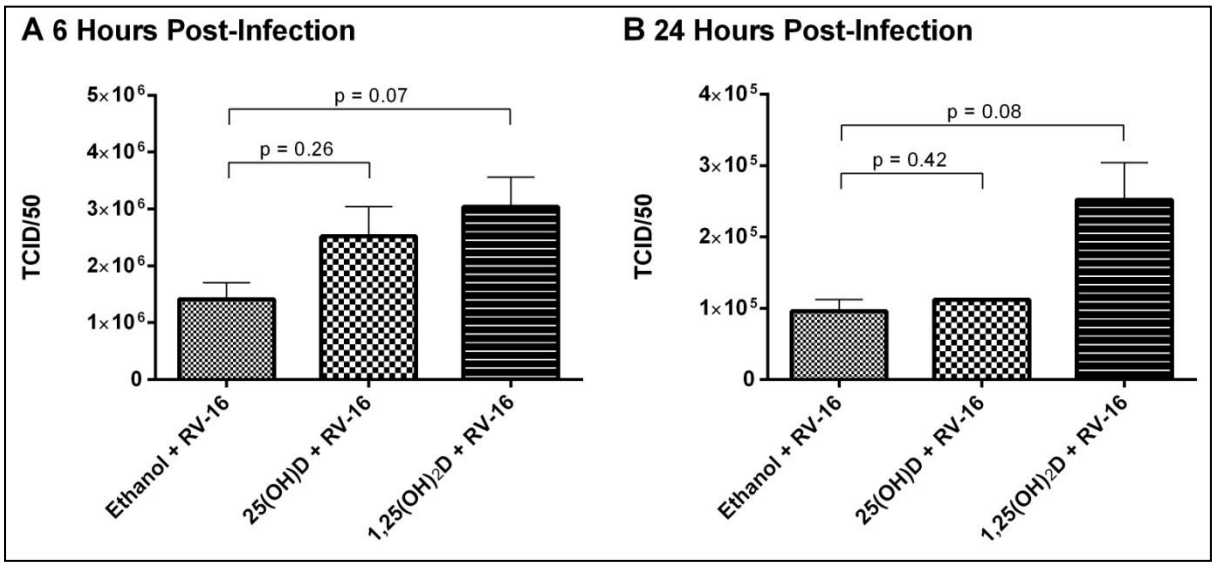


Figure 4.10: Effect of co-culture with vitamin D metabolites on supernatant viral titre following infection with RV. A549 cells were cultured overnight, before co-culture with vitamin D metabolites or control for 48 hours. Following this, cells were incubated with RV-16 for 6 (A) or 24 (B) hours and supernatants were aspirated. A cytopathic effect assay was carried out using supernatants harvested at each time point from which TCID/50 values were calculated. Data are expressed as mean + SEM, and P-values were calculated using a paired T-test.

4.2.10 CYP24A1 is highly expressed in A549 cells compared to CYP27B1

Vitamin D metabolism is regulated by the cytochrome p450 superfamily of enzymes. CYP27B1 is a 1-α-hydroxylase which is able to convert circulating 25(OH)D into the active form of $1,25(OH)_2D$. CYP24A1, a 24-hydroxylase enzyme, acts as part of a feedback mechanism to catabolise $1,25(OH)_2D$ into $1,24,25(OH)_3D$, as well as metabolising 25(OH)D to the relatively inactive metabolite $24,25(OH)_2D$. As such, the amount of active $1,25(OH)_2D$ is decreased. Therefore, the relative expression of these two enzymes has an impact on the activity of vitamin D. It has been described elsewhere that A549 cells express relatively high levels of CYP24A1, and relatively low levels of CYP27B1 [430], suggesting that vitamin D may not be able to exert any effects in A549 cells. As such, the relative expression of CYP24A1 compared to CYP27B1 was measured. A549 cells cultured overnight, before co-culture with vitamin D metabolites or vehicle (0.1% ethanol) for 48 hours. Cells

were lysed and, following RNA extraction and reverse transcription, RT-PCR was used to quantify CYP27B1 and CYP24A1 mRNA (Figure 4.11). Three technical replicates were used for each sample, from which an average was taken, and the experiment was repeated 3 times to provide three biological replicates. Due to only having n = 3 biological replicates, statistical tests could not be performed on these data. Using the CYP27B1 expression in the overnight culture of A549 cells as the denominator, it was evident that expression of CYP24A1 was much greater, with a 10^5-fold increases in CYP24A1 expression. 48 hour culture with vehicle or vitamin D metabolites did not affect expression of either CYP27B1 or CYP24A1.

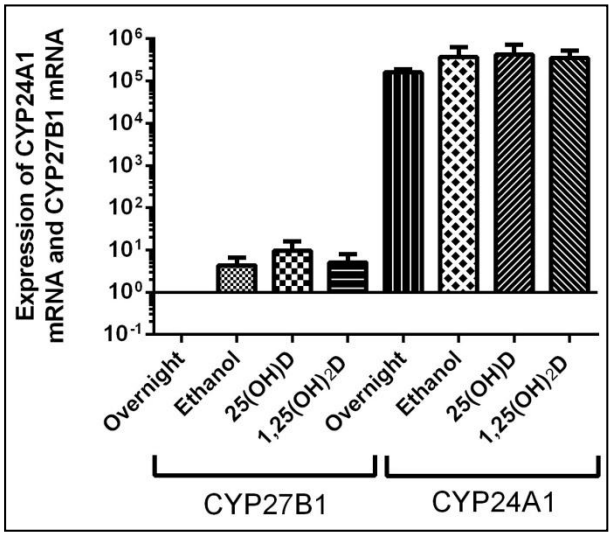


Figure 4.11: Expression of CYP27B1 and CYP24A1 in A549 cells. A549 cells were cultured overnight, before co-culture with vitamin D metabolites or control for 48 hours. CYP27B1 and CYP24A1 mRNA were quantified by RT-PCR and analysed using the ΔΔCT method. Each reaction was carried out in triplicate and a mean value was taken of these technical replicates. Data are from 3 repeats of the same experiment, represented as mean + SEM. P-values could not be calculated due to the small sample size of n = 3.

4.3 Discussion

It has been demonstrated here that co-culture with a physiological concentration of 25(OH)D renders A549 cells transiently resistant to infection with rhinovirus-16 following incubation with RV-16 for 6 hours. This effect was associated with attenuation of RV-induced ICAM-1 expression. Additionally, 25(OH)D was able to transiently inhibit RV-induced up-regulation of PafR and transiently reduce A549 cytotoxicity following RV infection.

On observing the significant reduction in viral mRNA following co-culture of A549 cells with vitamin D metabolites 6 hours post-infection (Figure 4.1), it was hypothesized that this outcome may be due to effects on the receptor ICAM-1, the main receptor for the major serotypes of rhinovirus, such as RV-16 [524, 525]. Infection with rhinovirus was seen to significantly increase ICAM-1 expression, both 6 hours and 24 hours post-infection, as also observed in other studies [457, 543-545], with vitamin D metabolites attenuating this RV-induced increase (Figure 4.2). Previously, RV-induced ICAM-1 expression has been demonstrated to be NF-κB-dependent [457], with ICAM-1 known to be one of the numerous genes regulated by this transcription factor [111, 457, 526]. The NF-κB family of transcription factors has a major role in the regulation of innate and adaptive immunity and inflammatory responses, with the p50/p65 heterodimer being the most ubiquitously expressed. In un-stimulated cells NF-κB dimers reside primarily in the cytoplasm in an inactive state due to association with members of the IκB (inhibitors of κB) family of proteins. While several non-receptor-mediated pathways such as UV-irradiation and oxidative stress have been demonstrated to activate NF-κB, the most common and best studied activation pathway used by the majority of stimuli is the canonical pathway. Ligation of receptors such as TLRs and TNF-R1 (TNF receptor-1) results in recruitment and activation of the IκB kinase (IKK) complex, which consists of two catalytically active kinases (IKKα and IKKβ) and a regulatory scaffold protein (IKKγ/NEMO). The activated IKK complex phosphorylates IκBα, leading to its polyubiquitination and subsequent proteasomal degradation. The degradation of IκBα results in the exposure of a strong nuclear localisation sequence in the p65 subunit of NF-κB, thus facilitating its translocation to the nucleus, where it can bind to κB motifs present in the promoter regions of many genes and regulate transcription [546-548]. It has previously been demonstrated that $1,25(OH)_2D$ is able to increase expression of the inhibitor IκBα in airway epithelial cells in a dose-dependent manner [327]. As such, it was hypothesized that the attenuation of RV-induced ICAM-1 expression resulting in decreased detection of viral mRNA may be due to effects of vitamin D metabolites on IκBα (Figure 4.12). Co-culture of A549 cells with vitamin D metabolites and subsequent incubation with RV-16 for 6 hours resulted in a 3-fold increase in IκBα mRNA expression, although statistical significance was not reached, and no increase was seen following incubation for 24 hours, suggesting potential time-dependent effects of vitamin D metabolites (Figure 4.3). Previous studies have also demonstrated an effect of $1,25(OH)_2D$ on inhibition of NF-κB activity, with an association with increased IκBα [327, 549].

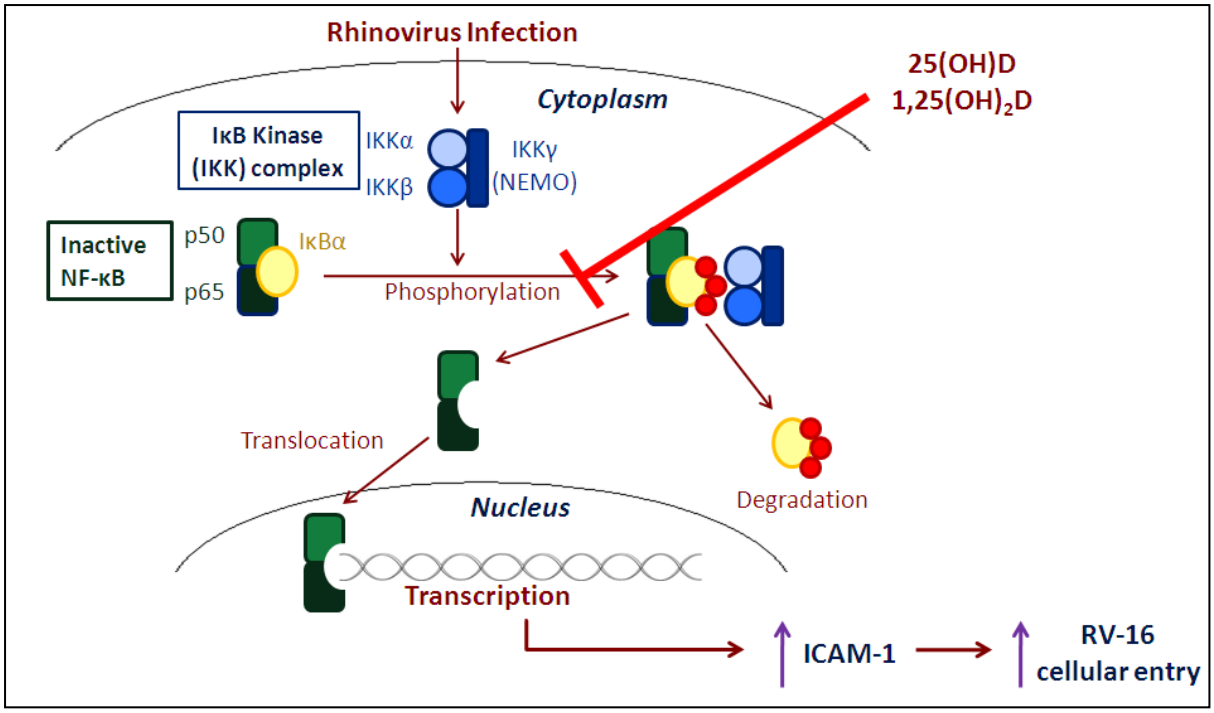


Figure 4.12: Hypothesized inhibition of NF-κB activation by vitamin D metabolites. Following rhinovirus infection, the IKK complex is activated to phosphorylate the inhibitor IκBα, resulting in its degradation. This allows NF-κB to translocate to the nucleus and bind to promoter regions, resulting in transcription of target genes such as ICAM-1, which in turn allows increased viral cellular entry. In the presence of vitamin D metabolites, the degradation of IκBα is inhibited, thus leaving NF-κB in its inactive state in the cytoplasm, attenuating RV-induced ICAM-1 expression and thus decreasing RV-16 cellular entry.

Abbreviations used: 1,25(OH)$_2$D: 1,25 di-hydroxyvitamin D; 25(OH)D: 25-hydroxyvitamin D; ICAM-1: intercellular adhesion molecule-1; IκBα: nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor alpha; IKK: inhibitor of NF-κB kinase; NF-κB: nuclear factor kappa B; RV: rhinovirus.

Other pathways known to be involved in anti-viral immunity were also investigated. The type I IFNs represent the major molecules associated with anti-viral immunity, with numerous functions including activation of DCs, induction of MHC class I expression, and stimulation of NK cells [550]. Incubation of A549 cells with RV-16 for 6 hours or 24 hours resulted in significantly reduced expression of IFN-α2, with the 24 hour time-point also resulting in decreased IFN-β mRNA (Figure 4.5). In contrast to this, other studies have demonstrated RV-induced production of type I IFNs in primary human bronchial epithelial cells (HBECs) [172, 551], BEAS-2B cells [482, 552], bronchoalveolar lavage (BAL) fluid [553], PBMCs [554], and bronchial smooth muscle cells [555]. However, the majority of these studies used minor group rhinoviruses [551, 552, 554, 555], which have been demonstrated to induce greater production of IFN-β in primary HBECs, while major group rhinovirus infection resulted in diminished production of IFN-β, with levels comparable to cells

stimulated with serum-free media [556]. Therefore, the differences seen in the study here compared to other published studies may be due to the serotype of rhinovirus used, with minor group rhinoviruses suggested to be more efficient at infecting cultured epithelial cells [557], and thus eliciting a stronger anti-viral immune response. In addition to this, while not finding a decrease in type I IFN production, two studies in A549 cells using major group rhinoviruses observed no induction of IFN-β following rhinovirus infection, which was attributed to impairment of IRF-3 activation due to inhibition of phosphorylation, prevention of homodimer formation and absence of nuclear accumulation [558, 559]. The inhibition of type I IFN production by rhinovirus may be an important evasion mechanism to allow replication and survival in host cells [560].

This study also demonstrated that A549 cells co-cultured with 25(OH)D resulted in increased expression of IFN-α2 6 hours post infection with RV-16, and co-culture with $1,25(OH)_2D$ resulted in increased IFN-β 24 hours post infection (Figure 4.5). Conversely, IFN-α2 expression was decreased by 25(OH)D 24 hours post-infection, and IFN-β expression was decreased by vitamin D metabolites 6 hours post-infection. These differences with the length of incubation with RV-16 may reflect the kinetics of the production of these type I IFNs, with IFN-α typically produced early on in the immune response, and IFN-β at a later time-point [482]. Together, this data suggests that the beneficial effects of vitamin D on the immune response to viruses may in part be due to an increase in type I IFN production. Conversely, vitamin D has been demonstrated elsewhere to decrease transcription of NF-κB driven genes such as IFN-β following infection of human tracheobronchial epithelial cells with RSV, with an associated reduction in IFN-stimulated proteins. However, in this case this was not observed to be detrimental, with the anti-viral state maintained and viral clearance unaffected [327].

While not typically associated with anti-viral immunity, LL-37 has been demonstrated to be important in the immune response to viruses [344, 356-358], and has been shown to be regulated by vitamin D [359, 530, 531]. Here it was demonstrated that co-culture with A549 cells with 25(OH)D and $1,25(OH)_2D$ significantly increased constitutive *CAMP* mRNA expression (Figure 4.4). Additionally, the positive control of $1,25(OH)_2D$ significantly increased *CAMP* expression following incubation with RV-16 for both 6 and 24 hours.

Another receptor, PafR, which has implications for virus-induced secondary bacterial infections, was also studied. The most cited example of the devastating impact of secondary bacterial infections is that of the 1918 influenza A pandemic, where an estimated 40-50 million deaths were attributable to secondary bacterial pneumonia [537, 561, 562]. Numerous mechanisms have been proposed to

explain this phenomenon, with one being the viral-induced up-regulation of receptors required for bacterial adhesion and colonization. Increasing evidence has demonstrated that vulnerability to bacterial pneumonia is mediated by the expression of bacterial receptors on lower airway cells, with blocking of PafR shown to attenuate infection [563]. PafR has been shown to be a receptor for *S.pneumoniae* and *H.influenzae*, and its expression has been shown to be increased by RSV, RV-14, influenza and coronavirus infections [88, 101, 102]. This study demonstrated RV-induced up-regulation of PafR at 6 hours post-infection, although infection for 24 hours resulted in decreased PafR expression (Figure 4.6). Both 25(OH)D and 1,25$(OH)_2$D were able to inhibit RV-induced up-regulation of PafR 6 hours post-infection. Viral-induced PafR has been associated with NF-κB activation [102, 109], suggesting that, similar to ICAM-1, vitamin D metabolites may be having their effects by increasing IκBα expression and thus inhibiting NF-κB activation.

Supernatants were also collected to measure the concentrations of a panel of inflammatory mediators. Rhinovirus infection induced up-regulation of the pro-inflammatory cytokines IL-6, IL-12 and IFN-γ, the chemokines IL-8, MIP-1α, MIP-1β, MCP-1, eotaxin and RANTES, the growth factors EGF, HGF and VEGF, and the anti-viral agent IFN-α, all important mediators of the immune response to acute viral infection (Figures 4.7 and 4.8). Co-culture of A549 cells with 1,25$(OH)_2$D demonstrated decreased concentrations of IL-2R, IFN-γ, RANTES, MIP-1β, EGF, HGF and VEGF 6 hours post-infection, compared to those cells cultured with vehicle. However, 1,25$(OH)_2$D was used as a positive control at concentrations 1000-times greater than seen in the circulation, with these responses not seen following co-culture with physiological concentrations of 25(OH)D. In addition to this, the magnitude of the differences observed was minimal, so, while effects such as vitamin D decreasing secretion of RANTES, VEGF and MIP-1β have been demonstrated elsewhere [564-566], these data are not conclusive.

Finally, supernatants were also utilised to assess the effects of vitamin D metabolites of cytotoxicity and viral release following RV infection. Viral incubation for both 6 and 24 hours resulted in a significant increase in A549 cytotoxicity (Figure 4.9). However, while co-culture with vitamin D metabolites had no effect on cytotoxicity 6 hours post-infection, at 24 hours post-infection both 25(OH)D and 1,25$(OH)_2$D inhibited RV-mediated cytotoxicity, although only by a small magnitude (1.4- and 1.2-fold respectively). Cell death following infection may be damaging to the host, with virions using the disrupted cell remnants as vehicles to spread and infect more cells. Conversely, cell death may also be beneficial to the host by killing virions by destroying the reservoir in which the virus replicates in [567]. Picornaviruses (the family of viruses which rhinoviruses belong to) have been demonstrated to initiate cell death upon infection, suggesting that they utilise this mechanism

to facilitate viral progeny release [568]. As such, the 25(OH)D- and 1,25$(OH)_2$D-mediated reduction in cytotoxicity at 24 hours post-infection reported here may be advantageous to the host, with increased cellular viability reflecting less invasion by virions and thus containing the spread of disease. A cytopathic effect assay was also carried out to quantify the viral titre present in the cell culture supernatants following infection. Co-culture with vitamin D metabolites was demonstrated to have no effect on viral titre present in the supernatant (Figure 4.10). The disconnect between the observed reduction in intracellular RV mRNA expression 6 hours post-infection following co-culture with vitamin D metabolites, and the lack of any effect of vitamin D metabolites on the viral titre in culture supernatants at 6 hours post-infection could be due to a lack of power. The effect on viral mRNA was subtle, and as such a larger sample size for the cytopathic effect assay may have been required to detect any vitamin D metabolite-induced changes in supernatant viral titre.

The use of A549 cells in this study offers an advantage over the other work carried out in this PhD using blood, since they represent the site of infection. Additionally, with the discovery that epithelial cells are able to convert inactive 25(OH)D to 1,25$(OH)_2$D [430], the respiratory epithelium is likely to be a key component of the effects of vitamin D on the immune response to respiratory pathogens. However, A549 cells are a transformed cell-line, and as such are not as physiologically accurate as using primary cells. They are also derived from the alveoli and are a model of type II pneumocytes, which are typically associated with surfactant production, although they have also been demonstrated to have a key role in the immune response [569-572]. Additionally, they represent cells of the lower respiratory tract, although, while typically thought of as a pathogen of the upper respiratory tract, rhinovirus has been demonstrated to cause bronchiolitis and pneumonia in the lower respiratory tract [16, 17, 573]. Furthermore, the immune response mounted by different cell-lines may be dissimilar, with distinct pathways involved in LPS-induced activation of A549 cells and the bronchiolar-derived BEAS-2B cells [574]. This may play a role in observed differences in RV induction of type I IFNs in BEAS-2B and A549 cells as described above. Finally, A549 cells have been shown to have relatively high levels of CYP24A1 (the enzyme which catabolises active 1,25$(OH)_2$D), and relatively low levels of CYP27B1 (the enzyme which hydroxylases 25(OH)D to produce the active metabolite) [430], suggesting that vitamin D may not be able to exert any effects in A549 cells. As such, expression of CYP24A1 and CYP27B1 were measured in this study, demonstrating markedly increased expression of CYP24A1 compared to CYP27B1 (Figure 4.11). Nevertheless, CYP27B1 was detectable, and the effects shown following co-incubation of A549 cells with vitamin D metabolites suggest that 25(OH)D is able to be hydroxylated to the active form, and that 1,25$(OH)_2$D is not being catabolised before it can exert any effect. However, the abundance of CYP24A1 may explain why, even though 1,25$(OH)_2$D was being used as a positive control in this

study, its effects on expression of RV mRNA, and ICAM-1 were diminished compared to that exerted by 25(OH)D. High expression of CYP24A1 may also explain the transient effects of co-culture with vitamin D metabolites on viral mRNA expression, RV-induced PafR expression and A549 viability observed in this study. By 24 hours post-infection, the CYP24A1 may have catabolised all of the active $1,25(OH)_2D$, thus not replicating the results seen at 6 hours post-infection. Therefore, due to all of the factors described, to accurately determine the effects of vitamin D metabolites on the immune response to RV infection, it would be beneficial to utilise different cell-lines, such as BEAS-2B cells, in addition to primary cells.

There are also a number of additional experiments which would be useful to carry out to further elucidate the effects of vitamin D metabolites in the protection against acute respiratory tract infections. Aside from using different cell types, a wider variety of respiratory pathogens could also be utilised. As already discussed, different rhinovirus serotypes elicit distinct immune responses, so the effect of vitamin D metabolites on other common respiratory viruses such as RSV, influenza and coronavirus could also be investigated. In addition, bacterial pathogens could also be studied, especially with respect to the observed effects of vitamin D on PafR expression. An adhesion assay looking into the effects of vitamin D metabolites on virus-induced bacterial adhesion would help to establish the importance of vitamin D metabolites in preventing secondary bacterial infections. The involvement of the NF-κB activation pathway in the effects of vitamin D could also be further elucidated in a variety of ways. Firstly, phosphorylation of IκBα could be determined by western blot, to assess whether vitamin D is increasing IκBα mRNA by inhibiting its phosphorylation and degradation, thus resulting in reduced activation of NF-κB. Silencing and antagonism of IκBα, ICAM-1, type I IFNs and LL-37 would also help to determine how important each of these components are in the observed vitamin D mediated resistance to RV infection. Finally, detection of the type III IFN, IFN-λ, would be advantageous in addition to the observed effects on type I IFNs. Type III IFNs have been demonstrated to be induced by viruses and play an important role in anti-viral immunity [575-578]. Many of the studies already discussed demonstrated RV-induced production of IFN-λ [482, 551, 552, 555], with its expression shown to be higher and more prolonged than that of the type I IFNs, and also more commonly associated with bronchial epithelial cells [482, 551]. As such, the effect of vitamin D on RV-induced IFN-λ would be interesting.

Therefore, here it has been demonstrated that co-culture with a physiological concentration of 25(OH)D, using $1,25(OH)_2D$ as a positive control, renders A549 cells transiently resistant to infection with rhinovirus-16 6 hours post-infection. This effect was associated with attenuation of RV-induced ICAM-1 expression, as well as an increase in *CAMP* expression and an altered type I IFN response.

Co-culture with 25(OH)D was also able to transiently significantly inhibit RV-induced up-regulation of PafR, with implications in the prevention of secondary bacterial infections.

5. Characterisation of the effects of vitamin D supplementation on the cellular profile of the lower airways and the production of induced sputum inflammatory mediators in people with asthma or COPD

5.1 Introduction

Sputum is the expectorated secretion of the lower respiratory tract, containing fluid saliva, cellular components, and inflammatory mediators such as cytokines and chemokines. It is a useful tool for profiling the airways, and is safer, less invasive and better tolerated than BAL (bronchoalveolar lavage) fluid, which involves a bronchoscope being passed into the airways, whist still providing reliable results [579-583]. Asthma and COPD are diseases of chronic airway inflammation, and thus assessment of the cells and inflammatory mediators present in the respiratory tract is important in determining the pathophysiology of these diseases and risk of exacerbation [584-587]. In recent years improvements in the methodology of collecting and processing sputum have aided in the importance of this technique. The use of hypertonic saline nebulisation has enabled individuals unable to spontaneously produce a sample to expectorate an induced sample, safety has been enhanced with monitoring of lung function throughout the procedure and pre-medication with β2-agonists, and methodology has improved with the identification of DL-Dithiothreitol (DTT) as an agent to disperse cells, the introduction of selecting cells from the expectorate rather than using the whole sample to reduce the confounding influence of saliva, and the use of cytospins to examine the cells microscopically [587-591]. With these advances, the use of induced sputum to assess airway inflammation is reproducible, and provides accurate and valid results [455].

As described in Chapter 1, vitamin D is an immunomodulatory agent which has been linked to respiratory health. Numerous observational studies have indicated a beneficial effect of vitamin D, with deficiency associated with a greater risk of acute respiratory tract infections [260, 272, 274, 279], longer duration of respiratory illnesses [279, 282], increased risk of asthma exacerbations [290], and impaired lung function [272, 293]. Intervention studies have also demonstrated that vitamin D supplementation reduces the incidence of respiratory tract infections [300, 303-305], and asthma and COPD exacerbations [303, 306-308]. However, where the immunomodulatory actions of *in vivo* supplementation have been assessed, peripheral blood has been used, which does not represent the site of infection when investigating respiratory health. The one study which did carry out sputum inductions on trial participants to determine whether the clinical effects of vitamin D

supplementation observed were associated with changes in the underlying inflammatory phenotype of the airways only looked at differential cell counts, with no analysis of inflammatory mediators in sputum supernatants [315]. Additionally, mechanistic studies into the effects of vitamin D on the immune response have largely been carried out in mice or using cell lines, and thus may not accurately represent the changes occurring in an *in vivo* system.

Therefore, the effect of vitamin D supplementation on the underlying inflammatory profile of the respiratory tract was investigated in people with asthma or COPD, in order to help determine whether it may be a useful therapy in improving respiratory health and preventing exacerbations and acute upper respiratory tract infections.

For the analysis of sputum inflammatory mediators and the subsequent analysis of peripheral blood as presented in later chapters, a 30-plex ELISA platform was used. Vitamin D has been demonstrated to have pleiotropic effects, and as such it was desirable to asses a range of inflammatory mediators to include Th1 and Th2 cytokines, innate cytokines, chemokines, type I IFNs and growth factors. Therefore, a 30-plex panel was chosen, allowing quantification of the following: IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8 (CXCL8), IL-10, IL-12, IL-13, IL-15, IL-17, IL-1RA, IL-2R, IFN-α, IFN-γ, TNF-α, MCP-1 (CCL2), MIP-1α (CCL3), MIP-1β (CCL4), RANTES (CCL5), eotaxin (CCL11), MIG (CXCL9), IP-10 (CXCL10), EGF (epidermal growth factor), FGF-basic (basic fibroblast growth factor), HGF (hepatocyte growth factor), VEGF (vascular endothelial growth factor), G-CSF (granulocyte colony-stimulating factor) and GM-CSF (granulocyte macrophage colony-stimulating factor).

In this chapter, in order to increase the power with which to characterise the effects of vitamin D supplementation on the cellular profile of the lower airways and the production of induced sputum inflammatory mediators, data from asthma and COPD patients were pooled. Asthma and COPD were also analysed separately, with no differences demonstrated, and this data is not presented for presentational ease. Therefore, data from asthma and COPD patients were pooled, accepting that these are markedly different diseases.

Primary clinical results for the ViDiAs and ViDiCO trials

While not in the scope of this thesis to expand on, the primary results of the clinical trials provide important information on the physical effects of vitamin D supplementation from which to compare the immunological results to. We aimed here to carry out a study whereby any effect of vitamin D

supplementation on primary outcomes such as time to first upper respiratory tract infection (URTI) could be mechanistically associated with observed changes in inflammatory markers and cellular profiles of induced sputum samples. As such, a summary of the primary outcomes of the ViDiAs (Table 5.1) and ViDiCO (Table 5.2) trials is provided (results not yet published). In addition to these primary findings, when stratifying by baseline vitamin D deficiency (defined as a serum 25(OH)D concentration < 50nmol/L), vitamin D supplementation significantly reduced the risk of moderate or severe exacerbation in the n = 148 COPD patients who were vitamin D deficient at baseline (aHR 0.57, 95% CI 0.35 to 0.92, p = 0.021).

Table 5.1: Clinical and biochemical outcomes by allocation in the ViDiAs trial

		Intervention	Placebo	Adjusted hazard ratio / odds ratio / incidence rate ratio / mean difference	95% CI	P-value
Median days to first severe exacerbation (IQR)		-- (192 to --)	-- (136 to --)	1.02	0.69 to 1.53	0.91
Percentage of patients with ≥1 severe exacerbation		46	41	1.20	0.70 to 2.05	0.52
Rate of severe exacerbations per participant-year		1.16	1.24	0.93	0.57 to 1.51	0.77
Percentage of moderate/severe exacerbations associated with URI		34	30	1.04	0.40 to 2.70	0.96
Mean peak asthma symptom score per severe exacerbation (s.d.)		1.60 (1.22)	1.67 (1.13)	-0.13	-0.52 to 0.26	0.51
Median days to first URI (IQR)		128 (42 to --)	112 (40 to 309)	0.87	0.64 to 1.16	0.34
Percentage of patients with ≥1 URI		74	79	0.71	0.38 to 1.32	0.28
Rate of URI per participant-year		1.99	2.14	0.89	0.70 to 1.14	0.35
Mean peak Jackson symptom score per URI (s.d.)		11.5 (5.1)	10.5 (4.7)	0.76	-0.47 to 1.99	0.23
ACT score, mean (s.d.)	2 mo	20.1 (3.9)	19.5 (4.4)	0.52	-0.27 to 1.31	0.56
	6 mo	20.6 (3.5)	20.6 (3.8)	0.00	-0.81 to 0.81	
	12 mo	20.4 (4.0)	20.4 (4.2)	0.00	-0.82 to 0.82	
FEV1 (% predicted), mean (s.d.)	2 mo	81.5 (18.7)	81.6 (20.4)	-0.95	-3.71 to 1.81	0.53
	6 mo	80.6 (21.0)	81.0 (20.8)	-1.56	-4.37 to 1.26	
	12 mo	81.6 (18.5)	80.1 (22.8)	0.44	-2.42 to 3.30	
Morning PEFR (mean over period since preceding study visit, mean (s.d.)	2 mo	380.6 (108.6)	375.2 (121.2)	-0.2	-8.1 to 7.7	0.38
	6 mo	385.0 (118.6)	382.9 (122.4)	-5.3	-13.4 to 2.8	
	12 mo	388.1 (116.8)	387.7 (122.9)	-5.4	-13.6 to 2.8	
Fractional exhaled nitric oxide, ppb, mean (s.d.)	2 mo	36.1 (25.7)	36.5 (31.7)	-1.3	-6.4 to 3.8	0.71
	6 mo	36.7 (28.8)	34.6 (32.4)	1.6	-3.7 to 6.8	
	12 mo	37.5 (26.9)	38.5 (36.5)	-1.4	-6.8 to 3.9	
Median SGRQ total score (IQR)	2 mo	16.0 (8.5 to 26.2)	17.3 (8.9 to 30.1)	0.87	0.79 to 0.96	0.03
	6 mo	14.7 (7.5 to 24.4)	14.6 (7.1 to 29.7)	0.90	0.82 to 0.99	
	12 mo	13.6 (7.1 to 24.7)	13.9 (7.2 to 25.0)	0.91	0.82 to 1.00	
Mean serum 25(OH)D, nmol/L (s.d.)	2 mo	61.2 (22.1)	48.5 (25.0)	12.5	7.6 to 17.3	<0.001
	12 mo	69.4 (21.0)	46.5 (24.6)	23.0	17.9 to 28.0	
Mean serum PTH, pmol/L (s.d.)	2 mo	5.69 (2.51)	6.19 (2.56)	-0.62	-1.35 to 0.12	0.04
	12 mo	5.50 (2.31)	6.31 (2.87)	-0.89	-1.66 to -0.13	

-- represents undefined values

Asthma symptoms scored from 0 (no symptoms) to 3 (severe symptoms); Jackson symptoms (sneezing, sore throat, headache, subjective sensation of fever or chilliness, malaise, nasal discharge, nasal obstruction, cough) each scored from 0 (no symptoms) to 3 (severe symptoms) and summed for each day of URI.

Abbreviations used: CI: confidence interval; IQR: inter-quartile range; URI: upper respiratory infection; ACT: asthma control test; s.d.: standard deviation; FEV1: forced expiratory volume in one second; PEFR: peak expiratory flow rate; ppb: parts per billion; PTH: parathyroid hormone; SGRQ: St George's Respiratory Questionnaire

Table 5.2: Clinical and biochemical outcomes by allocation in the ViDiCO trial

		Intervention	Placebo	Adjusted hazard ratio / odds ratio / incidence rate ratio / mean difference	95% CI	P-value
Median days to first moderate/severe exacerbation (IQR)		-- (87 to --)	278 (89 to --)	0.86	0.60 to 1.24	0.42
Percentage of patients with ≥1 moderate/severe exacerbation		54	64	0.69	0.39 to 1.23	0.21
Rate of moderate/severe exacerbations per participant-year		1.04	1.11	0.94	0.67 to 1.30	0.70
Percentage of moderate/severe exacerbations associated with URI		36	45	0.39	0.13 to 1.18	0.10
Mean peak exacerbation symptom score per moderate/severe exacerbation (s.d.)		5.47 (1.53)	5.94 (1.42)	-0.50	-0.97 to -0.02	0.04
Median days to first URI (IQR)		212 (44 to --)	152 (44 to --)	0.95	0.69 to 1.31	0.75
Percentage of patients with ≥1 URI		75	73	1.15	0.61 to 2.17	0.66
Rate of URI per participant-year		1.63	1.97	0.85	0.64 to 1.15	0.29
Mean peak Jackson symptom score per URI (s.d.)		10.1 (5.0)	11.2 (5.1)	-1.0	-2.3 to 0.4	0.16
Mean post-bronchodilator FEV1, % predicted (s.d.)	2 mo	62.9 (20.5)	65.5 (22.9)	-1.1	-2.9 to 0.7	0.68
	6 mo	60.9 (20.0)	63.5 (21.6)	-0.5	-2.4 to 1.4	
	12 mo	63.3 (19.6)	64.2 (21.8)	-0.5	-2.4 to 1.5	
Mean post-bronchodilator FVC, % predicted (s.d.)	2 mo	94.5 (17.6)	98.5 (22.4)	-2.6	-5.4 to 0.3	0.34
	6 mo	93.8 (18.4)	95.6 (19.5)	-1.1	-4.0 to 1.9	
	12 mo	93.4 (18.5)	94.9 (19.6)	-0.5	-3.6 to 2.6	
Mean SGRQ total score (s.d.)	2 mo	43.6 (18.5)	47.5 (19.7)	0.37	-2.42 to 3.16	0.74
	6 mo	44.1 (18.5)	46.6 (19.4)	1.28	-1.63 to 4.19	
	12 mo	43.4 (19.0)	45.7 (21.2)	1.45	-1.55 to 4.45	
Mean serum 25(OH)D, nmol/L (s.d.)	2 mo	57.9 (22.7)	47.1 (25.2)	10.8	5.6 to 16.1	< 0.001
	12 mo	67.4 (27.5)	47.1 (26.9)	19.8	14.0 to 25.5	
Mean serum PTH, pmol/L (s.d.)	2 mo	5.98 (2.46)	6.39 (2.79)	-0.49	-0.95 to -0.03	0.03
	12 mo	4.16 (2.09)	4.81 (2.21)	-0.58	-1.07 to -0.08	

-- represents undefined values

Exacerbation symptom score calculated by coding 7 exacerbation symptoms (increase in dyspnoea, sputum volume, sputum purulence, nasal congestion/discharge, wheeze, sore throat or cough) as 0 (symptom absent) or 1 (symptom present) and summing the scored to give a daily symptom score between 0 and 7; Jackson symptoms (sneezing, sore throat, headache, subjective sensation of fever or chilliness, malaise, nasal discharge, nasal obstruction, cough) each scored from 0 (no symptoms) to 3 (severe symptoms) and summed for each day of URI.

Abbreviations used: CI: confidence interval; IQR: inter-quartile range; URI: upper respiratory infection; s.d.: standard deviation; FEV1: forced expiratory volume in one second; FVC: forced vital capacity; PTH: parathyroid hormone; SGQR: St George's Respiratory Questionnaire

5.2 Results

5.2.1 Study recruitment

As described in chapter 2.1, a subset of participants recruited onto the ViDiAs and ViDiCO clinical trials were invited to give induced sputum samples. These samples were collected during randomisation (month 0), and at 2 and 12 month follow-up visits, to enable characterisation of baseline characteristics, and determination of any effect caused by supplementation with vitamin D_3. Figure 5.1 and 5.2 show the recruitment profiles for ViDiAs and ViDiCO respectively, with randomisation, the number of samples collected at each time point, and the number of participants lost to follow-up represented. Of those randomised into the sputum arm in the ViDiAs trial, 48% of people were on the intervention arm, and 52% on the control (placebo) arm. Similar numbers of follow-up samples were collected at each time point between the two arms, with n = 19 and n = 16 producing a 2 month sample in each arm, and n = 18 and n = 15 producing a 12 month sample in each arm. Over the duration of the study, 6 participants in the intervention arm and 11 participants in the control arm withdrew consent, left the sputum arm or were lost to follow-up, with reasons including poor lung function results, inability to expectorate, and being unable to attend morning study visits to provide a sample. Rates of differential cell count success were also similar between arms and at each time-point, with success rates of over 90% at all time-points in the intervention arm, and at month 12 in the control arm. The success rate was marginally lower in the control arm at baseline (70%) and at 2 month follow-up (88%), with differential cell count failures due to high levels of squamous cell contamination obscuring other cell types and rendering cell counts impossible. The success rate of being able to carry out flow cytometry was more varied, ranging from 37% in the intervention arm at baseline, to 0% in the control arm at month 2. The most common reason for not being able to carry out flow cytometry was an insufficient cell count, with a minimum of 1 million cells needed after sputum processing and cytospin preparation to ensure enough cells for staining to be successful and accurate results to be yielded. Additionally, a subset of samples were taken before the implementation of flow cytometry.

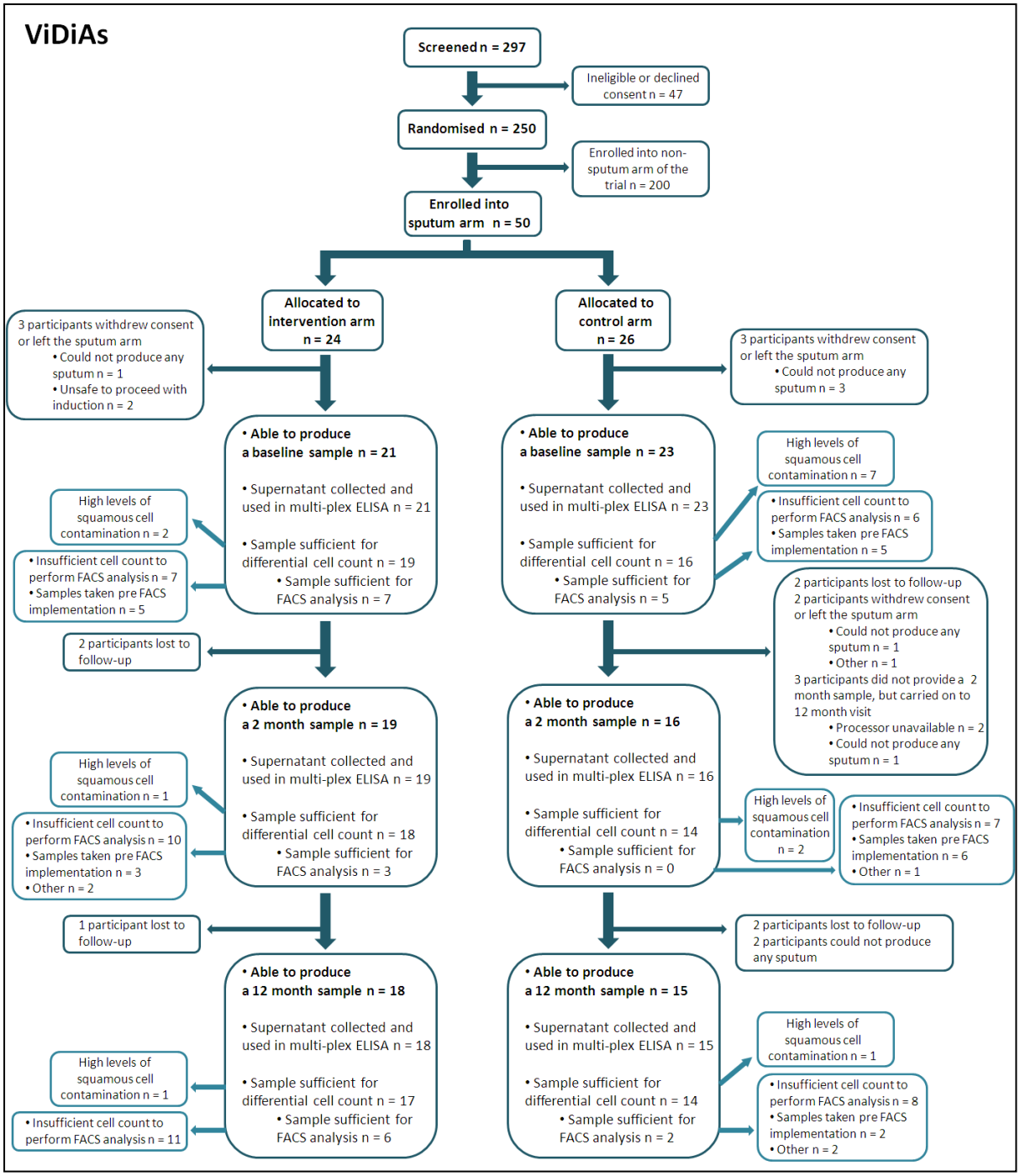


Figure 5.1: Recruitment profile for the sputum sub-study in asthma patients at baseline, month 2 and month 12, separated by allocation.

Of those randomised into the sputum arm in the ViDiCO trial (Figure 5.2), 56% of people were on the intervention arm, and 44% on the control arm. Similar numbers of follow-up samples were collected at each time point between the two arms, although numbers in the control arm were marginally smaller, with n = 21 and n = 16 producing a 2 month sample in each arm, and n = 18 and n = 12 producing a 12 month sample in each arm. Over the duration of the study, 10 participants in the intervention arm and 10 participants in the control arm withdrew consent, left the sputum arm or were lost to follow-up. Rates of differential cell count success were 100% at all time-points for both arms of the study. The success rate of being able to carry out flow cytometry was more varied, ranging from 65% in the control arm at baseline, to 33% in the intervention arm at month 2. The most common reason for not being able to carry out flow cytometry was an insufficient cell count, with a minimum of 1 million cells needed after sputum processing and cytospin preparation to ensure enough cells for staining to be successful and accurate results to be yielded. Additionally, a subset of samples were taken before the implementation of flow cytometry, and some were omitted due to time constraints, with study visits being carried out at an alternative site resulting in transportation times not allowing for the additional steps involved in preparing the samples for flow cytometric analysis. Rates of flow cytometry success were higher than in the asthma study, with COPD patients able to produce larger samples of sputum, thus containing higher numbers of cells.

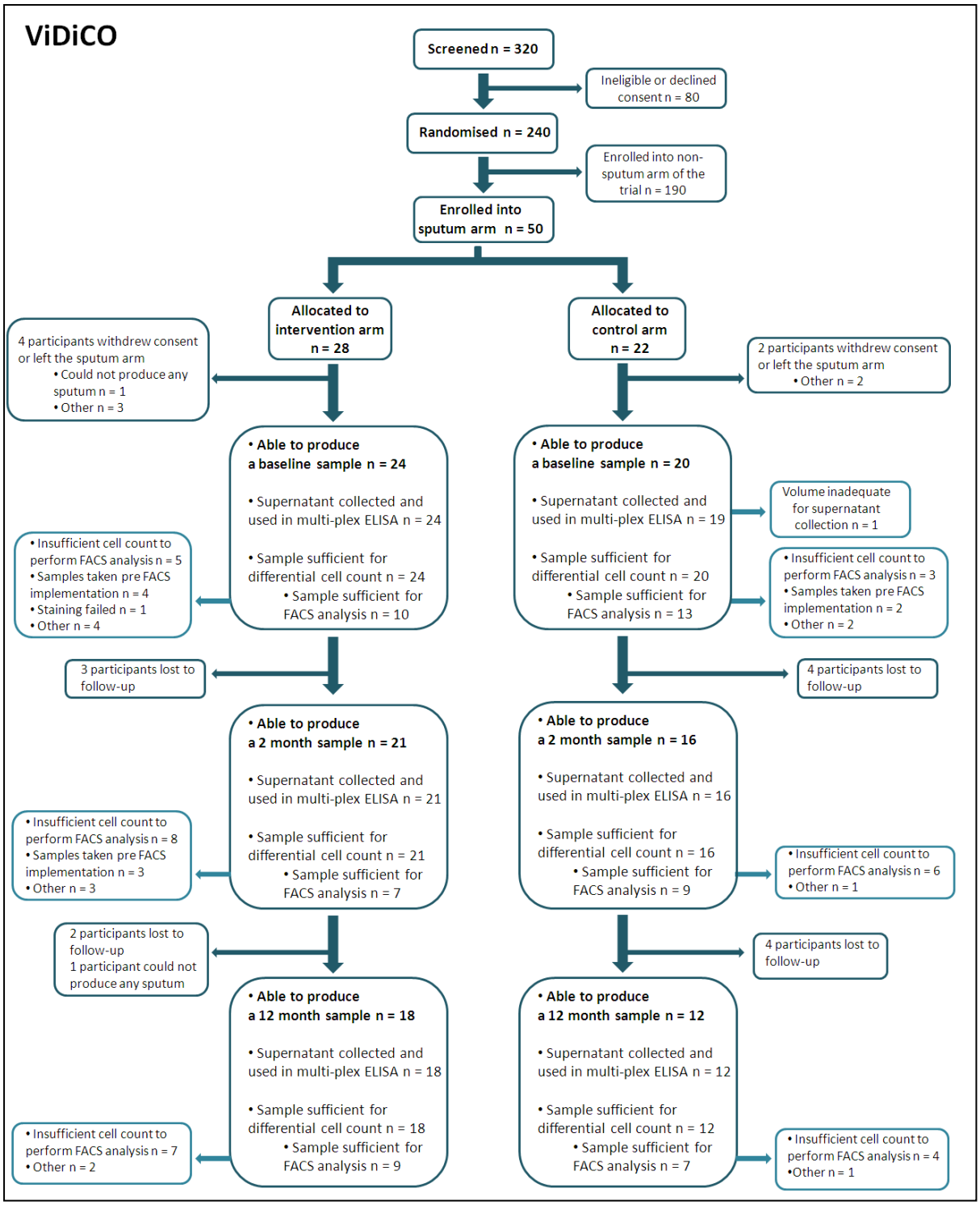


Figure 5.2: Recruitment profile for the sputum sub-study in COPD patients at baseline, month 2 and month 12, separated by allocation

5.2.2 Vitamin D supplementation results in a higher concentration of serum 25(OH)D in asthma patients, but not COPD patients in the sputum sub-study

The serum concentration of 25(OH)D was measured in all participants at baseline, 2 month and 12 month follow-up by liquid-chromatography-tandem mass spectrometry, as described in chapter 2.1.6 and elsewhere [456]. This was used to determine whether randomisation into the intervention arm of the study resulted in a significant increase in circulating serum 25(OH)D over the time-course of the study, and whether it resulted in a significantly higher concentration of serum 25(OH)D compared to participants in the placebo arm. In the ViDiAs study (Figure 5.3 A), there was a significantly higher concentration of mean serum 25(OH)D in the intervention arm compared to the placebo arm at both 2 months (mean 17.86 nmol/L difference 95% confidence interval [CI] 4.44 to 31.27, $p = 0.01$) and 12 months (mean 28.75 nmol/L difference, 95% CI 15.52 to 41.98, $p < 0.0001$). When compared to baseline concentrations, mean serum 25(OH)D was significantly higher at 2 month (mean 15.57 nmol/L difference, 95% CI 5.01 to 26.13, $p = 0.006$) and 12 month (mean 22.43 nmol/L difference, 95% CI 9,63 to 35.23, $p = 0.002$) follow-up in the intervention arm, with no change seen in the control arm. In the ViDiCO study (Figure 5.3 B), there was no difference in mean serum 25(OH)D concentration between the intervention arm and the control arm at each time-point (2 month: mean 2.27 nmol/L difference, 95% CI -9.91 to 14.45, $p = 0.71$; 12 month: mean 8.51 nmol/L difference 95% CI -7.13 to 24.15, $p = 0.28$). When compared to baseline concentrations, mean serum 25(OH)D concentration was significantly higher at 2 month (mean 15.20 difference, 95% CI 8.69 to 21.71, $p < 0.0001$) and 12 month (mean 20.50 nmol/L difference, 95% CI 9.86 to 31.14, $p = 0.0006$) follow-up in the intervention arm, and at 2 month follow-up in the control arm (mean 8.65 difference, 95% CI 1.14 to 16.16, $p = 0.03$).

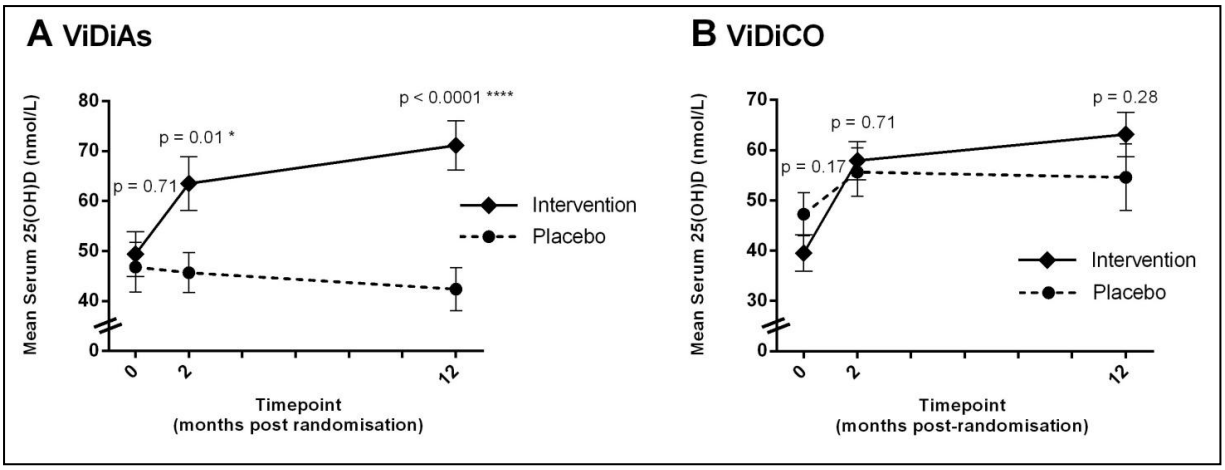


Figure 5.3: Change in serum 25(OH)D concentration over the course of the study for those enrolled in the intervention and placebo arms of the sputum sub-studies of the ViDiAs and ViDiCO trials. Serum samples were collected from trial participants at 0, 2 and 12 month visits and the concentration of 25(OH)D was measured by liquid-chromatography-tandem mass spectrometry. Data are expressed as means + SEM, and are only from participants who were enrolled into the sputum sub-study (n = 50 for ViDiAs and n = 50 for ViDiCO). P-values were calculated using an unpaired T-test.

As well as being an immunomodulator, vitamin D has a well established role in calcium homeostasis. The active metabolite $1,25(OH)_2D$ is able to increase intestinal calcium absorption, thus raising the concentration of calcium in the extracellular fluid. Extracellular calcium concentration is monitored by the calcium-sensing receptor (CaSR). Decreased calcium stimulates the secretion of parathyroid hormone (PTH) from the parathyroid glands. PTH is able to stimulate osteoclastic activity to promote bone reabsorption, increase tubular calcium reabsorption in the kidneys, and stimulate the transcription of the CYP27B1 gene, while also suppressing renal CYP24 mRNA expression, resulting in an increased concentration of $1,25(OH)_2D$. The resulting increase in extracellular calcium inhibits PTH secretion, thus making a negative feedback loop [592-594]. There is an inverse relationship between PTH and 25(OH)D, and therefore PTH is a useful alternative marker for vitamin D status. As such, PTH was analysed in COPD patients in the sputum sub-study to determine whether, despite observing a non-significant increase in serum 25(OH)D in the intervention arm, supplementation did significantly decrease serum PTH concentration (Figure 5.4). Blood was collected from all trial participants at baseline, 2 month and 12 month follow-up visits and serum PTH was quantified using an Architect ci8200 analyser (Abbott Diagnostics, Chicago, Illinois, USA). While the serum PTH concentration appeared to be lower in the intervention arm compared to the placebo arm at 12 months, the difference was not statistically significant (mean 1.10 pmol/L difference, 95% CI -0.15 to 2.35, p = 0.08). Compared to baseline, PTH concentration was significantly lower at 12 months in

the intervention arm (mean -1.46 pmol/L difference, 95% CI -2.21 to -0.71, p = 0.0005), while there was no difference in the placebo arm (mean -0.9 pmol/L difference, 95% CI -2.07 to 0.27, p = 0.12).

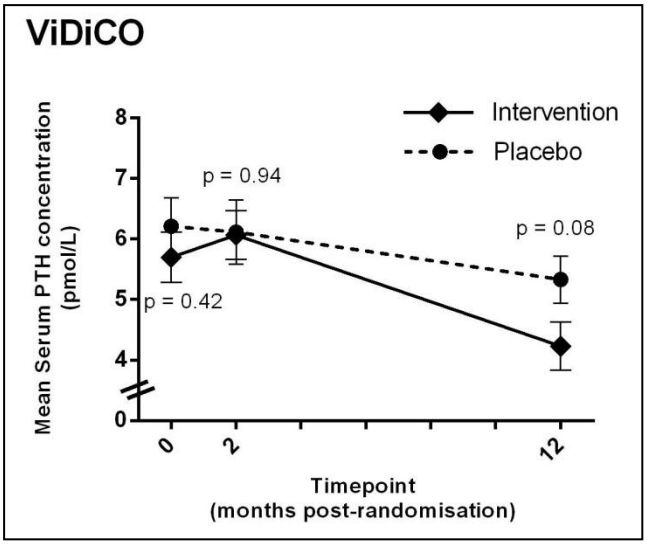


Figure 5.4: Change in serum PTH concentration over the course of the study for those enrolled in the intervention and placebo arms of the sputum sub-study of the ViDiCO trial. Serum samples were collected from trial participants at 0, 2 and 12 month visits and the concentration of PTH was measured using an Architect ci8200 analyser. *Data are expressed as means + SEM, and are only from participants who were enrolled into the sputum sub-study (n = 50). P-values were calculated using an unpaired T-test.*

5.2.3 Vitamin D supplementation has no effect on the cellular profile of the lower airways in patients with asthma or COPD

Induced sputum samples were collected and processed as described in Chapter 2.3 for participants in the sputum sub-study of ViDiAs and ViDiCO, at baseline, month 2 and month 12. Following cytospin preparation, differential cell counts were carried out, with averages taken from counts of 400 non-squamous cells from 3 cytospins for each participant at each time-point. Results were calculated as the percentage of each cell type counted, and also as absolute cell counts. Additionally, flow cytometry was carried out on a subset of samples at each time-point, as represented in Figures 5.1 and 5.2, to determine percentages and numbers of B-cells, $CD4^+$ T-cells, $CD8^+$ T-cells, myeloid dendritic cells (mDCs), plasmacytoid dendritic cells (pDCs) and regulatory T-cells (Tregs). The methodology is described in Chapter 2.4, with the gating techniques

demonstrated. Each data point was assigned an interaction variable for the time-point at which the sample was taken, and whether that participant was on the intervention or control arm of the study. Data for asthma and COPD patients were pooled for this analysis to provide greater power with which to assess any affect of vitamin D supplementation on the cellular profile of the lower airways. As described in Chapter 2.10, Qlucore Omics Explorer 2.3 was used to analyse the data, with the model using a multi-group analysis (i.e. repeated measures ANOVA) on interaction variables using log-transformed data, and including adjustment for time-point, allocation and study. P-values were deemed significant when less than 0.05 and with a corresponding q-value of less than 0.1. No statistically significant effect of vitamin D supplementation was observed on the cellular profile of the lower airways (Table 5.3).

Table 5.3: Effect of vitamin D supplementation on the cellular profiles of induced sputum in pooled data from asthma and COPD patients

Cell Type	Placebo 2 Month		Intervention 2 Month		Placebo 12 Month		Intervention 12 Month		Statistics		
	n	median	n	median	n	median	n	median	F-statistic	p-value	q-value
% Tregs	9	8.430	9	9.260	9	15.000	14	14.750	2.700	0.069	0.978
% Lymphocytes	30	0.540	39	0.500	26	0.625	35	0.500	2.162	0.118	0.978
% mDC	9	45.200	9	53.000	9	51.500	15	44.800	2.074	0.128	0.978
% B-cells	9	3.540	10	2.335	9	2.550	15	3.180	2.003	0.137	0.978
Absolute Epithelial Cells	30	16232.933	39	15994.080	26	23133.982	35	10794.420	1.317	0.270	0.978
% Epithelial Cells	30	0.830	39	0.580	26	0.750	35	0.750	1.023	0.361	0.978
Absolute CD4 T-cells	9	5730.361	10	1565.635	9	2714.450	15	1284.048	0.732	0.482	0.978
Absolute B-cells	9	1230.978	10	360.983	9	490.672	15	644.248	0.694	0.501	0.978
% Neutrophils	30	67.000	39	61.750	26	65.000	35	68.330	0.539	0.584	0.978
% CD8 T-cells	9	15.100	10	17.650	9	19.900	15	23.800	0.518	0.597	0.978
%pDC	9	6.400	9	6.430	9	3.730	15	8.080	0.464	0.629	0.978
Absolute CD8 T-cells	9	1191.692	10	516.758	9	1372.350	15	663.232	0.348	0.707	0.978
% Macrophages	30	24.625	39	32.420	26	30.625	35	25.830	0.332	0.718	0.978
Absolute mDC	9	403.248	9	275.865	9	378.444	15	245.050	0.323	0.724	0.978
% CD4 T-cells	9	70.300	10	71.150	9	61.300	15	57.500	0.281	0.756	0.978
Absolute Lymphocytes	30	8323.163	39	9028.418	26	12157.095	35	9441.530	0.245	0.783	0.978
% Eosinophils	30	3.040	39	1.580	26	2.290	35	1.250	0.222	0.801	0.978
Absolute Macrophages	30	468785.400	39	534737.650	26	626307.259	35	557710.610	0.219	0.804	0.978
Absolute pDC	9	65.478	9	33.487	9	41.670	15	38.272	0.217	0.805	0.978
Absolute Tregs	9	505.082	9	304.272	9	491.588	14	207.399	0.104	0.902	0.989
Absolute Neutrophils	30	1476323.563	39	823600.000	26	1263463.975	35	1273235.088	0.079	0.925	0.989
Absolute Eosinophils	30	43352.186	39	29859.000	26	32960.690	35	42593.250	0.011	0.989	0.989

5.2.4 Vitamin D supplementation has no effect on the concentrations of inflammatory mediators in induced sputum supernatants

Induced sputum samples were collected and processed as described in Chapter 2.3 for participants in the sputum sub-study of ViDiAs and ViDiCO, at baseline, month 2 and month 12. Supernatants were aspirated and stored at -80^{o}C, before being analysed by multiplex ELISA. As before, each data point was assigned an interaction variable for the month the sample was taken, and whether that participant was on the intervention or control arm of the study. Data for asthma and COPD patients were pooled for this analysis to provide greater power with which to assess any effect of vitamin D supplementation on induced sputum inflammatory mediators. Qlucore Omics Explorer 2.3 was used to analyse the data, modelling using a multi-group analysis (i.e. repeated measures ANOVA) on interaction variables with log-transformed data, and including adjustment for time-point, allocation and study. P-values were deemed significant when less than 0.05 and with a corresponding q-value of less than 0.1. No statistically significant effect of vitamin D supplementation was observed on induced sputum inflammatory mediators (Table 5.4).

Table 5.4: Effect of vitamin D supplementation on the concentrations of inflammatory mediators in induced sputum in pooled data from asthma and COPD patients

Analytes	Placebo 2 Month		Intervention 2 Month		Placebo 12 Month		Intervention 12 Month		Statistics		
	n	median	n	median	n	median	n	median	F-statistic	p-value	q-value
IFN-α	32	0.000	40	0.000	27	0.000	36	0.000	1.345	0.263	0.978
TNF-α	32	0.000	40	0.000	27	0.000	36	0.000	1.320	0.269	0.978
IL-4	32	1.251	40	4.303	27	1.188	36	6.259	1.305	0.273	0.978
MIG	32	0.000	40	0.000	27	0.000	36	0.000	1.138	0.322	0.978
IP-10	32	6.069	40	6.635	27	6.925	36	6.625	0.999	0.370	0.978
G-CSF	32	45.069	40	53.906	27	44.534	36	38.271	0.908	0.405	0.978
IL-15	32	0.000	40	5.968	27	6.841	36	6.841	0.902	0.407	0.978
IL-10	32	1.263	40	0.475	27	1.120	36	0.000	0.805	0.448	0.978
IL-6	32	28.159	40	23.443	27	26.815	36	21.054	0.771	0.464	0.978
IL-2R	32	18.744	40	13.335	27	11.676	36	16.138	0.627	0.535	0.978
HGF	32	38.641	40	24.043	27	45.178	36	28.965	0.598	0.551	0.978
IL-1RA	32	155.055	40	196.666	27	170.234	36	112.111	0.518	0.597	0.978
FGF-Basic	32	0.000	40	0.000	27	0.000	36	0.000	0.454	0.636	0.978
IL-8	32	681.177	40	445.793	27	1234.211	36	250.297	0.421	0.657	0.978
MIP-1β	32	13.872	40	10.993	27	12.010	36	12.511	0.408	0.666	0.978
IL-5	32	0.252	40	0.000	27	0.750	36	0.000	0.390	0.677	0.978
IFN-γ	32	0.000	40	0.631	27	0.000	36	0.557	0.368	0.692	0.978
MCP-1	32	17.967	40	18.259	27	29.679	36	18.948	0.322	0.725	0.978
IL-13	32	6.814	40	6.195	27	6.780	36	7.037	0.304	0.738	0.978
EGF	32	13.799	40	11.583	27	10.739	36	10.772	0.300	0.741	0.978
RANTES	32	0.000	40	0.000	27	0.000	36	0.000	0.281	0.755	0.978
IL-17	32	0.000	40	0.000	27	0.000	36	0.000	0.280	0.756	0.978
MIP-1α	32	0.000	40	3.711	27	0.000	36	3.276	0.268	0.765	0.978
VEGF	32	11.115	40	10.485	27	11.265	36	10.581	0.221	0.802	0.978
IL-1β	32	0.000	40	0.000	27	0.000	36	0.000	0.205	0.815	0.978
IL-12	32	0.000	40	0.000	27	0.000	36	0.000	0.123	0.885	0.989
IL-7	32	5.202	40	0.000	27	0.000	36	0.000	0.095	0.909	0.989
IL-2	32	0.000	40	0.000	27	0.000	36	0.000	0.063	0.939	0.989
Eotaxin	32	0.000	40	0.000	27	0.000	36	0.000	0.033	0.968	0.989
GM-CSF	32	3.117	40	3.219	27	3.195	36	3.226	0.012	0.989	0.989

All medians are given in pg/ml

5.2.5 Sub-group analysis by baseline 25(OH)D deficiency and genotype shows no effect of vitamin D supplementation on supernatant concentrations of inflammatory markers

Other studies have demonstrated a beneficial effect of vitamin D supplementation, but only in those participants who were profoundly deficient at baseline [308]. As such, it was decided to stratify results by baseline 25(OH)D deficiency, with a concentration of < 50nmol/L used as the cut-off. As before, data for asthma and COPD patients were pooled to provide greater power with which to assess any affect of vitamin D supplementation. Qlucore Omics Explorer 2.3 was used to analyse the data, modelling using a multi-group analysis (i.e. repeated measures ANOVA) on interaction variables with log-transformed data, and including adjustment for time-point, allocation and study. P-values were deemed significant when less than 0.05 and with a corresponding q-value of less than 0.1. No statistically significant effect of vitamin D supplementation was observed on induced sputum inflammatory mediators or the cellular profile of the lower airways in participants with a baseline vitamin D status of less than 50nmol/L (Table 5.5)

Table 5.5: Effect of vitamin D supplementation on the cellular profile and presence of induced sputum inflammatory mediators in pooled data from asthma and COPD patients with baseline vitamin D deficiency

Analytes	Placebo 2 Month		Intervention 2 Month		Placebo 12 Month		Intervention 12 Month		Statistics		
	n	median	n	median	n	median	n	median	F-statistic	p-value	q-value
% Tregs	3	8.300	7	9.075	5	10.500	8	13.650	2.957	0.056	0.978
% B-cells	3	3.540	7	2.530	5	1.880	9	3.320	2.332	0.101	0.978
MIP-1α	17	8.537	25	7.422	17	0.000	21	9.093	1.886	0.156	0.978
% Epithelial Cells	15	0.830	24	0.420	17	1.250	20	0.665	1.701	0.187	0.978
HGF	17	55.458	25	20.605	17	0.000	21	28.042	1.588	0.208	0.978
TNF-α	17	0.000	25	0.000	17	0.000	21	0.000	1.464	0.235	0.978
Absolute Epithelial Cells	15	17733.365	24	11931.215	17	33056.595	20	12192.511	1.283	0.281	0.978
% CD8 T-cells	3	24.600	7	20.100	5	26.400	9	38.200	1.231	0.295	0.978
Absolute Lymphocytes	15	7032.883	24	9931.050	17	10026.423	20	9003.165	1.205	0.303	0.978
MIG	17	0.000	25	0.000	17	0.000	21	0.000	1.147	0.321	0.978
RANTES	17	0.000	25	0.000	17	0.000	21	0.000	1.116	0.331	0.978
Absolute Neutrophils	15	1526500.000	24	1792475.711	17	1100258.384	20	1099655.747	1.075	0.344	0.978
IP-10	17	6.106	25	5.860	17	10.037	21	6.088	0.949	0.390	0.978
% Neutrophils	15	66.750	24	62.250	17	62.250	20	69.790	0.903	0.408	0.978
IL-7	17	11.642	25	0.000	17	0.000	21	0.000	0.855	0.428	0.978
G-CSF	17	70.043	25	53.906	17	44.534	21	38.392	0.769	0.466	0.978
IL-15	17	0.000	25	0.000	17	6.361	21	6.841	0.754	0.473	0.978
Absolute Macrophages	15	467264.550	24	606841.960	17	488416.500	20	486351.338	0.745	0.477	0.978
MIP-1β	17	12.123	25	10.391	17	8.218	21	13.130	0.692	0.502	0.978
IL-6	17	16.880	25	20.981	17	21.936	21	23.403	0.685	0.506	0.978
VEGF	17	11.194	25	10.002	17	11.239	21	11.649	0.682	0.507	0.978
% mDC	3	38.800	7	50.400	5	47.200	9	44.800	0.624	0.538	0.978
IFN-α	17	0.000	25	0.000	17	0.000	21	0.000	0.595	0.553	0.978
IL-8	17	662.004	25	347.589	17	864.140	21	204.028	0.594	0.554	0.978
FGF-Basic	17	0.000	25	0.000	17	0.000	21	0.000	0.589	0.557	0.978
% Lymphocytes	15	0.500	24	0.540	17	0.670	20	0.500	0.581	0.561	0.978
IL-4	17	0.671	25	5.986	17	1.006	21	6.259	0.544	0.582	0.978
% CD4 T-cells	3	61.700	7	70.000	5	61.300	9	45.400	0.540	0.584	0.978
Absolute mDC	3	386.394	7	300.274	5	348.208	9	130.293	0.426	0.654	0.978
IL-2R	17	13.418	25	13.418	17	10.732	21	13.252	0.424	0.655	0.978
% Macrophages	15	25.000	24	31.375	17	35.420	20	24.000	0.343	0.710	0.978
IL-12	17	0.000	25	0.000	17	0.000	21	0.000	0.329	0.720	0.978
IL-1RA	17	180.160	25	197.224	17	124.848	21	101.729	0.321	0.726	0.978
IL-1β	17	0.000	25	0.000	17	0.000	21	0.000	0.270	0.764	0.978
MCP-1	17	17.807	25	15.920	17	26.159	21	12.310	0.258	0.773	0.978
%pDC	3	6.400	7	6.130	5	10.800	9	9.090	0.223	0.800	0.978
IL-10	17	1.120	25	0.000	17	0.000	21	0.000	0.223	0.801	0.978
Absolute CD4 T-cells	3	4885.942	7	1653.991	5	2506.588	9	1259.155	0.199	0.820	0.978
Absolute Tregs	3	400.595	7	226.850	5	221.846	8	141.450	0.160	0.852	0.978
IL-17	17	0.000	25	0.000	17	0.000	21	0.000	0.158	0.854	0.978
IL-5	17	0.503	25	0.000	17	0.000	21	0.000	0.148	0.863	0.978
IL-13	17	6.849	25	5.673	17	6.092	21	7.037	0.145	0.865	0.978
Absolute B-cells	3	939.143	7	308.667	5	437.821	9	556.117	0.132	0.876	0.978
Absolute CD8 T-cells	3	1184.332	7	595.306	5	1372.350	9	663.232	0.130	0.878	0.978
EGF	17	14.445	25	8.804	17	15.077	21	10.118	0.113	0.893	0.978
Absolute pDC	3	68.591	7	32.548	5	41.670	9	25.122	0.112	0.895	0.978
Absolute Eosinophils	15	49939.512	24	28964.950	17	31405.077	20	39445.700	0.102	0.903	0.978
% Eosinophils	15	3.420	24	1.375	17	4.750	20	1.415	0.099	0.905	0.978
IL-2	17	0.000	25	0.000	17	0.000	21	0.000	0.043	0.958	0.986
Eotaxin	17	0.000	25	0.000	17	0.000	21	0.000	0.027	0.974	0.986
IFN-γ	17	0.000	25	0.557	17	0.000	21	0.000	0.023	0.978	0.986
GM-CSF	17	3.176	25	3.192	17	3.206	21	3.208	0.014	0.986	0.986

Analyte medians are given in pg/ml, cell medians are given as absolute numbers or percentages

Other studies have also demonstrated that effects of vitamin D supplementation on respiratory diseases are modified by genotype [328, 329, 336]. As such, it was decided to stratify analysis by genotype, looking specifically at single nucleotide polymorphisms (SNPs) in the vitamin D receptor (VDR) and retinoid x receptor (RXRA) genes. The clinical analysis of the ViDiCO trial showed an interaction effect, with genotype modifying the effect of allocation on the primary outcome of time to upper respiratory tract infection in people with COPD (methodology described in Chapter 2.7). Vitamin D was demonstrated to be protective by increasing the time to experiencing an upper respiratory tract infection in COPD patients with the minor alleles for the VDR SNPs rs4334089, rs11568820, rs7976091 and rs7970314, and the RXRA SNP rs7861779, and the major homozygous alleles for the VDR SNP rs10783219 (results not yet published, but summarised in Table 5.6). As such, these genotypes were used for further stratification of the analysis. Qlucore Omics Explorer 2.3 was used as before for analysis, modelling using a multi-group analysis (i.e. repeated measures ANOVA) on interaction variable, with adjustment for time-point and allocation. However, the number of participants with each genotype for each SNP was deemed to be too small to accurately make any significant conclusions on whether the effect of vitamin D supplementation on inflammatory markers of induced sputum and the lower airways was modified by genotype (Table 5.7).

Table 5.6: Hazard ratios for time to URI and time to exacerbation by allocation and genetic sub-group – statistically significant results

Gene	SNP	Clinical Outcome	Genotype	HR (95% CI)	P-Value	HR for Interaction Term (95% CI)	P-Value for Interaction Term	Q-Value for Interaction Term
VDR	rs4334089	Time to URI	GG	1.80 (1.11 to 2.91)	0.02	0.39 (0.24 to 0.64)	0.0002	0.002
			AG	0.55 (0.34 to 0.91)	0.02			
			AA	0.38 (0.10 to 1.44)	0.16			
VDR	rs10783219	Time to URI	AA	0.52 (0.31 to 0.87)	0.01	2.46 (1.44 to 4.21)	0.0009	0.003
			AT	1.42 (0.87 to 2.31)	0.16			
			TT	5.20 (1.41 to 19.15)	0.01			
VDR	rs11568820	Time to URI	CC	1.44 (0.92 to 2.24)	0.11	0.37 (0.21 to 0.65)	0.0005	0.002
			CT	0.70 (0.42 to 1.18)	0.19			
			TT	0.18 (0.02 to 1.72)	0.14			
VDR	rs7976091	Time to URI	CC	1.40 (0.90 to 2.18)	0.13	0.38 (0.22 to 0.65)	0.0004	0.002
			CT	0.70 (0.42 to 1.18)	0.18			
			TT	0.17 (0.02 to 1.56)	0.12			
VDR	rs7970314	Time to URI	AA	1.33 (0.85 to 2.09)	0.22	0.48 (0.29 to 0.82)	0.007	0.017
			AG	0.68 (0.41 to 1.13)	0.13			
			GG	0.59 (0.11 to 3.24)	0.54			
RXRA	rs7861779	Time to URI	GG	1.17 (0.80 to 1.72)	0.41	0.43 (0.20 to 0.95)	0.04	0.08
			AA + AG	0.53 (0.27 to 1.07)	0.08			
RXRA	rs7861779	Time to exacerbation	GG	1.10 (0.71 to 1.69)	0.67	0.40 (0.16 to 0.99)	0.049	0.098
			AA + AG	0.45 (0.20 to 1.03)	0.06			

Abbreviations used: VDR: vitamin D receptor; RXRA retinoid X receptor; URI: upper respiratory infection; HR: hazard ratio; CI: confidence interval; SNP: single nucleotide polymorphism

Table 5.7: The number of COPD patients in the sputum sub-study in each genetic sub-group, separated by time-point and allocation

Gene	SNP	Genotype	Placebo 0 Month	Placebo 2 Month	Placebo 12 Month	Intervention 0 Month	Intervention 2 Month	Intervention 12 Month
VDR	rs4334089	GG	14	11	8	14	13	11
		AG	2	2	2	10	8	7
		AA	4	3	2	0	0	0
VDR	rs10783219	AA	9	8	6	9	7	6
		AT	9	6	5	11	11	9
		TT	2	2	1	1	1	1
VDR	rs11568820	CC	14	11	8	12	11	9
		CT	3	3	3	12	10	9
		TT	2	1	1	0	0	0
VDR	rs7976091	CC	15	12	9	12	11	9
		CT	2	2	2	12	10	9
		TT	3	2	1	0	0	0
VDR	rs7970314	AA	13	10	7	10	9	7
		AG	3	3	3	13	11	10
		GG	3	2	1	1	1	1
RXRA	rs7861779	GG	17	14	11	19	18	16
		AG	2	1	1	4	2	1
		AA	1	1	0	0	0	0

Abbreviations used: VDR: vitamin D receptor; RXRA retinoid X receptor; SNP: single nucleotide polymorphism

5.3 Discussion

It has been demonstrated here that supplementation with vitamin D resulted in a significant increase in serum 25(OH)D concentration throughout the course of the ViDiAs study. However, this increase was not demonstrated in the ViDiCO study, and was not associated with any effect on the cellular and inflammatory profiles of sputum samples from participants with asthma or COPD.

Most intervention studies looking into the effects of vitamin D supplementation on acute respiratory tract infections have not analysed sputum samples to go alongside the clinical findings. We aimed here to carry out a study whereby any effect of vitamin D supplementation on the primary outcomes of time to URI and time to exacerbation could be mechanistically associated with observed changes in inflammatory markers and cellular profiles of induced sputum samples. For two clinical trials carried out in asthma and COPD, 50 participants from each were recruited into the sputum arm of the study, entailing undergoing sputum induction at months 0, 2 and 12. Samples were processed, and supernatants were collected to undergo multiplex ELISA, cytospins were produced to perform

differential cell counts, and a subset of samples were stained for flow cytometric analysis. In pooled data from asthma and COPD samples, there was shown to be no effect of vitamin D supplementation on any of these inflammatory mediators over the course of the study. For the asthma trial this corroborates the primary findings from the clinical trial, which saw no effect of vitamin D supplementation on time to first severe exacerbation or time to first URI (data not yet published, but summarised in Table 5.1). Additionally, the trial results indicated no effect of supplementation on asthma symptom control (measured by asthma control test scores), health service or medication use, or measures of respiratory physiology such as FEV_1, exhaled nitric oxide and peak expiratory flow rate (PEFR), although a modest improvement in quality of life as determined by St George's Respiratory Questionnaire (SGRQ) scores was observed ($p = 0.03$). Thus the immunological findings presented in this chapter support the clinical findings from the ViDiAs trial.

For the COPD trial, the primary clinical findings also demonstrated no effect of vitamin D supplementation on time to first exacerbation or time to first URI (data not yet published, but summarised in Table 5.2). Additionally, no effect was seen on symptom scores, quality of life (via SGRQ scores), health service or medication use, or measures of respiratory function such as FEV_1 and FVC. However, when stratifying by baseline vitamin D deficiency (defined as a serum 25(OH)D concentration < 50nmol/L), vitamin D supplementation significantly reduced the risk of moderate or severe exacerbation in the n = 148 people who were vitamin D deficient at baseline (aHR 0.57, 95% CI 0.35 to 0.92, $p = 0.021$). Similar stratification was attempted in the sputum sub-study, to determine if supplementation would have any effect on inflammatory markers in those people who were vitamin D deficient at baseline. Data were pooled from the asthma and COPD trials to increase power, but no effect of vitamin D supplementation on induced sputum inflammatory markers was observed. However, even after pooling the data, numbers for the flow cytometric cellular analysis were still small and unlikely to provide sufficient power to determine any significant effects. Likewise, when stratifying by genotype in the ViDiCO study population as a whole, an interaction effect was demonstrated with genotype modifying the effect of allocation on time to first URTI (as summarised in Table 5.6). However, the numbers in the sputum sub-study for the genotypes associated with each SNP were too small to provide sufficient power to carry out the analysis. Therefore, while the pooled immunological findings presented in this chapter support the clinical findings from the ViDiAs and ViDiCO trials as a whole, they are unable to support the clinical findings obtained from stratifying by baseline vitamin D deficiency or genotype.

In the studies which found a protective effect of vitamin D supplementation in preventing exacerbations in asthmatic subjects [303, 306, 307], sputum samples were not collected and analysed in any of them. As such, any protective effect of vitamin D in these studies cannot be associated with mechanistic data on the effect of vitamin D on inflammatory mediators and the cellular profile of the respiratory tract. Similarly in COPD, the study which demonstrated a protective effect of vitamin D supplementation in reducing incidence of exacerbations in profoundly deficient subjects, only offered the mechanistic explanation that there was an increase in monocyte phagocytic capacity in the blood [308]. While sputum samples were collected, they were only used for analysis of the bacterial cultures present in the airways and not to determine whether phagocytosis by the cells present in the lower airways, and thus at the site of infection, was improved as was shown in the blood. One study which did collect sputum samples showed no effect of vitamin D supplementation on differential cell counts, while inflammatory mediators were not looked at [315]. As with the ViDiAs trial, the overall clinical findings of this trial demonstrated no effect of vitamin D supplementation on rate of exacerbations, lung function, quality of life, or treatment failure in asthma patients. Therefore, the main strength of the study carried out here was in the collection and analysis of induced sputum samples to determine the effects of vitamin D supplementation at the site of infection.

Observational studies have also investigated the association between serum 25(OH)D concentration and markers of airway inflammation. One study, looking at children with asthma, collected sputum samples and BAL fluid, but failed to show any association between vitamin D status and eosinophilic or neutrophilic airway inflammation [595]. Another study, however, did show an association between vitamin D deficiency in people with bronchiectasis, with higher concentrations of neutrophil elastase, IL-8, TNF-α and IL-1β in sputum samples [596]. Therefore, while observational studies have indicated a potential association between vitamin D status and markers of respiratory inflammation, causal inference cannot be made without robust intervention studies.

The main limitation of this study was in the inability to have sufficient power when stratifying by baseline vitamin D deficiency or genotype, and in the study group as a whole for flow cytometric analysis, which may have enabled corroboration with the clinical findings. A number of participants withdrew their consent or were lost to follow-up in both studies. This was in part due to being unable to expectorate and thus not being willing to undergo further sputum induction at subsequent visits after finding nebulisation an unpleasant experience. As such, only 66% of asthma and 60% of COPD patients attended the final visit at month 12. To attempt to compensate for this lack of power, data from the ViDiAs and ViDiCO trials were pooled during analysis, with study (i.e. which

trial the participants were in) eliminated as a confounder. To allow for this rate of drop-out, and to enable stratification of analysis, and thus increase power, larger numbers would have needed to have been recruited into the sputum sub-study. Alternatively, recruitment would have needed to focus on those who were vitamin D deficient and thus might benefit more from supplementation, or those with a specific genotype. The pooling of data from the 2 trials also raises another potential limitation of the study, with asthma and COPD defined as distinct diseases with different inflammatory profiles. There is a large degree of heterogeneity within asthmatic and COPD patient populations, with, for example, both neutrophilic and eosinophilic airway inflammation reported in both asthma and COPD patients [597-599], resulting in the phenotypic distinction between the two populations being obscured. However, in this study asthma and COPD were well-defined due to the specific inclusion and exclusion criteria used during recruitment. As such, "study" was eliminated as a confounder during analysis, to remove any influence of having asthma or COPD on the outcome of the analysis.

Another limitation is the observation that, while there was an increase in mean serum 25(OH)D concentration over the course of the study in the intervention arm of ViDiCO, there was not a significant difference between the 25(OH)D concentration between the 2 arms at both months 2 and 12. This appears to be due to the fact that, by chance, participants in the control arm of the sputum sub-study had a higher baseline 25(OH)D concentration compared to the intervention arm. As such, the effect of intervention resulted in serum 25(OH)D concentrations only marginally greater than those in the control arm. This lack of a difference in serum 25(OH)D concentration between the two arms may have obscured any effect of vitamin D supplementation in the intervention arm. Since it is the trough levels which were measured here, with participants taking the IMP dose before returning 2 months later when a blood sample was taken and the next dose given, serum 25(OH)D concentration was likely to be higher immediately after IMP administration. It has been shown elsewhere that peak vitamin D status is achieved 1 week after supplementation [316], so any difference in serum 25(OH)D concentration between the two arms of the study may have been masked. However, there was also no statistically significant difference in PTH concentration between the two arms at 12 months, further indicating that supplementation in the intervention arm of the ViDiCO trial was not sufficient to significantly increase vitamin D status compared to the control arm. In the COPD study group as a whole, as summarised in Table 5.2, a significantly higher serum 25(OH)D concentration was observed in the intervention group compared to the placebo group, which was also associated with a lower concentration of PTH.

Therefore, here it has been demonstrated that, while supplementation was successful in increasing the vitamin D status of ViDiAs trial participants, this was not associated with any change in the cellular profile or concentration of inflammatory markers in induced sputum from patients with asthma or COPD. There may be a beneficial effect of vitamin D supplementation in those with profound baseline deficiency or in people with certain genotypes, but we were not powered to accurately assess these hypotheses in this study.

6. Characterisation of the effects of vitamin D supplementation on the inflammatory response in peripheral whole blood following stimulation with TLR ligands or whole pathogens

6.1 Introduction

As reviewed in Chapter 1, some intervention studies have demonstrated that vitamin D supplementation reduces the incidence of respiratory tract infections [300, 303, 305] and asthma and COPD exacerbations [303, 306-308]. However, while blood samples were collected in these trials to measure 25(OH)D concentration and safety markers such as corrected calcium and creatinine, an analysis of the inflammatory markers present was not carried out. Blood provides a useful and readily available tool in assessing the immune response to pathogens, since many of the leukocytes which are required to mount an effective immune response are represented in this sample. As well as the cellular components such as lymphocytes, neutrophils, monocytes, dendritic cells and eosinophils, blood also contains plasma which contains complement components and inflammatory mediators. Additionally blood platelets, as well as causing blood coagulation to limit the spread of infection, express TLRs, and when activated can secrete the contents of their granules, containing over 300 proteins including antimicrobial peptides, cytokines and chemokines [600-603]. As such, blood samples collected from clinical trial participants undergoing vitamin D supplementation were stimulated *ex vivo* with TLR ligands and whole pathogens to enable determination of the effects of vitamin D supplementation on the inflammatory response mounted, by analysis of the cellular profile and quantification of the inflammatory mediators released.

As described in Chapter 5, a multiplex ELISA platform was used in order to asses a range of inflammatory mediators which may be encompassed by the pleiotropic immunomodulatory actions of vitamin D. Therefore, a 30-plex panel was chosen, allowing quantification of the following: IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8 (CXCL8), IL-10, IL-12, IL-13, IL-15, IL-17, IL-1RA, IL-2R, IFN-α, IFN-γ, TNF-α, MCP-1 (CCL2), MIP-1α (CCL3), MIP-1β (CCL4), RANTES (CCL5), eotaxin (CCL11), MIG (CXCL9), IP-10 (CXCL10), EGF (epidermal growth factor), FGF-basic (basic fibroblast growth factor), HGF (hepatocyte growth factor), VEGF (vascular endothelial growth factor), G-CSF (granulocyte colony-stimulating factor) and GM-CSF (granulocyte macrophage colony-stimulating factor).

In this chapter, in order to increase the power with which to characterise the effects of vitamin D supplementation on the inflammatory response in peripheral whole blood following stimulation with TLR ligands or whole pathogens, data from participants on the same dosing regimen were pooled. ViDiAs, ViDiCO, ViDiFlu staff and ViDiFlu residents were also analysed separately, with no differences demonstrated, and this data is not presented for presentational ease.

Primary clinical results for the ViDiAs, ViDiCO and ViDiFlu trials

As explained in Chapter 5, while not in the scope of this thesis to expand on the primary clinical results of the trials, these results are undoubtedly important for comparing the immunological results to, in order to determine whether any effects of vitamin D supplementation on primary outcomes such as time to first upper respiratory tract infection (URTI) could be mechanistically associated with observed changes in inflammatory markers and cellular profiles. Since the results of these trials have not yet been published, a summary of the ViDiAs and ViDiCO trials was provided in chapter 5. The only statistically significant outcomes were increased mean serum 25(OH)D concentration, decreased PTH concentration, and an improved SGRQ score in the intervention arm of ViDiAs, and in ViDiCO increased mean serum 25(OH)D concentration, decreased PTH concentration and an improved symptom score during exacerbation. When stratifying by baseline vitamin D deficiency (defined as a serum 25(OH)D concentration < 50nmol/L), vitamin D supplementation also significantly reduced the risk of moderate or severe exacerbations in COPD patients who were vitamin D deficient at baseline (adjusted hazard ratio [aHR] 0.57, 95% CI 0.35 to 0.92, p = 0.021).

A summary of the primary outcomes of the ViDiFlu trial is provided in Table 6.1. Among ViDiFlu participants, there was no difference in time to acute respiratory infection between high and low-dose intervention (aHR 1.18, 95% CI 0.84 to 1.66, p = 0.34). Surprisingly, allocation to the high-dose intervention arm was associated with an increased risk of upper respiratory tract infection (URTI) (aHR 1.48, 95% CI 1.02 to 2.16, p = 0.04), but there was no effect on lower respiratory tract infection (LRTI) (aHR 1.12, 95% CI 0.75 to 1.66, p = 0.57). A significant difference in serum 25(OH)D concentration was observed between the two arms of the study (p < 0.001).

Table 6.1: Clinical and biochemical outcomes by allocation in the ViDiFlu trial

		High-Dose	Low-Dose	Adjusted hazard ratio / odds ratio / incidence rate ratio / mean difference	95% CI	P-value
Median days to first ARI (IQR)		203 (55 to --)	227 (83 to --)	1.18	0.84 to 1.66	0.34
Percentage of patients with ≥1 ARI		66	63	1.21	0.68 to 2.15	0.52
Rate of ARI per participant-year		2.15	1.98	1.15	0.84 to 1.58	0.37
Median duration of symtpoms per ARI, days (IQR)		6 (3 to 13)	5 (3 to 11)	1.17	0.92 to 1.49	0.21
Median days to first URI (IQR)		247 (75 to --)	-- (107 to --)	1.48	1.02 to 2.16	0.039
Percentage of patients with ≥1 URI		62	49	1.83	0.99 to 3.41	0.055
Rate of URI per participant-year		1.15	1.00	1.34	0.94 to 1.90	0.10
Median duration of symtpoms per URI, days (IQR)		7 (4 to 12)	5 (3 to 10)	1.34	1.09 to 1.65	0.005
Median peak Jackson symptom score per URI (IQR)		9 (6 to 14)	8 (5 to 12)	1.11	0.91 to 1.35	0.30
Median days to first LRI (IQR)		-- (109 to --)	-- (137 to --)	1.12	0.75 to 1.66	0.57
Percentage of patients with ≥1 LRI		51	49	1.11	0.64 to 1.92	0.72
Rate of LRI per participant-year		1.00	0.97	1.06	0.71 to 1.58	0.77
Median duration of symtpoms per LRI, days (IQR)		6 (2 to 13)	5 (3 to 11)	1.07	0.76 to 1.51	0.70
Median peak MacFarlane symptom score per LRI (IQR)		4 (3 to 7)	4 (3 to 6)	1.11	0.92 to 1.33	0.27
Mean serum 25(OH)D, nmol/L (s.d.)	2 mo	65.5 (19.8)	52.9 (21.7)	13.2	8.3 to 18.0	< 0.001
	12 mo	85.3 (24.3)	59.1 (26.0)	25.7	20.6 to 30.7	
Mean serum PTH, pmol/L (s.d.)	2 mo	5.53 (2.51)	5.89 (2.67)	-0.16	-0.69 to 0.37	0.004
	12 mo	5.89 (2.56)	7.27 (3.34)	-0.93	-1.49 to -0.37	

-- Represents undefined values

Jackson symptoms (sneezing, sore throat, headache, subjective sensation of fever or chilliness, malaise, nasal discharge, nasal obstruction, cough) each scored from 0 (no symptoms) to 3 (severe symptoms) and summed for each day of URI.

Abbreviations used: CI: confidence interval; IQR: interquartile range; ARI: acute respiratory infection; URTI: upper respiratory tract infection; LRTI: lower respiratory tract infection; s.d.: standard deviation; PTH: parathyroid hormone

6.2 Results

6.2.1 Study recruitment

As described in Chapter 2.1, participants from all three trials provided blood samples at baseline, 2 month and 12 month follow-up. A subset of these samples was used in a whole blood assay to determine the inflammatory response mounted in peripheral blood to a range of TLR ligands and pathogens. Figures 6.1, 6.2 and 6.3 show the recruitment profiles for all three trials, and the number of samples included in the whole blood assay at each time-point. For this analysis, residents and staff in the ViDiFlu trial were analysed separately, due to different dosing regimens being used (low dose vs. high dose vitamin D in residents; placebo vs. high dose in staff – as described in Chapter 2).

ViDiAs Trial Recruitment

Of those randomised in the ViDiAs trial who provided a sodium heparin blood sample for whole blood assay, 53% were in the intervention arm and 47% were in the control arm (Figure 6.1). Similar numbers of follow-up samples were collected at each time-point between the two arms, with n = 50 and n = 42 producing a 2 month sample, and n = 42 and n = 42 producing a 12 month sample in each arm respectively. Over the course of the study, 9 participants in the intervention arm and 3 participants in the control arm were lost to follow-up. From the offset of the whole blood assay, all samples were stimulated with a panel of TLR ligands (LPS, Pam2CSK4, Pam3CSK4, PolyI:C and R848), as well as providing unstimulated whole blood and serum samples. At a later date, following procurement of the whole pathogens from collaborators at Imperial College London and optimisation of the conditions needed (as described in chapter 3), the pathogens RV-16, RV-1B, RSV, *Haemophilus influenzae* and *Streptococcus pneumoniae* were added as stimuli in the whole blood assay. Due to budgetary constraints, not all plasma samples generated during the whole blood assay could be analysed by multiplex ELISA. As such, the decision on which samples to analyse was based on having both baseline and 12 month samples stimulated with the whole range of stimuli. This resulted in 0 month and 12 month samples being analysed for n = 22 people in the intervention arm, and n = 23 people in the control arm. 12 month samples were analysed rather than 2 month samples due to a larger inter-arm difference in 25(OH)D concentration at this later time-point, with more time for 25(OH)D levels to impact the inflammatory response.

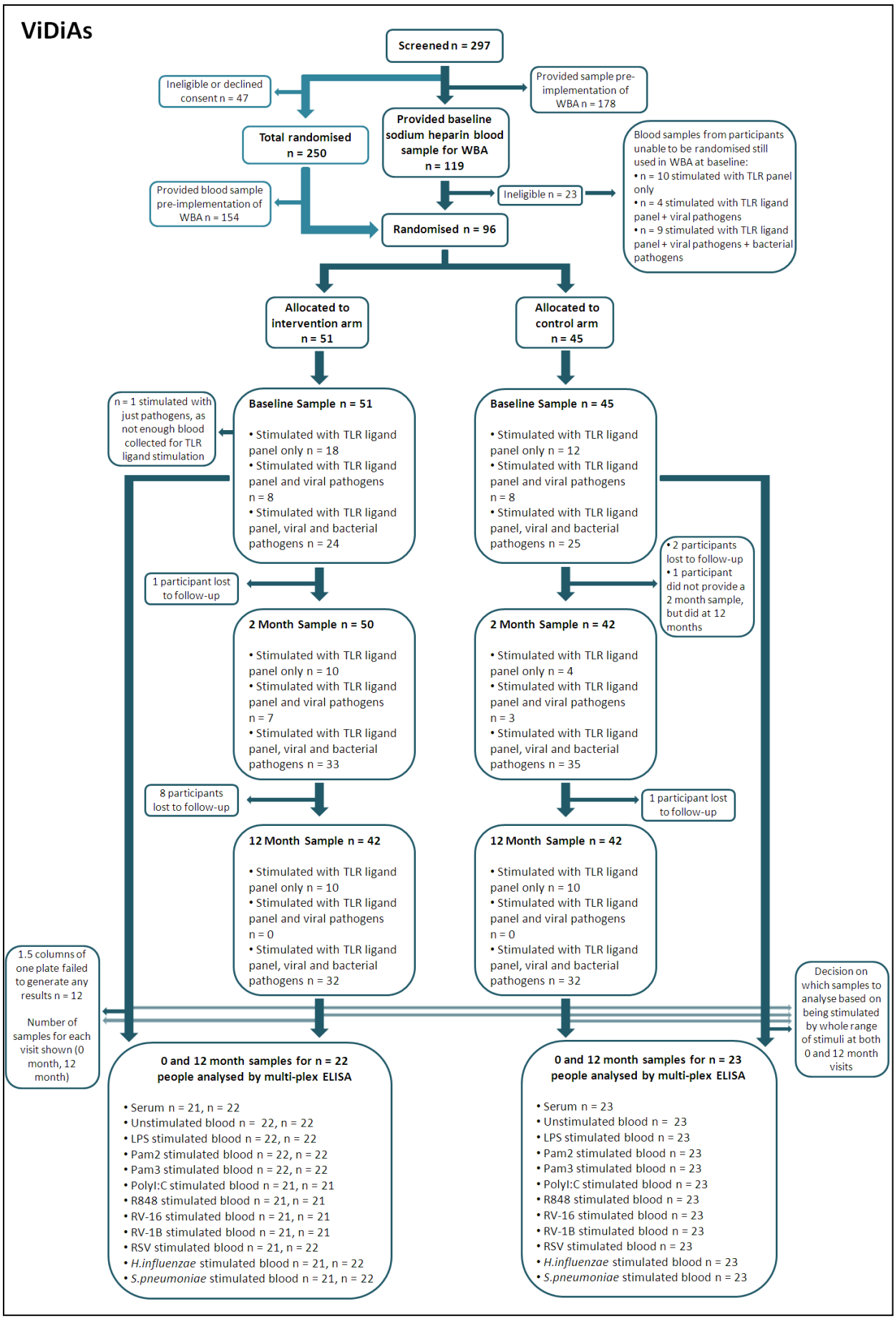


Figure 6.1: Recruitment profile for the whole blood assay in asthma patients at baseline, month 2 and month 12, separated by allocation

ViDiCO Trial Recruitment

Of those randomised in the ViDiCO trial who provided a sodium heparin blood sample for whole blood assay, 51% were in the intervention arm and 49% were in the control arm (Figure 6.2). Similar numbers of follow-up samples were collected at each time-point between the two arms, with n = 41 and n = 40 producing a 2 month sample, and n = 31 and n = 35 producing a 12 month sample in each arm. Over the course of the study, 14 participants in the intervention arm and 9 participants in the control arm were lost to follow-up. As with ViDiAs, not all plasma samples generated during the whole blood assay could be analysed by multiplex ELISA. As such, 0 month and 12 month samples were analysed for n = 8 people in the intervention arm, and n = 14 people in the control arm.

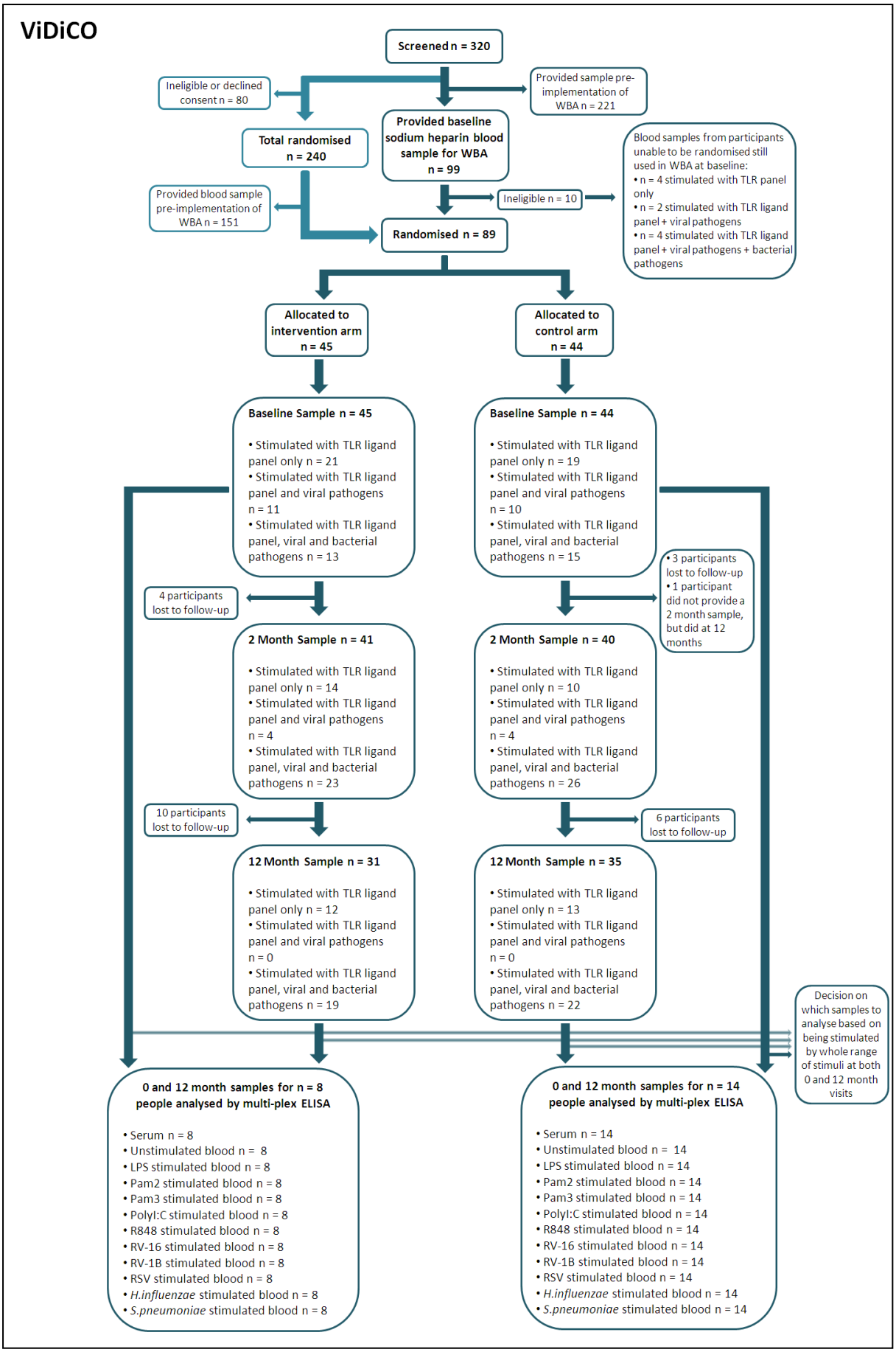


Figure 6.2: Recruitment profile for the whole blood assay in COPD patients at baseline, month 2 and month 12, separated by allocation

ViDiFlu Trial Recruitment

Of those randomised in the ViDiFlu trial who provided a sodium heparin blood sample for whole blood assay, 57% were in the intervention arm and 43% were in the control arm (Figure 6.3). Analysis was separated into participants living in sheltered accommodation schemes (labelled as residents) and participants working as staff or carers in sheltered accommodation schemes (labelled as staff). This was due to the differing dosing regimens used between staff and residents, with residents randomised to daily low dose + 2-monthly placebo vs. intermittent bolus dose vitamin D, and staff randomised to 2-monthly placebo vs. 2-monthly bolus dose vitamin D.

For the residents, 61% of those whose samples underwent *ex vivo* stimulation were in the intervention arm and 39% were in the control arm. Slightly more participants in the intervention arm provided follow-up samples at each time-point compared to the control arm, with n = 38 and n = 24 respectively producing a 2 month sample, and n = 34 and n = 24 respectively producing a 12 month sample in each arm. Over the course of the study, 6 participants in the intervention arm and 2 participants in the control arm were lost to follow-up. For the staff members, 41% were on the intervention arm and 59% were on the control arm. Similar numbers of follow-up samples were collected at each time-point between the two arms, with n = 7 and n = 10 respectively producing a 2 month sample, and n = 7 and n = 9 respectively producing a 12 month sample in each arm. Over the course of the study, 0 participants in the intervention arm and 1 participant in the control arm were lost to follow-up.

Unlike ViDiAs and ViDiCO, all samples with both a baseline and 12 month sample were analysed by multiplex ELISA, regardless of whether the whole panel of stimuli were used. This was due to the fact that the primary results of the ViDiFlu clinical trial indicated an effect of vitamin D supplementation on risk of URTI, whereas no such effect was observed in ViDiAs and ViDiCO. Therefore, it was desirable to attempt to offer a mechanistic explanation behind this finding by way of analysis of inflammatory mediators released in the blood following *ex vivo* stimulation. As such, 0 month and 12 month samples were analysed for n = 34 in the intervention arm for residents, n = 24 in the control arm for residents, n = 7 in the intervention arm for staff members, and n = 9 in the control arm for staff members.

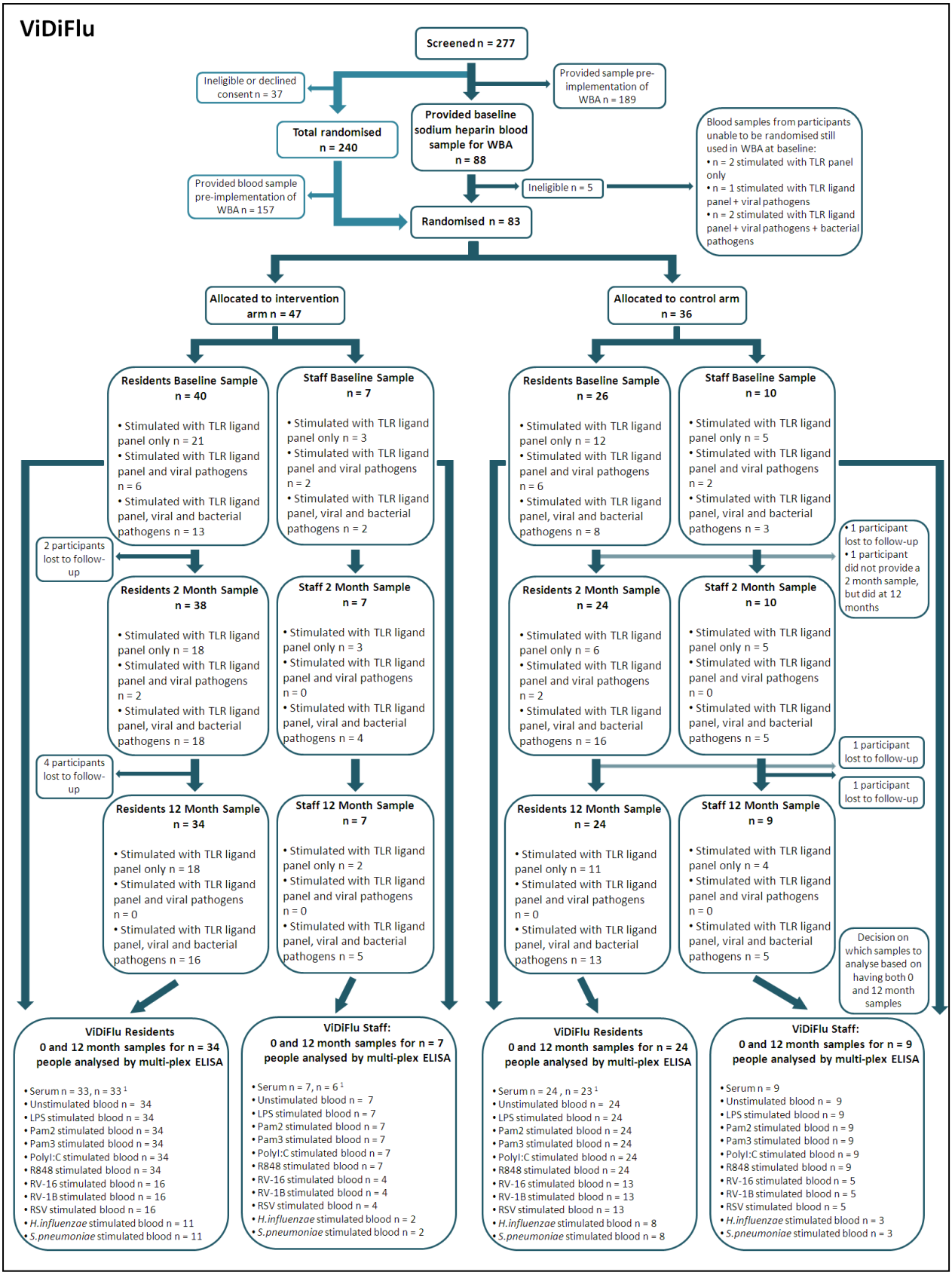


Figure 6.3: Recruitment profile for the whole blood assay in healthy patients at baseline, month 2 and month 12, separated by allocation and into staff and residents

[1]Two n values represents 0 month and 12 month samples – some serum samples had insufficient volume to be assayed

Flow Cytometry Study

The sodium heparin blood samples used for the whole blood assay at 12 months were also used to analyse the cellular profile of the blood in a subset of participants. Flow cytometry was used to determine numbers of B-cells, $CD4^+$ T-cells, $CD8^+$ T-cells, plasmacytoid dendritic cells (pDCs), myeloid dendritic cells (mDCs) and regulatory T-cells (Tregs) and Figures 6.4, 6.5 and 6.6 show the recruitment profiles for the flow cytometry sub-study. Due to the late implementation of this technique during the progress of the clinical trials, only 12 month blood samples were analysed, thus baseline data for this outcome were unavailable. However, it was reasoned that any significant effect of vitamin D supplementation would still be observable, with the assumption that all baseline values would be similar between the two arms of the trials, thus any difference at the 12 month time-point should be attributable to the intervention.

In the ViDiAs trial, 51% of people who provided a 12 month blood sample were in the intervention arm, and 49% were in the control arm. 74% of 12 month blood samples in the intervention arm were analysed by flow cytometry, compared to 76% of those in the control arm.

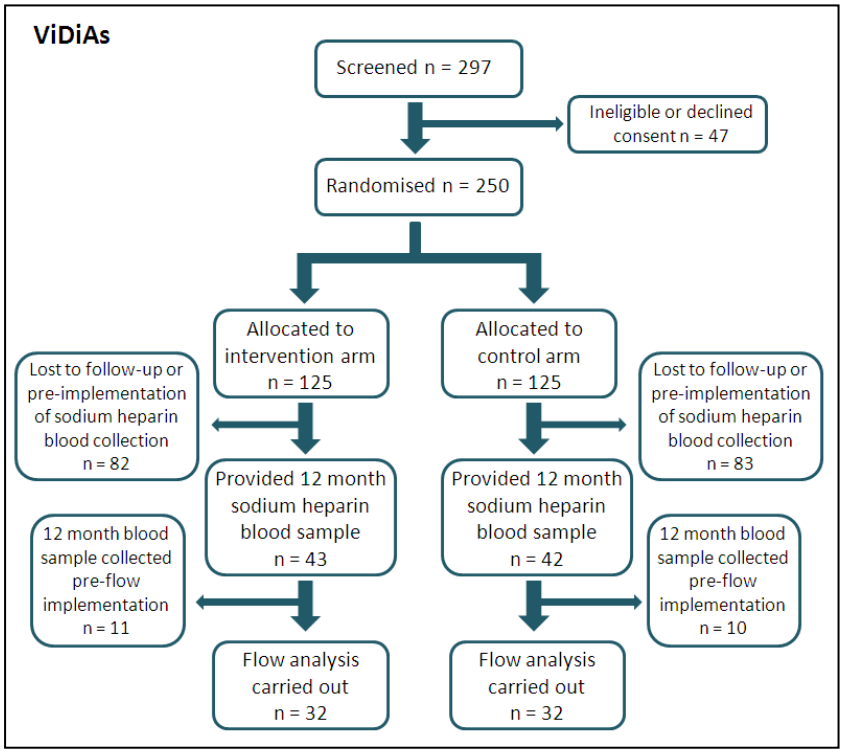


Figure 6.4: Recruitment profile for flow cytometric analysis of whole blood in asthma patients at month 12, separated by allocation

In the ViDiCO trial, 48% of people who provided a 12 month blood sample were in the intervention arm, and 52% were in the control arm. 53% of 12 month blood samples in the intervention arm were analysed by flow cytometry, compared to 71% of those in the control arm.

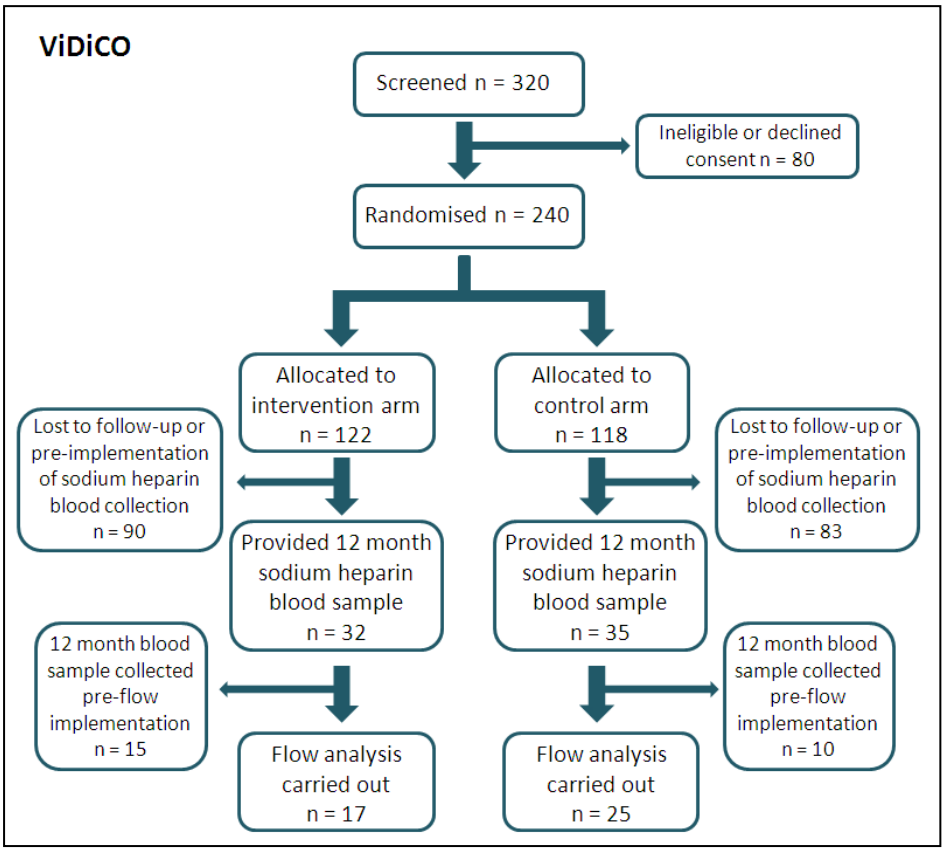


Figure 6.5: Recruitment profile for flow cytometric analysis of whole blood in COPD patients at month 12, separated by allocation

In the ViDiFlu trial, 60% of residents who provided a 12 month blood sample were in the intervention arm, and 40% were in the control arm. 54% of residents 12 month blood samples in the intervention arm were analysed by flow cytometry, compared to 72% of those in the control arm. In staff members, 48% of participants who provided a 12 month blood sample were in the intervention arm, and 52% were in the control arm. 43% of 12 month blood samples in the intervention arm were analysed by flow cytometry, compared to 40% of those in the control arm.

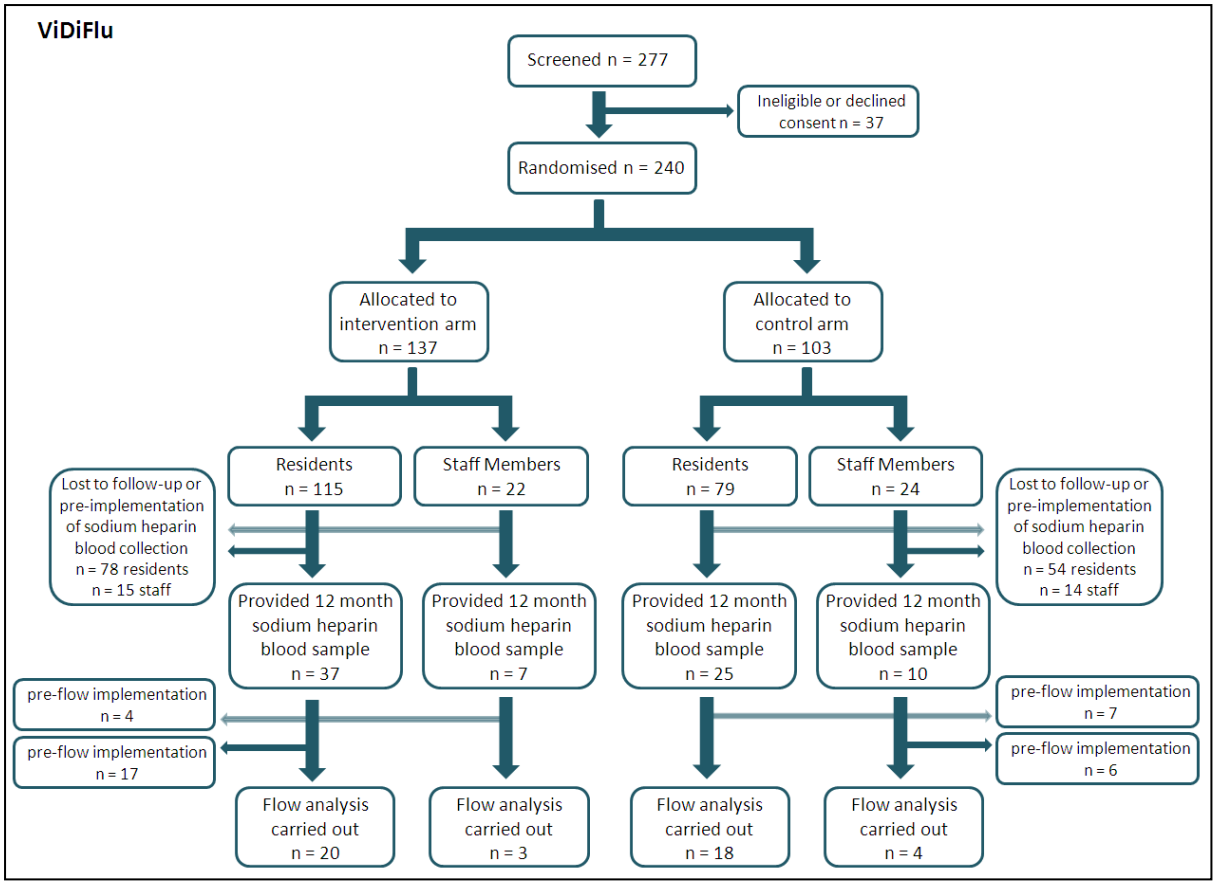


Figure 6.6: Recruitment profile for flow cytometric analysis of whole blood in controls at month 12, separated by allocation and into staff and residents

6.2.2 Vitamin D supplementation results in a higher concentration of serum 25(OH)D

The serum concentration of 25(OH)D was measured in all participants at baseline, 2 month and 12 month follow-up by liquid-chromatography-tandem mass spectrometry, as described in Chapter 2.1.6. This was used to determine whether randomisation into the intervention arm of the study resulted in a significant increase in circulating serum 25(OH)D over the time-course of the study, and whether it resulted in a significant difference between the control and intervention arms of the study. Since multiplex ELISA analysis was only carried out on 0 and 12 month samples, only these time-points are presented in Figure 6.7. Additionally, results are only presented for the sub-set of participants who had immunoassays carried out on their blood samples. In the ViDiAs study, the concentration of serum 25(OH)D was significantly higher at the 12 month time-point compared to baseline in the intervention arm (mean 25.27 nmol/L difference, 95% CI 16.30 to 34.24, $p < 0.0001$), while there was no difference over time in the control arm (mean -1.13 nmol/L difference, 95% CI -8.71 to 6.45, $p = 0.76$). There was also no difference between the two arms at baseline (mean -6.62

nmol/L difference, 95% CI -18.89 to 5.64, p = 0.28), but at 12 month follow-up 25(OH)D in the intervention arm was significantly higher than in the control arm (mean 33.03 nmol/L difference, 95% CI 23.39 to 42.76, p < 0.0001). The same was seen in the ViDiCO study, with 25(OH)D significantly higher at 12 months compared to 0 months in the intervention arm (mean 40.00 nmol/L difference, 95% CI 20.32 to 59.68, p = 0.002), but not in the control arm (mean -5.46 nmol/L difference, 95% CI -12.61 to 1.68, p = 0.12). There was also no difference between the two arms at baseline (mean 16.68 nmol/L difference, 95% CI -1.85 to 35.21, p = 0.08), but at 12 month follow-up 25(OH)D in the intervention arm was significantly higher than in the control arm (mean 28.79 nmol/L difference, 95% CI 8.16 to 49.42, p = 0.009).

Analysis of the ViDiFlu study was separated into residents and staff members due to the different dosing regimens used for the two groups. For ViDiFlu residents, the concentration of serum 25(OH)D was significantly higher at the 12 month time-point compared to baseline in both the intervention arm (mean 35.48 nmol/L difference, 95% CI 26.69 to 44.28, p < 0.0001) and the control arm (mean 12.75 nmol/L difference, 95% CI 4.73 to 20.77, p = 0.003). This is due to the fact that in the control arm participants were receiving low-dose vitamin D supplementation, as it was deemed unethical to withhold vitamin D in older adults with limited sunlight exposure. As such, within the ViDiFlu residents population, the comparison is between low-dose supplementation (4.8ml of placebo every 2 months + daily 10µl of active IMP for a year) and high-dose supplementation (4.8ml of active IMP every 2 months + daily 10µl of active IMP for a year). However, there was still a difference observed between the two arms at 12 month follow-up, with serum 25(OH)D significantly higher in the intervention arm (high-dose vitamin D) compared to the control arm (low-dose vitamin D) (mean 24.13 nmol/L difference, 95% CI 12.28 to 35.99, p = 0.0001), while there was no difference at baseline (mean -0.63 nmol/L difference, 95% CI -13.49 to 12.24, p = 0.92). Staff members participating in ViDiFlu underwent the same dosing regimen as that used in ViDiAs and ViDiCO. The concentration of serum 25(OH)D was higher at the 12 month time-point compared to baseline in the intervention arm, although statistical significance was not quite obtained, likely due to the small numbers present in this sub-group (mean 34.33 nmol/L difference, 95% CI -9.31 to 77.98, p = 0.09), while no difference over time in the control arm was observed (mean 0.22 nmol/L difference, 95% CI -13.38 to 13.83, p = 0.97). There was also no difference between the two arms at baseline (mean 4.76 nmol/L difference, 95% CI -24.56 to 34.09, p = 0.73), but at 12 month follow-up 25(OH)D in the intervention arm was significantly higher than in the control arm (mean 37.28 nmol/L difference, 95% CI 4.64 to 69.91, p = 0.03).

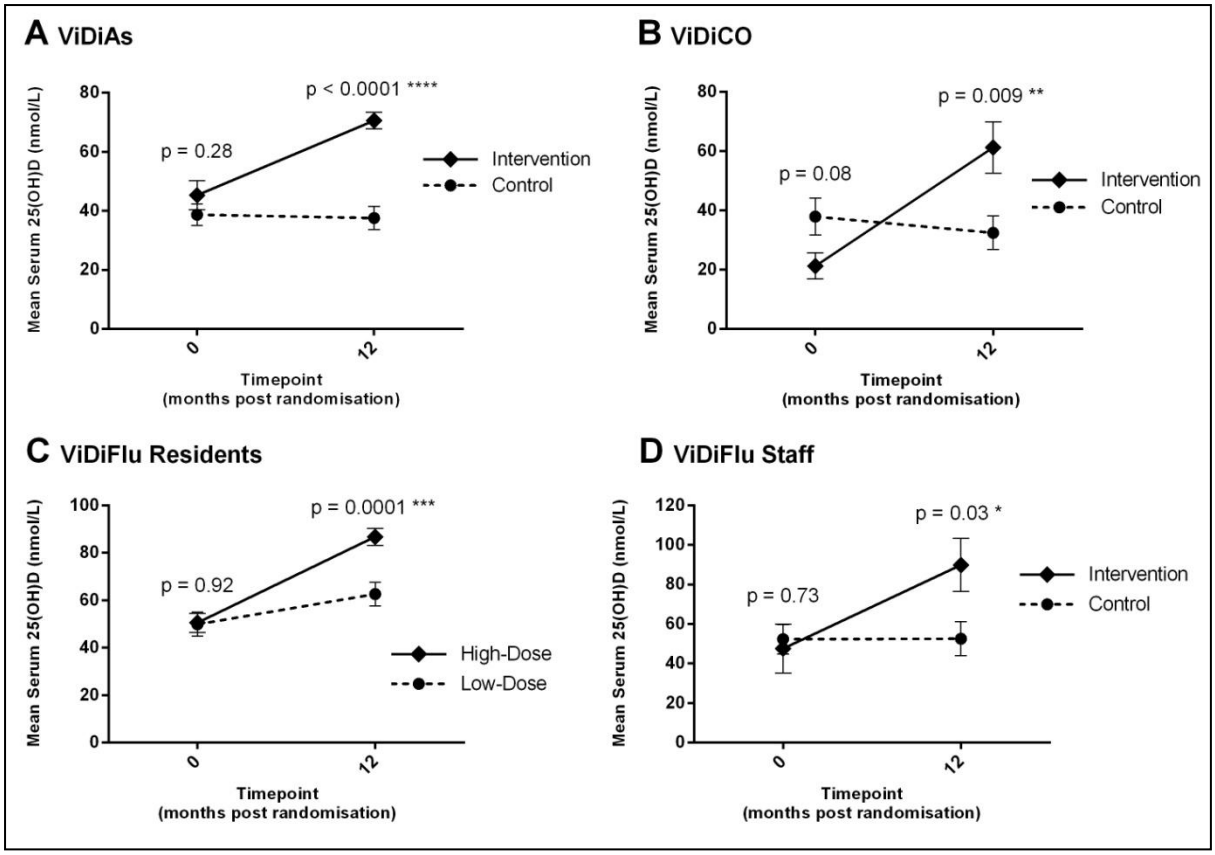


Figure 6.7: Change in serum 25(OH)D concentration over the course of the study for those enrolled in the intervention and control arms of all three trials, who provided samples for multiplex ELISA analysis. Serum samples were collected from trial participants at 0 and 12 month visits and the concentration of 25(OH)D was measured by liquid-chromatography-tandem mass spectrometry. Data are expressed as means + SEM, and are only from those participants who provided blood samples for the whole blood assay which underwent subsequent analysis by multiplex ELISA (intervention n = 22, control n = 23 for ViDiAs; intervention n = 8, control n = 14 for ViDiCO; high-dose intervention n = 34, low-dose intervention n = 24 for ViDiFlu residents; intervention n = 7, control n = 9 for ViDiFlu staff). P-values were calculated using an unpaired T-test.

6.2.3 High-dose vitamin D supplementation results in decreased absolute and percentage of B-cells in older adults without asthma or COPD, but has no effect in asthma and COPD patients

Peripheral whole blood samples were collected from participants in all 3 trials at baseline, 2 month and 12 month follow-up. As shown in figures 6.4 to 6.6, a subset of 12 month samples underwent flow cytometric analysis, to determine percentages and absolute numbers of B-cells, $CD4^+$ T-cells, $CD8^+$ T-cells, mDCs, pDCs and Tregs. The methodology and gating strategies are described in Chapter

2.4. Additionally, coulter counts were carried out in all participants to determine the number of basophils, eosinophils, lymphocytes, monocytes and neutrophils circulating in peripheral blood. To allow corroboration with the other variables analysed in this chapter, only the counts from people who provided sodium heparin samples which were analysed by multiplex ELISA or flow cytometry are included, rather than using results from all participants from all trials.

Each data point was assigned an interaction variable for time-point at which the sample was taken (i.e. months post randomisation), and whether the participant was in the intervention or control arm of the study. As for the sputum analysis presented in Chapter 5, and for all subsequent data presented in this chapter, data for participants on the same dosing regimen were pooled for this analysis to provide greater power with which to assess any affect of vitamin D supplementation on the cellular profile of peripheral whole blood. As such, data from asthma patients, COPD patients and staff on the ViDiFlu trial were pooled, while data from the residents of the ViDiFlu trial (i.e. older adults living in sheltered accommodation with neither asthma nor COPD) were analysed separately. Qlucore Omics Explorer 2.3 was used to analyse the data, modelling using a multi-group analysis (i.e. repeated measures ANOVA) on interaction variables with log-transformed data, and including adjustment for time-point, allocation and study (i.e. whether participants had asthma, COPD or neither condition). P-values were deemed significant when less than 0.05 and with a corresponding q-value of less than 0.1. No statistically significant effect of vitamin D supplementation was observed in pooled data from asthma and COPD patients and ViDiFlu staff members (Table 6.2) on the cellular profile of whole blood.

Table 6.2: Effect of vitamin D supplementation on the cellular profile of peripheral whole blood in data pooled from asthma patients, COPD patients and carers or staff members of sheltered accommodation schemes

Analyte	Placebo 12 Month		Intervention 12 Month		Statistics		
	n	median	n	median	F-statistic	P-value	Q-value
Absolute Basophils	68	0.100	60	0.050	1.807	0.180	0.985
Absolute mDCs	61	0.010	53	0.010	0.923	0.338	0.985
Absolute pDCs	61	0.004	53	0.004	0.174	0.677	0.985
% pDCs (% of DCs)	61	28.000	53	26.500	0.147	0.702	0.985
Absolute Eosinophils	68	0.200	60	0.200	0.098	0.754	0.985
% mDCs (% of DCs)	61	58.600	53	60.300	0.047	0.829	0.985
% CD4 T-cells (% of lymphocytes)	61	36.400	52	36.850	0.043	0.836	0.985
Absolute Neutrophils	68	3.800	60	3.850	0.032	0.857	0.985
Absolute CD4 T-cells	61	0.719	52	0.687	0.019	0.891	0.985
% Tregs (% of CD4 T-cells)	61	6.500	52	6.350	0.018	0.894	0.985
% B-cells (% of lymphocytes)	61	6.500	52	6.650	0.016	0.900	0.985
Absolute Lymphocytes	68	2.050	60	1.900	0.013	0.910	0.985
Absolute Tregs	61	0.045	52	0.046	0.011	0.918	0.985
% CD8 T-cells (% of lymphocytes)	61	16.800	52	17.850	0.009	0.923	0.985
Absolute Monocytes	68	0.600	60	0.500	0.005	0.945	0.985
Absolute B-cells	61	0.131	52	0.139	0.003	0.960	0.985
Absolute CD8 T-cells	61	0.324	52	0.368	0.000	0.985	0.985

Absolute cell counts are given as x 10^9 cells/L

Analysis of ViDiFlu residents provides a population of older adults living in sheltered accommodation without either asthma or COPD, to assess the effects of low-dose vitamin D supplementation versus high-dose vitamin D supplementation. As with the other trials, each data point was assigned an interaction variable for the time-point at which the sample was taken, and whether the participant was in the intervention or control arm of the study. Qlucore Omics Explorer 2.3 was used to analyse the data, modelling using a multi-group analysis (i.e. repeated measures ANOVA) on interaction variables with log-transformed data, and including adjustment for time-point and allocation. P-values were deemed significant when less than 0.05 and with a corresponding q-value of less than 0.1. High-dose vitamin D supplementation resulted in significantly fewer B-cells (Table 6.3, Figure 6.8), both in terms of percentage of total lymphocytes (median difference of -4.3%, p = 0.001, q = 0.01) and absolute numbers (median difference of -0.08 x 10^9 cells/ml, p = 0.0005, q = 0.008). No statistically significant effect of vitamin D supplementation was observed in any other cellular components of the blood in ViDiFlu residents (Table 6.3).

Table 6.3: Effect of high-dose vitamin D supplementation on the cellular profile of peripheral whole blood in older adults living in sheltered accommodation

Analyte	Low-Dose 12 Month		High-Dose 12 Month		Statistics		
	n	median	n	median	F-statistic	P-value	Q-value
Absolute B-cells	18	0.174 (0.11 to 0.28)	20	0.099 (0.07 to 0.13)	12.871	0.0005	0.008
% B-cells (% of lymphocytes)	18	10.050 (5.73 to 13.90)	20	5.750 (3.98 to 7.58)	10.930	0.001	0.011
Absolute Tregs	18	0.055	20	0.044	2.055	0.154	0.675
% mDCs (% of DCs)	18	65.900	20	56.250	1.751	0.188	0.675
Absolute CD4 T-cells	18	0.650	20	0.571	1.280	0.260	0.675
% CD4 T-cells (% of lymphocytes)	18	36.750	20	33.750	1.170	0.282	0.675
% pDCs (% of DCs)	18	23.400	20	31.050	1.123	0.292	0.675
Absolute pDCs	18	0.004	20	0.004	1.008	0.317	0.675
Absolute Basophils	24	0.000	34	0.000	0.783	0.378	0.714
% CD8 T-cells (% of lymphocytes)	18	17.200	20	18.250	0.509	0.477	0.797
Absolute mDCs	18	0.008	20	0.007	0.425	0.516	0.797
Absolute CD8 T-cells	18	0.342	20	0.320	0.266	0.607	0.854
% Tregs (% of CD4 T-cells)	18	7.500	20	6.350	0.186	0.667	0.854
Absolute Eosinophils	24	0.200	34	0.100	0.146	0.703	0.854
Absolute Neutrophils	24	3.750	34	3.150	0.092	0.762	0.857
Absolute Lymphocytes	24	2.050	34	1.750	0.060	0.807	0.857
Absolute Monocytes	24	0.500	34	0.400	0.012	0.912	0.912

Absolute cell counts are given as x 10^9 cells/L

IQRs are given for statistically significantly different results only

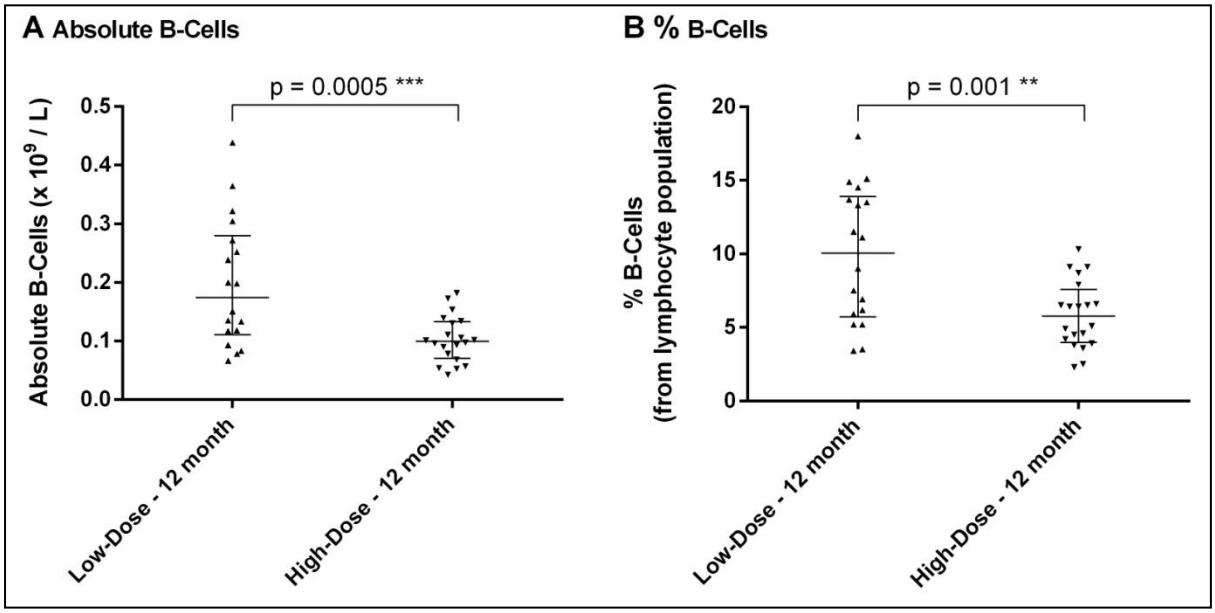


Figure 6.8: Effect of high-dose vitamin D supplementation on the B-cell population in peripheral whole blood of older adults living in sheltered accommodation. Peripheral blood was collected for n = 38 residents of sheltered accommodation schemes at their 12 month visit. Flow cytometry was used to determine the cellular profile, with B-cells defined as being $CD3^{-}CD19^{+}$ cells. Percentages were calculated from the total lymphocyte population, and absolute numbers were calculated using lymphocyte values obtained from coulter counts. Data are represented as scatter plots with median + IQR, with p-values calculated using a multi-group comparison on an interaction variable for time-point and allocation, adjusting for time-point and allocation using the programme Qlucore Omics Explorer 2.3. P-values were deemed significant when less than 0.05, with a corresponding q-value of less than 0.1.

6.2.4 Vitamin D supplementation has no effect on concentrations of inflammatory mediators present in the serum of asthma and COPD patients, or older adults with neither condition

Peripheral blood samples were collected from all participants at baseline, 2 month and 12 month follow-up visits into serum separating tubes (SST) to obtain serum samples. Serum samples from baseline and 12 month visits were analysed by multiplex ELISA, giving the concentrations of a panel of 30 inflammatory mediators. Each data point was assigned an interaction variable for the time-point at which the sample was taken, and whether the participant was in the intervention or control arm of the study. Qlucore Omics Explorer 2.3 was used to analyse the data, modelling using a multi-group analysis (i.e. repeated measures ANOVA) on interaction variables with log-transformed data, and including adjustment for time-point, allocation and study. P-values were deemed significant

when less than 0.05 and with a corresponding q-value of less than 0.1. No statistically significant effect of vitamin D supplementation on the serum concentrations of inflammatory mediators was observed in data pooled from asthma and COPD patients and ViDiFlu staff members (Table 6.4), or in data from older adults living in sheltered accommodation (Table 6.5).

Table 6.4: Effect of vitamin D supplementation on the serum concentration of inflammatory mediators in data pooled from asthma patients, COPD patients and carers or staff members of sheltered accommodation schemes

Analyte	Placebo 12 Month		Intervention 12 Month		Statistics		
	n	median	n	median	F-statistic	P-value	Q-value
RANTES [2]	29	6742.526	24	4414.131	1.344	0.248	0.999
EGF	46	22.962	36	18.863	1.288	0.258	0.999
IP-10	46	24.877	36	20.927	1.025	0.313	0.999
IL-4	46	9.013	36	7.581	0.809	0.370	0.999
G-CSF	46	43.389	36	40.426	0.754	0.387	0.999
TNF-α	46	4.387	36	2.715	0.411	0.522	0.999
IL-6	46	1.857	36	1.983	0.401	0.527	0.999
IL-17	46	1.995	36	1.542	0.333	0.564	0.999
MIP-1β	46	41.040	36	41.920	0.251	0.617	0.999
IL-8	46	8.941	36	11.102	0.221	0.639	0.999
GM-CSF	46	3.266	36	2.487	0.155	0.694	0.999
IL-1β	46	0.000	36	0.000	0.146	0.702	0.999
MIG	46	35.014	36	35.058	0.126	0.723	0.999
IL-13	46	13.071	36	14.880	0.123	0.727	0.999
IFN-γ	46	0.000	36	0.000	0.069	0.794	0.999
IL-15	46	34.780	36	28.861	0.059	0.808	0.999
IL-12	46	78.457	36	81.300	0.055	0.816	0.999
IL-2R	46	178.745	36	163.718	0.053	0.819	0.999
IL-7	46	5.202	36	11.997	0.051	0.822	0.999
VEGF	46	7.800	36	8.303	0.038	0.845	0.999
IL-2	46	0.760	36	0.323	0.037	0.848	0.999
MCP-1	46	295.190	36	257.977	0.031	0.860	0.999
HGF	46	149.857	36	141.356	0.028	0.867	0.999
IL-10	46	5.141	36	2.488	0.027	0.870	0.999
FGF-Basic	46	5.066	36	0.000	0.017	0.895	0.999
MIP-1α	46	34.357	36	33.449	0.002	0.965	0.999
IFN-α	46	39.895	36	33.475	0.002	0.966	0.999
IL-5	46	0.577	36	0.991	0.001	0.970	0.999
IL-1RA	46	793.315	36	761.486	0.000	0.988	0.999
Eotaxin	46	53.938	36	41.994	0.000	0.999	0.999

All medians are given in pg/ml

[2] For one analyte numbers were smaller due to mean fluorescence index (MFI) values exceeding the upper limit of detection, resulting in concentrations being unable to be calculated.

Table 6.5: Effect of high-dose vitamin D supplementation on the serum concentration of inflammatory mediators in older adults living in sheltered accommodation

Analyte	Low-Dose 12 Month		High-Dose 12 Month		Statistics		
	n	median	n	median	F-statistic	P-value	Q-value
G-CSF	23	30.886	33	43.037	2.487	0.118	0.991
MIP-1α	23	26.447	33	22.781	1.823	0.180	0.991
TNF-α	23	2.022	33	0.000	1.554	0.215	0.991
IP-10 [2]	23	35.668	32	38.604	1.406	0.238	0.991
RANTES [2]	21	4014.198	23	3712.770	0.691	0.408	0.991
MIG	23	35.322	33	40.323	0.556	0.458	0.991
IL-12	23	74.180	33	94.674	0.429	0.514	0.991
IL-5	23	0.623	33	0.586	0.421	0.518	0.991
IL-13	23	11.168	33	12.386	0.314	0.576	0.991
IFN-γ	23	1.024	33	0.627	0.309	0.580	0.991
GM-CSF	23	3.606	33	3.265	0.304	0.582	0.991
IL-7	23	0.000	33	0.000	0.252	0.617	0.991
MCP-1	23	284.070	33	368.421	0.178	0.674	0.991
IL-10	23	4.701	33	1.916	0.155	0.694	0.991
VEGF	23	5.237	33	0.000	0.127	0.722	0.991
IL-15	23	15.347	33	19.338	0.126	0.723	0.991
IL-8	23	14.049	33	15.241	0.125	0.724	0.991
EGF	23	47.288	33	55.489	0.124	0.725	0.991
IL-2	23	0.956	33	0.000	0.093	0.761	0.991
IL-4	23	8.054	33	6.214	0.082	0.775	0.991
IFN-α	23	22.655	33	29.430	0.053	0.819	0.991
IL-2R	23	130.589	33	154.260	0.043	0.837	0.991
HGF	23	141.410	33	144.590	0.036	0.850	0.991
IL-17	23	1.049	33	0.000	0.028	0.867	0.991
IL-1RA	23	510.715	33	555.900	0.020	0.887	0.991
IL-1β	23	0.000	33	0.000	0.014	0.905	0.991
MIP-1β	23	54.164	33	43.590	0.009	0.923	0.991
IL-6	23	2.438	33	2.393	0.005	0.945	0.991
FGF-Basic	23	15.440	33	11.782	0.000	0.986	0.991
Eotaxin	23	44.761	33	64.370	0.000	0.991	0.991

All medians are given in pg/ml

[2] For some analytes numbers were smaller due to MFI values exceeding the upper limit of detection, resulting in concentrations being unable to be calculated.

6.2.5 Vitamin D supplementation has no effect on the inflammatory response to bacterial TLR ligands in the peripheral whole blood of asthma and COPD patients, or older adults with neither condition

Peripheral whole blood samples were stimulated with a panel of 3 bacterial TLR ligands (LPS, Pam2CSK4 and Pam3CSK4), as described in chapter 2 and 3, at baseline, 2 month and 12 month follow-up visits in participants in all three clinical trials. Plasma samples were aspirated and baseline and 12 month samples were analysed by multiplex ELISA for 30 inflammatory mediators. Each data point was assigned an interaction variable for the time-point at which the sample was taken, and whether the participant was in the intervention or control arm of the study. Qlucore Omics Explorer 2.3 was used to analyse the data, modelling using a multi-group analysis (i.e. repeated measures ANOVA) on interaction variables with log-transformed data, and including adjustment for time-point, allocation and study. P-values were deemed significant when less than 0.05 and with a corresponding q-value of less than 0.1. No statistically significant effect of vitamin D supplementation on the concentrations of inflammatory mediators released following stimulation with LPS, Pam2CSK4 or Pam3CSK4 was observed in data pooled from asthma and COPD patients and ViDiFlu staff members (Table 6.6), or in data from older adults living in sheltered accommodation (Table 6.7). Stimuli were analysed separately, but are all presented in the same table.

Table 6.6: Effect of vitamin D supplementation on the release of inflammatory mediators following bacterial TLR stimulation of peripheral whole blood in data pooled from asthma patients, COPD patients and carers or staff members of sheltered accommodation schemes

Analyte	LPS-Stimulated Blood							Pam2CSK4-Stimulated Blood							Pam3CSK4-Stimulated Blood						
	Placebo 12 Month		Intervention 12 Month		Statistics			Placebo 12 Month		Intervention 12 Month		Statistics			Placebo 12 Month		Intervention 12 Month		Statistics		
	n	median	n	median	F-statistic	P-value	Q-value	n	median	n	median	F-statistic	P-value	Q-value	n	median	n	median	F-statistic	P-value	Q-value
EGF	46	63.272	37	48.760	0.770	0.382	0.988	46	47.698	37	36.708	0.165	0.685	0.997	46	46.548	37	43.146	0.278	0.599	0.979
Eotaxin	46	73.844	37	61.034	0.746	0.389	0.988	46	70.606	37	60.783	0.311	0.578	0.997	46	74.153	37	65.605	0.187	0.666	0.979
FGF-Basic	46	59.121	37	57.030	0.857	0.356	0.988	46	32.607	37	30.486	0.012	0.914	0.997	46	35.865	37	33.517	0.293	0.589	0.979
G-CSF	46	200.888	37	191.586	0.005	0.943	0.988	46	98.687	37	107.716	0.288	0.592	0.997	46	101.966	37	106.787	0.362	0.548	0.979
GM-CSF	46	3.841	37	3.815	0.006	0.939	0.988	46	2.961	37	2.801	0.017	0.897	0.997	46	3.272	37	2.980	0.549	0.460	0.979
HGF	46	231.055	37	241.821	0.231	0.631	0.988	46	174.683	37	177.617	0.000	0.997	0.997	46	194.589	37	193.324	0.040	0.842	0.979
IFN-α	46	200.484	37	184.875	0.089	0.765	0.988	46	199.169	37	188.122	0.001	0.981	0.997	46	205.985	37	184.267	0.001	0.979	0.979
IFN-γ	46	5.704	37	5.275	0.479	0.490	0.988	46	0.878	37	0.000	0.867	0.353	0.997	46	0.969	37	0.634	0.495	0.483	0.979
IL-10	46	442.983	37	456.496	0.067	0.797	0.988	46	41.172	37	35.255	0.591	0.443	0.997	46	71.107	37	65.874	0.372	0.543	0.979
IL-12	46	290.693	37	371.841	0.403	0.527	0.988	46	94.875	37	85.317	0.002	0.961	0.997	46	108.054	37	110.839	0.105	0.746	0.979
IL-13	46	29.992	37	28.138	0.087	0.769	0.988	46	16.764	37	15.066	0.377	0.540	0.997	46	17.990	37	17.312	0.007	0.931	0.979
IL-15	46	303.758	37	282.368	0.007	0.932	0.988	46	144.518	37	155.330	0.526	0.469	0.997	46	161.578	37	156.159	0.273	0.602	0.979
IL-17	46	6.971	37	8.050	0.316	0.575	0.988	46	4.407	37	4.039	0.385	0.536	0.997	46	4.401	37	4.567	0.421	0.517	0.979
IL-1β	46	215.894	37	252.360	0.323	0.571	0.988	46	32.126	37	27.277	0.015	0.904	0.997	46	33.586	37	29.709	0.022	0.882	0.979
IL-1RA	46	2596.107	37	2730.501	0.040	0.842	0.988	46	1215.744	37	1130.867	0.159	0.691	0.997	46	1266.004	37	1256.560	0.289	0.592	0.979
IL-2	46	6.378	37	6.191	0.390	0.533	0.988	46	2.266	37	2.730	0.299	0.585	0.997	46	2.646	37	3.685	0.108	0.743	0.979
IL-2R	46	374.259	37	360.047	1.176	0.280	0.988	46	264.703	37	246.369	0.361	0.549	0.997	46	255.626	37	266.409	3.593	0.060	0.979
IL-4	46	24.958	37	22.882	0.076	0.783	0.988	46	16.073	37	13.533	0.012	0.913	0.997	46	18.387	37	15.834	0.200	0.655	0.979
IL-5	46	4.394	37	3.644	0.167	0.683	0.988	46	2.105	37	1.617	0.707	0.402	0.997	46	2.104	37	2.068	0.028	0.867	0.979
IL-6 [2]	23	9114.264	21	11873.223	0.016	0.899	0.988	46	559.180	37	633.602	0.514	0.475	0.997	46	913.411	36	819.628	0.521	0.472	0.979
IL-7	46	76.072	37	79.161	0.003	0.959	0.988	46	38.711	37	37.501	0.662	0.417	0.997	46	43.151	37	42.315	0.003	0.956	0.979
IL-8 [2]	38	16958.791	33	17510.027	0.597	0.441	0.988	46	4451.871	37	5669.146	0.032	0.857	0.997	44	6092.960	36	6549.098	0.112	0.739	0.979
IP-10	46	182.213	37	248.095	0.023	0.878	0.988	46	49.432	37	49.838	1.277	0.260	0.997	46	55.411	37	52.733	0.495	0.483	0.979
MCP-1 [2]	44	14708.563	35	12132.999	0.351	0.554	0.988	44	17900.823	36	13808.395	0.123	0.727	0.997	44	17613.878	34	13139.492	0.022	0.881	0.979
MIG	46	64.166	37	70.806	1.192	0.277	0.988	46	50.628	37	48.300	1.385	0.241	0.997	46	52.249	37	55.386	0.158	0.691	0.979
MIP-1α [2]	45	5223.006	37	6026.641	0.000	0.992	0.992	46	168.465	37	137.063	0.282	0.596	0.997	46	299.052	37	199.748	0.328	0.568	0.979
MIP-1β [2]	30	20190.429	23	13751.303	0.528	0.468	0.988	46	1759.943	37	1830.232	0.033	0.856	0.997	46	2305.250	37	1896.575	0.096	0.757	0.979
RANTES [2]	21	4620.601	23	8174.674	4.099	0.045	0.988	25	5383.777	26	4616.125	0.159	0.691	0.997	24	4777.693	25	4757.865	0.631	0.428	0.979
TNF-α	46	116.384	37	126.752	0.025	0.874	0.988	46	13.758	37	13.870	0.084	0.772	0.997	46	18.662	37	18.119	0.019	0.892	0.979
VEGF	46	166.220	37	187.985	0.012	0.912	0.988	46	65.404	37	58.176	0.037	0.847	0.997	46	72.678	37	75.109	0.005	0.943	0.979

All medians are given in pg/ml

[2] For some analytes numbers were smaller due to MFI values exceeding the upper limit of detection, resulting in concentrations being unable to be calculated.

Table 6.7: Effect of high-dose vitamin D supplementation on the release of inflammatory mediators following bacterial TLR stimulation of peripheral whole blood in older adults living in sheltered accommodation

Analyte	LPS-Stimulated Blood							Pam2CSK4-Stimulated Blood							Pam3CSK4-Stimulated Blood						
	Low-Dose 12 Month		High-Dose 12 Month		Statistics			Low-Dose 12 Month		High-Dose 12 Month		Statistics			Low-Dose 12 Month		High-Dose 12 Month		Statistics		
	n	median	n	median	F-statistic	P-value	Q-value	n	median	n	median	F-statistic	P-value	Q-value	n	median	n	median	F-statistic	P-value	Q-value
EGF	24	42.004	34	41.797	0.160	0.690	0.990	24	31.053	34	30.404	0.136	0.713	0.990	24	33.663	34	30.564	0.747	0.389	0.962
Eotaxin	24	42.849	34	56.577	0.272	0.603	0.990	24	45.849	34	52.777	0.483	0.488	0.990	24	48.609	34	56.438	0.015	0.903	0.962
FGF-Basic	24	49.090	34	43.507	0.044	0.834	0.990	24	30.480	34	27.080	0.175	0.677	0.990	24	32.297	34	30.355	0.192	0.662	0.962
G-CSF	24	167.905	34	163.853	0.098	0.755	0.990	24	84.050	34	81.483	0.166	0.684	0.990	24	88.029	34	89.993	0.798	0.374	0.962
GM-CSF	24	4.278	34	3.454	0.480	0.490	0.990	24	3.199	34	2.899	0.614	0.435	0.990	24	3.147	34	2.976	0.762	0.385	0.962
HGF	24	174.030	34	167.141	0.138	0.711	0.990	24	128.424	34	125.680	0.085	0.772	0.990	24	123.364	34	133.788	0.015	0.902	0.962
IFN-α	24	141.700	34	139.920	0.024	0.877	0.990	24	135.245	34	128.359	0.002	0.964	0.990	24	131.364	34	138.535	0.432	0.512	0.962
IFN-γ	24	6.390	34	5.849	0.872	0.352	0.990	24	1.673	34	1.924	0.362	0.548	0.990	24	1.515	34	2.264	0.824	0.366	0.962
IL-10	24	223.686	34	286.530	0.138	0.711	0.990	24	34.566	34	20.977	0.722	0.397	0.990	24	42.952	34	41.731	0.223	0.638	0.962
IL-12	24	375.908	34	342.325	0.111	0.739	0.990	24	73.505	34	79.178	0.613	0.435	0.990	24	88.654	34	98.359	0.085	0.772	0.962
IL-13	24	23.805	34	22.123	0.729	0.395	0.990	24	13.404	34	12.537	0.665	0.417	0.990	24	14.977	34	12.577	0.923	0.339	0.962
IL-15	24	276.341	34	257.501	0.018	0.895	0.990	24	118.762	34	113.089	0.001	0.975	0.990	24	140.333	34	131.260	0.047	0.829	0.962
IL-17	24	6.084	34	5.672	0.374	0.542	0.990	24	1.579	34	0.000	0.348	0.557	0.990	24	1.314	34	0.000	0.160	0.690	0.962
IL-1β	24	249.374	34	212.410	0.364	0.548	0.990	24	21.110	34	18.216	0.951	0.332	0.990	24	28.141	34	18.739	0.780	0.379	0.962
IL-1RA	24	2267.938	34	2054.539	0.050	0.823	0.990	24	900.536	34	816.840	0.106	0.745	0.990	24	1024.002	34	935.158	0.045	0.833	0.962
IL-2	24	5.925	34	3.671	0.027	0.870	0.990	24	0.613	34	0.000	0.975	0.326	0.990	24	1.606	34	0.000	0.237	0.627	0.962
IL-2R	24	302.264	34	321.012	0.026	0.872	0.990	24	186.438	34	211.367	0.179	0.673	0.990	24	204.360	34	216.547	0.248	0.620	0.962
IL-4	24	13.858	34	15.841	0.035	0.851	0.990	24	7.746	34	10.285	0.075	0.784	0.990	24	11.251	34	10.292	0.004	0.952	0.980
IL-5	24	2.003	34	1.949	0.014	0.905	0.990	24	1.080	34	0.937	0.031	0.861	0.990	24	1.593	34	1.048	0.090	0.765	0.962
IL-6 [2]	15	8719.512	20	5726.877	0.010	0.919	0.990	24	513.205	34	549.656	0.644	0.424	0.990	21	622.804	34	524.494	0.028	0.867	0.962
IL-7	24	60.974	34	51.812	0.143	0.706	0.990	24	26.392	34	24.296	0.891	0.347	0.990	24	33.129	34	24.380	0.822	0.367	0.962
IL-8 [2]	21	16428.057	31	18170.461	0.201	0.655	0.990	24	4083.028	34	4108.477	0.003	0.958	0.990	23	5753.822	33	4988.821	0.065	0.799	0.962
IP-10 [2]	23	210.355	33	219.024	0.068	0.794	0.990	24	44.637	34	53.098	0.273	0.603	0.990	24	48.165	34	52.548	0.035	0.851	0.962
MCP-1 [2]	22	15588.260	32	15013.243	0.691	0.408	0.990	24	16486.173	30	15230.881	0.027	0.870	0.990	23	16962.927	30	19748.560	0.265	0.608	0.962
MIG	24	61.353	34	66.750	0.037	0.849	0.990	24	45.451	34	50.051	0.180	0.673	0.990	24	48.876	34	54.150	0.120	0.729	0.962
MIP-1α [2]	23	5304.089	34	4569.631	0.000	0.989	0.990	24	166.733	34	126.475	0.199	0.656	0.990	24	222.050	34	191.312	0.387	0.535	0.962
MIP-1β [2]	19	11390.066	21	14186.236	0.002	0.967	0.990	24	1855.232	34	1508.188	0.142	0.707	0.990	24	2140.887	34	1985.258	0.300	0.585	0.962
RANTES [2]	16	3643.494	23	3745.786	1.890	0.172	0.990	20	3005.843	26	2404.313	1.313	0.254	0.990	20	3177.786	28	2634.617	0.014	0.907	0.962
TNF-α	24	126.923	34	157.740	0.381	0.538	0.990	24	11.893	34	12.461	0.508	0.478	0.990	24	17.934	34	17.568	0.028	0.868	0.962
VEGF	24	131.785	34	145.957	0.019	0.892	0.990	24	55.872	34	54.463	0.150	0.699	0.990	24	68.400	34	58.984	0.040	0.842	0.962

All medians are given in pg/ml

[2] For some analytes numbers were smaller due to MFI values exceeding the upper limit of detection, resulting in concentrations being unable to be calculated

6.2.6 Vitamin D supplementation has no effect on the inflammatory response to viral TLR ligands in the peripheral whole blood of asthma and COPD patients, or older adults with neither condition

Peripheral whole blood samples were stimulated with a panel of viral TLR ligands (polyI:C and R848), as described in Chapters 2 and 3, at baseline, 2 month and 12 month follow-up visits in participants in all three clinical trials. Plasma samples were aspirated and baseline and 12 month samples were analysed by multiplex ELISA for 30 inflammatory mediators. Each data point was assigned an interaction variable for the time-point at which the sample was taken, and whether the participant was in the intervention or control arm of the study. Qlucore Omics Explorer 2.3 was used to analyse the data, modelling using a multi-group analysis (i.e. repeated measures ANOVA) on interaction variables with log-transformed data, and including adjustment for time-point, allocation and study. P-values were deemed significant when less than 0.05 and with a corresponding q-value of less than 0.1. No statistically significant effect of vitamin D supplementation on the concentrations of inflammatory mediators released following stimulation with polyI:C or R848 was observed in data pooled from asthma and COPD patients and ViDiFlu staff members (Table 6.8), or in data from older adults living in sheltered accommodation (Table 6.9). Stimuli were analysed separately, but are all presented in the same table.

Table 6.8: Effect of vitamin D supplementation on the release of inflammatory mediators following viral TLR stimulation of peripheral whole blood in data pooled from asthma patients, COPD patients and carers or staff members of sheltered accommodation schemes

Analyte	Poly I:C-Stimulated Blood							R848-Stimulated Blood						
	Placebo 12 Month		Intervention 12 Month		Statistics			Placebo 12 Month		Intervention 12 Month		Statistics		
	n	median	n	median	F-statistic	P-value	Q-value	n	median	n	median	F-statistic	P-value	Q-value
EGF	46	46.413	36	39.121	0.699	0.404	0.993	46	76.667	36	69.160	1.212	0.273	0.906
Eotaxin	46	19.442	36	18.854	1.328	0.251	0.993	46	58.496	36	53.606	2.291	0.132	0.906
FGF-Basic	46	34.614	36	38.403	0.573	0.450	0.993	46	110.983	36	123.411	1.988	0.161	0.906
G-CSF	46	93.599	36	97.503	0.027	0.870	0.993	46	237.563	36	249.365	0.232	0.631	0.906
GM-CSF	46	3.076	36	3.039	0.102	0.750	0.993	46	5.806	36	5.517	0.710	0.401	0.906
HGF	46	173.014	36	162.711	0.098	0.755	0.993	46	269.027	36	270.085	2.090	0.150	0.906
IFN-α	46	192.041	36	165.792	0.023	0.881	0.993	46	348.019	36	276.709	0.129	0.720	0.906
IFN-γ	46	3.249	36	3.875	0.739	0.391	0.993	46	184.115	36	202.253	0.141	0.707	0.906
IL-10	46	9.195	36	6.833	0.099	0.753	0.993	46	992.571	36	1127.910	0.616	0.434	0.906
IL-12 [2]	46	94.162	36	95.685	0.638	0.426	0.993	46	3044.744	34	3373.975	0.112	0.739	0.906
IL-13	46	12.472	36	12.780	2.439	0.120	0.993	46	32.902	36	33.775	0.117	0.733	0.906
IL-15	46	324.604	36	310.274	0.004	0.952	0.993	46	621.734	36	570.763	0.593	0.442	0.906
IL-17	46	3.706	36	3.544	3.286	0.072	0.993	46	8.037	36	9.202	0.255	0.614	0.906
IL-1β	46	24.697	36	25.161	1.363	0.245	0.993	46	1050.101	36	1231.554	0.613	0.435	0.906
IL-1RA	46	1512.207	36	1470.827	0.050	0.824	0.993	46	9230.802	36	9665.223	0.101	0.751	0.906
IL-2	46	3.354	36	3.917	0.008	0.930	0.993	46	7.875	36	7.897	1.267	0.262	0.906
IL-2R	46	239.068	36	249.956	2.285	0.133	0.993	46	428.457	36	429.691	2.253	0.135	0.906
IL-4	46	16.198	36	13.533	0.435	0.510	0.993	46	28.180	36	24.454	0.342	0.560	0.906
IL-5	46	1.580	36	1.390	0.003	0.959	0.993	46	5.032	36	4.898	0.042	0.837	0.906
IL-6 [2]	46	335.493	35	270.495	0.867	0.353	0.993	17	9256.145	15	13149.052	0.009	0.925	0.925
IL-7	46	28.620	36	26.180	0.408	0.524	0.993	46	96.098	36	95.268	0.031	0.859	0.906
IL-8 [2]	46	1284.730	36	1254.267	0.001	0.973	0.993	45	7264.994	34	7559.025	0.023	0.880	0.906
IP-10 [2]	42	438.609	30	710.958	0.281	0.597	0.993	16	1238.663	15	1758.062	0.555	0.457	0.906
MCP-1 [2]	44	18733.266	33	14512.085	0.000	0.989	0.993	44	17408.498	32	12800.339	0.024	0.876	0.906
MIG	46	55.482	36	60.178	0.137	0.712	0.993	46	86.780	36	112.896	1.297	0.257	0.906
MIP-1α [2]	46	206.095	36	148.299	0.017	0.896	0.993	43	9697.010	36	12301.902	0.524	0.470	0.906
MIP-1β [2]	45	2170.700	36	1897.009	0.011	0.915	0.993	29	15679.017	23	15907.578	0.942	0.333	0.906
RANTES [2]	44	1963.962	34	2281.582	1.275	0.260	0.993	28	5933.008	24	4626.947	1.265	0.262	0.906
TNF-α [2]	46	14.573	36	13.366	0.000	0.993	0.993	46	2144.856	35	2436.860	0.438	0.509	0.906
VEGF	46	55.175	36	51.004	0.000	0.990	0.993	46	333.682	36	363.447	0.254	0.615	0.906

All medians are given in pg/ml

[2] *For some analytes numbers were smaller due to MFI values exceeding the upper limit of detection, resulting in concentrations being unable to be calculated*

Table 6.9: Effect of high-dose vitamin D supplementation on the release of inflammatory mediators following viral TLR stimulation of peripheral whole blood in older adults living in sheltered accommodation

Analyte	PolyI:C-Stimulated Blood							R848-Stimulated Blood						
	Low-Dose 12 Month		High-Dose 12 Month		Statistics			Low-Dose 12 Month		High-Dose 12 Month		Statistics		
	n	median	n	median	F-statistic	P-value	Q-value	n	median	n	median	F-statistic	P-value	Q-value
EGF	24	34.109	34	26.428	0.713	0.400	0.812	24	70.688	34	61.401	0.132	0.717	0.977
Eotaxin	24	16.622	34	17.183	0.061	0.805	0.936	24	45.213	34	57.531	0.042	0.838	0.977
FGF-Basic	24	28.732	34	27.187	0.511	0.476	0.812	24	101.540	34	84.822	0.009	0.925	0.977
G-CSF	24	63.924	34	69.855	2.075	0.153	0.812	24	180.048	34	201.124	0.605	0.438	0.977
GM-CSF	24	3.936	34	3.496	1.133	0.289	0.812	24	5.622	34	4.931	0.406	0.525	0.977
HGF	24	118.078	34	120.591	0.177	0.675	0.842	24	217.290	34	213.103	0.358	0.551	0.977
IFN-α	24	134.208	34	130.150	0.273	0.603	0.842	24	211.434	34	213.643	1.034	0.311	0.977
IFN-γ	24	2.750	34	2.753	0.371	0.544	0.842	24	186.884	34	214.271	0.028	0.868	0.977
IL-10	24	7.998	34	5.598	1.243	0.267	0.812	24	777.029	34	816.084	0.054	0.817	0.977
IL-12	24	86.008	34	83.910	0.486	0.487	0.812	24	3649.981	34	2871.754	0.100	0.752	0.977
IL-13	24	11.039	34	11.469	0.921	0.339	0.812	24	29.517	34	30.619	0.764	0.384	0.977
IL-15	24	279.405	34	173.047	0.985	0.323	0.812	24	553.540	34	458.064	0.232	0.631	0.977
IL-17	24	2.099	34	0.000	2.737	0.101	0.812	24	7.224	34	7.452	0.722	0.397	0.977
IL-1β	24	24.972	34	14.406	1.385	0.242	0.812	24	1153.038	34	1200.248	0.224	0.637	0.977
IL-1RA	24	912.218	34	936.630	0.541	0.464	0.812	24	6854.931	34	6289.217	0.062	0.804	0.977
IL-2	24	2.683	34	0.000	2.793	0.098	0.812	24	7.122	34	6.240	0.033	0.857	0.977
IL-2R	24	177.479	34	207.954	1.297	0.257	0.812	24	346.183	34	403.896	0.046	0.831	0.977
IL-4	24	13.162	34	9.542	0.019	0.892	0.949	24	18.976	34	18.732	0.005	0.946	0.977
IL-5	24	1.100	34	1.240	0.252	0.617	0.842	24	4.043	34	3.263	0.067	0.797	0.977
IL-6 [2]	24	228.077	34	224.867	0.258	0.612	0.842	7	8833.411	10	9197.283	0.004	0.949	0.977
IL-7	24	18.575	34	20.253	2.291	0.133	0.812	24	64.143	34	70.051	0.105	0.747	0.977
IL-8 [2]	23	786.720	33	2054.620	0.707	0.402	0.812	21	8762.863	32	8986.964	0.268	0.605	0.977
IP-10 [2]	21	201.748	31	253.040	0.214	0.644	0.842	11	2050.420	20	1570.080	0.120	0.730	0.977
MCP-1 [2]	20	10037.817	29	15103.911	1.133	0.290	0.812	20	17840.684	30	20352.138	0.045	0.833	0.977
MIG	24	53.135	34	49.813	0.005	0.943	0.970	24	81.978	34	77.334	0.040	0.842	0.977
MIP-1α [2]	24	146.698	34	119.539	2.803	0.097	0.812	23	10324.786	33	10131.018	0.050	0.824	0.977
MIP-1β [2]	24	1498.407	34	1568.516	1.385	0.242	0.812	13	11637.306	20	14540.041	0.162	0.688	0.977
RANTES [2]	23	1277.811	33	1607.737	0.544	0.462	0.812	16	3841.058	20	4444.226	0.491	0.485	0.977
TNF-α [2]	24	11.922	34	9.966	1.265	0.263	0.812	20	2464.161	33	2770.427	0.302	0.584	0.977
VEGF	24	35.048	34	42.214	0.178	0.674	0.842	24	245.763	34	271.854	0.092	0.762	0.977

All medians are given in pg/ml

[2] For some analytes numbers were smaller due to MFI values exceeding the upper limit of detection, resulting in concentrations being unable to be calculated

6.2.7 Vitamin D supplementation has no effect on the inflammatory response to bacterial pathogens in the peripheral whole blood of asthma and COPD patients, or older adults with neither condition

Peripheral whole blood samples were infected with 2 bacterial pathogens (*Haemophilus influenzae* and *Streptococcus pneumoniae)*, as described in Chapters 2 and 3, at baseline, 2 month and 12 month follow-up visits in participants in all three clinical trials. Plasma samples were aspirated and baseline and 12 month samples were analysed by multiplex ELISA for 30 inflammatory mediators. Each data point was assigned an interaction variable for the time-point at which the sample was taken, and whether the participant was in the intervention or control arm of the study. Qlucore Omics Explorer 2.3 was used to analyse the data, modelling using a multi-group analysis (i.e. repeated measures ANOVA) on interaction variables with log-transformed data, and including adjustment for time-point, allocation and study. P-values were deemed significant when less than 0.05 and with a corresponding q-value of less than 0.1. No statistically significant effect of vitamin D supplementation on the concentrations of inflammatory mediators released following stimulation with *H-influenzae* or *S.pneumoniae* was observed in data pooled from asthma and COPD patients and ViDiFlu staff members (Table 6.10), or in data from older adults living in sheltered accommodation (Table 6.11). Stimuli were analysed separately, but are all presented in the same table.

Table 6.10: Effect of vitamin D supplementation on the release of inflammatory mediators following bacterial pathogen stimulation of peripheral whole blood in data pooled from asthma patients, COPD patients and carers or staff members of sheltered accommodation schemes

Analyte	*H.influenzae* -Stimulated Blood							*S.pneumoniae* -Stimulated Blood						
	Placebo 12 Month		Intervention 12 Month		Statistics			Placebo 12 Month		Intervention 12 Month		Statistics		
	n	median	n	median	F-statistic	P-value	Q-value	n	median	n	median	F-statistic	P-value	Q-value
EGF	40	77.135	32	66.803	1.936	0.166	0.948	40	106.120	32	84.738	1.827	0.178	0.915
Eotaxin	40	70.472	32	57.834	1.631	0.203	0.948	40	87.809	32	79.958	0.337	0.563	0.915
FGF-Basic	40	87.234	32	95.806	0.834	0.363	0.948	40	154.829	32	169.621	0.339	0.561	0.915
G-CSF	40	333.464	32	340.814	0.000	0.989	0.989	40	520.303	32	507.907	0.075	0.784	0.915
GM-CSF	40	5.236	32	5.050	0.358	0.550	0.948	40	19.845	32	16.237	0.194	0.660	0.915
HGF	40	269.084	32	259.543	0.017	0.895	0.979	40	339.019	32	351.291	0.931	0.336	0.915
IFN-α	40	210.396	32	189.449	0.156	0.693	0.948	40	220.986	32	203.726	0.064	0.801	0.915
IFN-γ	40	47.673	32	59.339	0.158	0.691	0.948	40	462.965	32	722.638	0.030	0.862	0.915
IL-10	40	1132.911	32	1074.293	0.165	0.685	0.948	40	248.765	32	226.324	0.178	0.674	0.915
IL-12	40	564.712	32	488.830	0.846	0.359	0.948	40	2291.424	32	2240.859	0.022	0.882	0.915
IL-13	40	34.016	32	31.442	1.220	0.271	0.948	40	36.140	32	34.962	0.937	0.334	0.915
IL-15	40	400.859	32	347.899	0.514	0.475	0.948	40	525.661	32	447.739	0.976	0.325	0.915
IL-17	40	8.534	32	9.394	1.003	0.318	0.948	40	11.343	32	12.461	1.834	0.178	0.915
IL-1β [2]	40	1130.344	32	1178.666	0.368	0.545	0.948	36	5941.831	27	5501.435	0.042	0.837	0.915
IL-1RA	40	3532.213	32	4075.383	0.984	0.323	0.948	40	5158.468	32	5886.761	0.610	0.436	0.915
IL-2	40	7.904	32	8.631	0.230	0.632	0.948	40	14.010	32	13.847	0.403	0.526	0.915
IL-2R	40	411.460	32	399.087	0.337	0.563	0.948	40	489.259	32	474.178	0.333	0.565	0.915
IL-4	40	27.945	32	26.478	0.001	0.977	0.989	40	31.271	32	29.857	1.060	0.305	0.915
IL-5	40	6.362	32	5.911	0.064	0.801	0.948	40	6.960	32	5.852	0.024	0.877	0.915
IL-6 [2]	18	12857.676	16	19032.900	0.042	0.837	0.948	16	15611.447	13	26208.943	0.024	0.877	0.915
IL-7	40	84.121	32	92.442	0.311	0.578	0.948	40	100.769	32	111.879	0.340	0.560	0.915
IL-8 [2]	34	17003.070	26	18644.345	0.369	0.545	0.948	33	20418.393	25	21484.110	0.097	0.756	0.915
IP-10 [2]	39	297.266	31	405.143	1.111	0.294	0.948	33	625.124	23	721.072	3.149	0.078	0.915
MCP-1 [2]	40	14501.348	31	10942.119	1.994	0.160	0.948	39	18699.243	30	13995.466	0.877	0.350	0.915
MIG	40	74.605	32	93.185	0.091	0.763	0.948	40	114.927	32	142.420	2.527	0.114	0.915
MIP-1α [2]	39	9311.637	31	9292.737	0.137	0.712	0.948	39	13720.846	30	20483.918	0.070	0.791	0.915
MIP-1β [2]	24	18082.764	23	20082.380	0.046	0.831	0.948	26	16567.900	20	14971.970	0.002	0.967	0.967
RANTES [2]	18	7188.639	20	7251.318	2.379	0.125	0.948	22	4903.688	24	4866.614	5.719	0.018	0.628
TNF-α [2]	40	417.779	32	382.622	0.181	0.671	0.948	31	4780.241	26	4938.045	0.020	0.889	0.915
VEGF	40	260.080	32	281.441	0.002	0.964	0.989	40	489.434	32	531.364	0.064	0.800	0.915

All medians are given in pg/ml

[2] For some analytes numbers were smaller due to MFI values exceeding the upper limit of detection, resulting in concentrations being unable to be calculated

Table 6.11: Effect of high-dose vitamin D supplementation on the release of inflammatory mediators following bacterial pathogen stimulation of peripheral whole blood in older adults living in sheltered accommodation

Analyte	*H.influenzae* -Stimulated Blood							*S.pneumoniae* -Stimulated Blood						
	Low-Dose 12 Month		High-Dose 12 Month		Statistics			Low-Dose 12 Month		High-Dose 12 Month		Statistics		
	n	median	n	median	F-statistic	P-value	Q-value	n	median	n	median	F-statistic	P-value	Q-value
EGF	8	57.052	11	57.840	1.524	0.220	0.990	8	97.237	11	97.232	0.032	0.858	0.998
Eotaxin	8	31.705	11	45.561	0.008	0.930	0.990	8	53.333	11	56.887	1.118	0.293	0.998
FGF-Basic	8	104.383	11	80.321	0.014	0.905	0.990	8	179.672	11	197.603	0.003	0.957	0.998
G-CSF	8	201.833	11	182.489	0.137	0.712	0.990	8	375.010	11	326.130	0.281	0.597	0.998
GM-CSF	8	4.575	11	4.568	0.040	0.843	0.990	8	16.485	11	15.454	0.009	0.924	0.998
HGF	8	194.669	11	185.160	0.122	0.727	0.990	8	262.798	11	221.302	0.005	0.943	0.998
IFN-α	8	174.400	11	174.186	0.004	0.950	0.990	8	188.778	11	192.813	0.245	0.621	0.998
IFN-γ	8	60.464	11	42.727	0.673	0.414	0.990	8	671.949	11	250.475	1.258	0.264	0.998
IL-10	8	415.239	11	460.621	0.065	0.799	0.990	8	122.609	11	142.562	0.006	0.936	0.998
IL-12	8	617.677	11	473.589	0.009	0.925	0.990	8	2036.033	11	1726.786	0.301	0.584	0.998
IL-13	8	23.684	11	24.962	0.224	0.637	0.990	8	28.234	11	27.902	1.104	0.296	0.998
IL-15	8	481.769	11	374.935	0.893	0.347	0.990	8	534.582	11	551.031	0.044	0.834	0.998
IL-17	8	7.032	11	7.120	0.669	0.415	0.990	8	7.886	11	8.594	0.022	0.881	0.998
IL-1β [2]	8	974.437	11	644.575	0.067	0.796	0.990	7	5056.543	10	5443.089	0.080	0.777	0.998
IL-1RA	8	3618.199	11	3255.416	0.000	0.984	0.990	8	5101.052	11	3375.860	0.222	0.638	0.998
IL-2	8	5.996	11	5.357	0.018	0.895	0.990	8	9.989	11	8.544	0.176	0.676	0.998
IL-2R	8	381.770	11	458.309	0.202	0.654	0.990	8	424.774	11	474.760	0.603	0.439	0.998
IL-4	8	23.059	11	23.713	0.000	0.985	0.990	8	27.216	11	27.577	0.000	0.998	0.998
IL-5	8	6.427	11	6.493	0.048	0.827	0.990	8	6.889	11	6.852	0.362	0.549	0.998
IL-6 [2]	3	26301.965	3	31121.647	1.487	0.225	0.990	1	34802.114	2	19052.634	0.297	0.587	0.998
IL-7	8	54.272	11	60.111	0.031	0.861	0.990	8	58.737	11	61.059	0.044	0.834	0.998
IL-8 [2]	8	23866.450	11	21215.646	0.441	0.508	0.990	7	24134.295	10	20899.655	2.263	0.135	0.998
IP-10 [2]	7	935.394	11	387.307	0.676	0.413	0.990	3	1180.792	9	335.269	0.476	0.492	0.998
MCP-1 [2]	8	14491.513	11	11371.131	0.177	0.675	0.990	7	14520.964	11	10151.394	0.016	0.900	0.998
MIG	8	63.363	11	74.633	0.546	0.462	0.990	8	86.091	11	107.687	0.005	0.945	0.998
MIP-1α	8	6821.709	11	8670.035	0.201	0.655	0.990	8	12159.825	11	9886.146	0.002	0.969	0.998
MIP-1β [2]	7	15763.189	6	11202.286	0.326	0.569	0.990	6	18003.251	7	11879.005	0.548	0.461	0.998
RANTES [2]	7	3452.343	6	6250.522	0.820	0.367	0.990	4	7830.596	7	6209.783	0.432	0.513	0.998
TNF-α [2]	8	622.405	11	320.114	1.130	0.290	0.990	6	5102.707	9	3490.737	2.129	0.147	0.998
VEGF	8	189.375	11	213.069	0.061	0.805	0.990	8	339.304	11	471.314	0.013	0.908	0.998

All medians are given in pg/ml

[2] *For some analytes numbers were smaller due to MFI values exceeding the upper limit of detection, resulting in concentrations being unable to be calculated*

6.2.8 Vitamin D supplementation has no effect on the inflammatory response to viral pathogens in the peripheral whole blood of asthma and COPD patients, or older adults with neither condition

Peripheral whole blood samples were infected with 2 viral pathogens (Rhinoviurs-16, Rhinovirus-1B, and respiratory syncytial virus), as described in Chapters 2 and 3, at baseline, 2 month and 12 month follow-up visits in participants in all three clinical trials. Plasma samples were aspirated and baseline and 12 month samples were analysed by multiplex ELISA for 30 inflammatory mediators. Each data point was assigned an interaction variable for the time-point at which the sample was taken, and whether the participant was in the intervention or control arm of the study. Qlucore Omics Explorer 2.3 was used to analyse the data, modelling using a multi-group analysis (i.e. repeated measures ANOVA) on interaction variables with log-transformed data, and including adjustment for time-point, allocation and study. P-values were deemed significant when less than 0.05 and with a corresponding q-value of less than 0.1. No statistically significant effect of vitamin D supplementation on the concentrations of inflammatory mediators released following stimulation with RV-16, RV-1B, or RSV was observed in data pooled from asthma and COPD patients and ViDiFlu staff members (Table 6.12), or in data from older adults living in sheltered accommodation (Table 6.13). Stimuli were analysed separately, but are all presented in the same table.

Table 6.12: Effect of vitamin D supplementation on the release of inflammatory mediators following viral pathogen stimulation of peripheral whole blood in data pooled from asthma patients, COPD patients and carers or staff members of sheltered accommodation schemes

Analyte	RV-16-Stimulated Blood							RV-1B-Stimulated Blood							RSV-Stimulated Blood						
	Placebo 12 Month		Intervention 12 Month		Statistics			Placebo 12 Month		Intervention 12 Month		Statistics			Placebo 12 Month		Intervention 12 Month		Statistics		
	n	median	n	median	F-statistic	P-value	Q-value	n	median	n	median	F-statistic	P-value	Q-value	n	median	n	median	F-statistic	P-value	Q-value
EGF	42	71.807	33	62.116	0.975	0.325	0.992	42	62.836	33	62.547	2.145	0.145	0.976	42	65.410	34	61.279	2.543	0.113	0.912
Eotaxin	42	50.594	33	51.703	0.573	0.450	0.992	42	52.897	33	54.119	0.625	0.430	0.976	42	48.806	34	44.499	0.465	0.496	0.949
FGF-Basic	42	60.755	33	56.537	0.633	0.427	0.992	42	60.926	33	52.407	0.796	0.373	0.976	42	51.897	34	49.415	2.061	0.153	0.912
G-CSF	42	143.132	33	153.641	0.034	0.854	0.992	42	161.503	33	157.229	0.549	0.460	0.976	42	133.571	34	142.008	0.741	0.391	0.912
GM-CSF	42	4.254	33	3.697	0.583	0.446	0.992	42	5.017	33	4.850	0.151	0.698	0.976	42	6.175	34	6.524	0.096	0.758	0.949
HGF	42	240.643	33	223.690	1.250	0.265	0.992	42	244.533	33	223.603	1.002	0.318	0.976	42	234.019	34	207.033	1.292	0.257	0.912
IFN-α [2]	42	747.390	33	627.220	0.031	0.861	0.992	41	832.540	33	827.110	0.033	0.856	0.976	42	627.675	34	393.254	0.127	0.722	0.949
IFN-γ	42	103.239	33	84.183	0.000	0.983	0.992	42	154.035	33	135.952	0.003	0.953	0.976	42	84.947	34	81.551	0.043	0.835	0.949
IL-10	42	111.925	33	159.228	0.219	0.640	0.992	42	167.927	33	112.999	0.116	0.734	0.976	42	18.616	34	16.972	1.090	0.298	0.912
IL-12	42	208.510	33	225.449	0.000	0.992	0.992	42	214.296	33	146.086	0.270	0.604	0.976	42	99.860	34	105.757	4.321	0.039	0.912
IL-13	42	25.639	33	25.845	0.004	0.951	0.992	42	27.968	33	24.962	0.214	0.644	0.976	42	28.597	34	29.031	0.000	0.989	0.989
IL-15	42	597.977	33	595.165	0.637	0.426	0.992	42	589.518	33	495.394	0.146	0.703	0.976	42	625.767	34	565.651	0.002	0.960	0.989
IL-17	42	5.786	33	6.619	0.015	0.901	0.992	42	6.824	33	7.488	0.124	0.725	0.976	42	6.013	34	6.107	1.529	0.218	0.912
IL-1β	42	94.209	33	95.212	0.055	0.815	0.992	42	116.343	33	110.465	0.002	0.969	0.976	42	25.044	34	21.929	1.969	0.162	0.912
IL-1RA	42	3997.979	33	3924.780	0.146	0.703	0.992	42	3569.823	33	3585.622	0.117	0.732	0.976	42	2863.930	34	2557.472	0.168	0.683	0.949
IL-2	42	14.375	33	11.963	1.004	0.318	0.992	42	13.943	33	14.795	0.467	0.495	0.976	42	33.144	34	46.707	0.002	0.960	0.989
IL-2R	42	331.975	33	333.122	0.546	0.461	0.992	42	351.538	33	359.494	0.176	0.675	0.976	42	297.323	34	286.913	1.195	0.276	0.912
IL-4	42	24.456	33	20.961	0.026	0.873	0.992	42	23.955	33	20.858	0.087	0.769	0.976	42	24.673	34	21.058	1.295	0.257	0.912
IL-5	42	3.291	33	4.241	0.003	0.959	0.992	42	3.775	33	3.905	0.070	0.791	0.976	42	5.086	34	4.669	0.149	0.700	0.949
IL-6 [2]	35	2285.342	28	2341.378	0.088	0.767	0.992	33	2627.379	27	3129.086	0.001	0.976	0.976	14	12446.547	15	13417.626	0.111	0.740	0.949
IL-7	42	64.867	33	64.380	0.160	0.689	0.992	42	70.810	33	70.337	0.017	0.897	0.976	42	77.652	34	87.587	0.041	0.840	0.949
IL-8 [2]	41	8041.475	31	3962.007	0.010	0.921	0.992	39	7004.204	31	8981.376	0.020	0.887	0.976	37	11864.094	30	10255.796	0.003	0.956	0.989
IP-10 [2]	7	1495.336	7	2008.095	0.728	0.395	0.992	10	1074.726	11	1873.535	1.268	0.262	0.976	8	2244.222	10	2127.778	0.326	0.569	0.949
MCP-1 [2]	39	18226.338	29	12518.084	0.095	0.758	0.992	40	19822.594	30	13689.007	0.006	0.940	0.976	40	19852.978	31	13663.212	0.835	0.362	0.912
MIG	42	90.375	33	97.305	0.000	0.988	0.992	42	108.103	33	110.611	0.044	0.834	0.976	42	85.403	34	83.168	0.854	0.357	0.912
MIP-1α	42	1410.187	33	1519.257	0.478	0.490	0.992	42	2222.742	33	1583.690	0.315	0.575	0.976	42	297.969	34	322.720	0.299	0.585	0.949
MIP-1β [2]	39	4254.513	31	4662.511	0.021	0.884	0.992	39	5229.846	28	3405.547	0.214	0.644	0.976	42	1569.431	34	2003.826	0.046	0.831	0.949
RANTES [2]	27	5571.371	21	4863.842	1.397	0.239	0.992	21	4486.328	19	6493.904	0.368	0.545	0.976	24	5218.661	19	4006.158	3.240	0.074	0.912
TNF-α	42	62.061	33	82.367	0.011	0.917	0.992	42	92.070	33	66.558	0.016	0.901	0.976	42	18.135	34	16.900	0.773	0.381	0.912
VEGF	42	128.708	33	159.123	0.078	0.780	0.992	42	141.312	33	135.850	0.323	0.571	0.976	42	179.025	34	201.106	0.468	0.495	0.949

All medians are given in pg/ml

[2] For some analytes numbers were smaller due to MFI values exceeding the upper limit of detection, resulting in concentrations being unable to be calculated

Table 6.13: Effect of high-dose vitamin D supplementation on the release of inflammatory mediators following viral pathogen stimulation of peripheral whole blood in older adults living in sheltered accommodation

Analyte	RV-16-Stimulated Blood							RV-1B-Stimulated Blood							RSV-Stimulated Blood						
	Low-Dose 12 Month		High-Dose 12 Month		Statistics			Low-Dose 12 Month		High-Dose 12 Month		Statistics			Low-Dose 12 Month		High-Dose 12 Month		Statistics		
	n	median	n	median	F-statistic	P-value	Q-value	n	median	n	median	F-statistic	P-value	Q-value	n	median	n	median	F-statistic	P-value	Q-value
EGF	13	68.071	16	66.505	0.728	0.395	1.000	13	70.422	16	66.048	0.001	0.970	0.990	13	69.439	16	64.912	0.328	0.568	0.961
Eotaxin	13	29.793	16	47.614	0.704	0.403	1.000	13	30.494	16	47.151	0.909	0.342	0.990	13	30.843	16	38.451	0.437	0.510	0.961
FGF-Basic	13	48.209	16	48.965	0.000	0.991	1.000	13	57.089	16	50.599	0.829	0.364	0.990	13	49.247	16	48.231	0.180	0.672	0.961
G-CSF	13	89.313	16	95.935	1.733	0.191	1.000	13	92.921	16	102.980	0.895	0.346	0.990	13	90.503	16	96.077	2.561	0.112	0.961
GM-CSF	13	4.895	16	4.152	0.916	0.341	1.000	13	5.559	16	4.708	0.511	0.476	0.990	13	6.758	16	6.678	1.490	0.225	0.961
HGF	13	200.185	16	187.348	1.165	0.283	1.000	13	195.290	16	204.723	0.179	0.673	0.990	13	191.241	16	194.325	0.953	0.331	0.961
IFN-α	13	533.472	16	491.822	0.000	1.000	1.000	13	621.285	16	464.009	0.771	0.382	0.990	13	405.223	16	317.202	0.001	0.981	0.990
IFN-γ	13	35.898	16	26.793	0.032	0.859	1.000	13	82.871	16	61.158	0.087	0.769	0.990	13	83.567	16	59.988	1.322	0.253	0.961
IL-10	13	80.661	16	70.364	0.151	0.698	1.000	13	219.091	16	158.947	0.010	0.922	0.990	13	13.394	16	11.899	1.262	0.264	0.961
IL-12	13	140.927	16	177.438	0.892	0.347	1.000	13	139.227	16	176.881	0.022	0.882	0.990	13	100.502	16	115.218	0.376	0.541	0.961
IL-13	13	26.778	16	28.509	3.664	0.058	1.000	13	28.831	16	29.888	0.490	0.485	0.990	13	29.076	16	30.651	3.216	0.076	0.961
IL-15	13	680.184	16	641.667	0.041	0.839	1.000	13	675.468	16	594.566	0.010	0.919	0.990	13	676.595	16	630.254	0.046	0.830	0.961
IL-17	13	4.214	16	5.382	0.001	0.970	1.000	13	4.379	16	5.223	0.001	0.971	0.990	13	4.214	16	5.032	0.035	0.851	0.961
IL-1β	13	40.021	16	39.324	0.033	0.856	1.000	13	90.542	16	101.118	0.005	0.943	0.990	13	27.212	16	25.623	0.110	0.740	0.961
IL-1RA	13	3167.497	16	3201.909	0.327	0.569	1.000	13	2971.725	16	2992.651	0.073	0.787	0.990	13	2663.100	16	2829.375	0.036	0.851	0.961
IL-2	13	9.864	16	13.889	0.348	0.556	1.000	2	10.801	16	10.767	1.227	0.270	0.990	13	50.658	16	40.411	0.878	0.351	0.961
IL-2R	13	299.933	16	317.971	0.447	0.505	1.000	13	324.659	16	360.368	0.156	0.694	0.990	13	246.156	16	296.847	0.882	0.350	0.961
IL-4	13	22.285	16	21.077	0.097	0.756	1.000	13	21.879	16	22.710	0.026	0.872	0.990	13	23.503	16	22.710	0.150	0.699	0.961
IL-5	13	3.837	16	3.831	0.199	0.656	1.000	13	4.677	16	4.326	0.101	0.751	0.990	13	4.363	16	5.095	0.006	0.938	0.990
IL-6 [2]	9	1352.660	13	862.321	0.032	0.859	1.000	8	2229.797	10	1152.521	0.593	0.443	0.990	1	2772.589	0	n/a	1.829	0.179	0.961
IL-7	13	49.134	16	46.400	0.417	0.520	1.000	13	50.164	16	54.047	0.114	0.736	0.990	13	68.296	16	69.859	0.225	0.636	0.961
IL-8 [2]	10	4415.088	15	4407.683	0.112	0.739	1.000	11	5574.035	15	5390.371	0.434	0.511	0.990	9	6235.638	12	12636.244	0.569	0.452	0.961
IP-10 [2]	0	n/a	4	2379.834	0.785	0.378	1.000	13	809.702	7	1448.571	0.094	0.759	0.990	1	1376.500	5	1790.657	0.694	0.407	0.961
MCP-1 [2]	9	29446.866	14	21355.671	0.669	0.415	1.000	10	26876.529	14	19465.024	0.022	0.881	0.990	10	25271.051	14	19861.988	0.169	0.682	0.961
MIG	13	68.718	16	56.641	0.103	0.749	1.000	13	59.802	16	55.369	0.247	0.620	0.990	13	59.334	16	51.668	0.775	0.380	0.961
MIP-1α [2]	13	806.647	16	726.748	0.149	0.700	1.000	13	1338.350	16	1997.940	0.061	0.805	0.990	13	186.781	16	314.606	0.496	0.483	0.961
MIP-1β [2]	11	3065.921	16	2827.837	0.149	0.700	1.000	10	4793.079	15	3965.397	0.137	0.712	0.990	12	1030.862	16	1278.308	0.055	0.816	0.961
RANTES [2]	5	5237.248	7	6216.103	0.095	0.758	1.000	5	4925.518	6	7897.327	1.635	0.204	0.990	7	2524.622	3	3948.604	0.302	0.584	0.961
TNF-α	13	41.622	16	37.836	0.061	0.806	1.000	13	90.766	16	85.686	0.001	0.977	0.990	13	14.631	16	13.585	1.816	0.181	0.961
VEGF	13	95.411	16	83.840	0.468	0.495	1.000	13	101.363	16	93.713	0.161	0.689	0.990	13	191.127	16	160.894	0.999	0.320	0.961

All medians are given in pg/ml

[2] For some analytes numbers were smaller due to MFI values exceeding the upper limit of detection, resulting in concentrations being unable to be calculated

6.2.9 Sub-group analysis by baseline deficiency and genotype shows no effect of vitamin D supplementation on the release of inflammatory mediators from stimulated whole blood

It has previously been demonstrated that an effect of vitamin D supplementation may only be observable in those people who were profoundly deficient at baseline [308]. As such, it was decided to stratify the results from the whole blood assay by baseline 25(OH)D deficiency, with a concentration of < 50nmol/L used as the cut-off. As with the main analysis, as already presented in this chapter, data for asthma and COPD patients and ViDiFlu staff were pooled, while ViDiFlu residents were evaluated separately. Results were analysed systematically, looking at the cellular profile, and inflammatory mediators in serum and blood stimulated by a range of TLR ligands and pathogens. Each data point was assigned an interaction variable for the time-point at which the sample was taken, and whether the participant was in the intervention or control arm of the study. Qlucore Omics Explorer 2.3 was used to analyse the data, modelling using a multi-group analysis (i.e. repeated measures ANOVA) on interaction variables with log-transformed data, and including adjustment for time-point, allocation and study.

The results are shown for all variables which had a p-value of less than 0.05 (Table 6.14), showing a potential effect of vitamin D supplementation in increasing LPS-stimulated RANTES concentration in data pooled from deficient COPD and asthma patients and ViDiFlu staff ($p = 0.04$), and PolyI:C-stimulated IL-2R ($p = 0.047$), and IL-12 ($p = 0.04$) and RSV-stimulated G-CSF ($p = 0.049$) concentrations in deficient ViDiFlu residents. There was also a potential effect of high-dose supplementation in decreasing PolyI:C-stimulated MIP-1α ($p = 0.01$), TNF-α ($p = 0.03$), IL-1β ($p = 0.03$), IL-2 ($p = 0.045$) and IL-8 ($p = 0.049$) concentrations in deficient ViDiFlu residents when compared to 12 month samples in the placebo arm. Additionally, the effect of high-dose vitamin D supplementation on reducing the number of B-cells in peripheral blood was demonstrated again in vitamin D deficient older adults living in sheltered accommodation ($p = 0.04$). However, all of these results were associated with a q value of greater than 0.1, meaning that there is a high likelihood of false positives among the discoveries, and thus they cannot reliably be deemed significant.

Table 6.14: Effect of vitamin D supplementation on the release of inflammatory mediators in the blood of people with baseline vitamin D deficiency (<50nmol/L)

Study	Sample type	Analyte	Placebo / Low-Dose 12 Month		Intervention / High-Dose 12 Month		Statistics		
			n	median (IQR)	n	median (IQR)	F-statistic	P-value	Q-value
Pooled	LPS-stimulated blood	RANTES [2]	12	5250.609 (2776 to 14434)	15	8174.674 (3400 to 24162)	4.142	0.044	0.962
ViDiFlu Residents	PolyI:C-stimulated blood	MIP-1α	12	229.176 (117 to 2272)	18	110.985 (75 to 234)	6.835	0.012	0.247
ViDiFlu Residents	PolyI:C-stimulated blood	TNF-α	12	18.925 (6 to 51)	18	9.240 (7 to 15)	4.754	0.034	0.247
ViDiFlu Residents	PolyI:C-stimulated blood	IL-1β	12	33.388 (14 to 104)	18	14.406 (9 to 31)	4.735	0.034	0.247
ViDiFlu Residents	PolyI:C-stimulated blood	IL-12	12	81.126 (68 to 103)	18	90.474 (65 to 96)	4.415	0.040	0.247
ViDiFlu Residents	PolyI:C-stimulated blood	IL-2	12	2.837 (2 to 4)	18	2.216 (0 to 3)	4.204	0.045	0.247
ViDiFlu Residents	PolyI:C-stimulated blood	IL-2R	12	182.433 (147 to 239)	18	208.417 (149 to 258)	4.114	0.047	0.247
ViDiFlu Residents	PolyI:C-stimulated blood	IL-8 [2]	11	3110.474 (630 to 15341)	18	2115.061 (685 to 3984)	4.035	0.049	0.247
ViDiFlu Residents	RSV-stimulated blood	G-CSF	8	79.496 (69 to 105)	12	96.077 (78 to 123)	4.052	0.049	0.919
ViDiFlu Residents	FACS analysis of blood	B-cells	9	0.135 (0.08 to 0.3)	11	0.096 (0.06 to 0.1)	4.395	0.041	0.691

Medians for analytes are given in pg/ml; absolute B-cells are given as x 10^9 / L

For pooled samples, comparison was between placebo and intervention, for ViDiFlu residents comparison was between low-dose and high-dose vitamin D supplementation.

[2] For some analytes numbers were smaller due to MFI values exceeding the upper limit of detection, resulting in concentrations being unable to be calculated

Other studies have also demonstrated that the various effects of vitamin D supplementation in respiratory diseases are modified by genotype [328, 329, 336]. As such, it was decided to stratify analysis by genotype, looking specifically at SNPs in the vitamin D receptor (VDR) and retinoid x receptor (RXRA) genes. The clinical analysis of the ViDiCO trial showed an interaction effect, with genotype modifying the effect of allocation on the primary outcome of time to upper respiratory tract infection (methodology described in Chapter 2.7). Vitamin D was demonstrated to be protective by increasing the time to experiencing an upper respiratory tract infection in COPD patients with the minor alleles for the VDR SNPs rs4334089, rs11568820, rs7976091 and rs7970314, and the RXRA SNP rs7861779, and the major homozygous alleles for the VDR SNP rs10783219 (results not yet published, but summarised in Table 5.6 of Chapter 5). As such, these genotypes were chosen for stratification of the whole blood assay analysis. Each data point was assigned an interaction variable for the month the sample was taken, and whether the participant was on the

intervention or control arm of the study as before. Qlucore Omics Explorer 2.3 was used to analyse the data, modelling using a multi-group comparison (i.e. repeated measures ANOVA) on interaction variable, and adjusting for time-point and allocation. However, the number of participants with each genotype for each SNP was deemed to be too small to accurately make any significant conclusions on whether the effect of vitamin D supplementation on the release of inflammatory mediators from stimulated blood was modified by genotype due to a lack of statistical power (Table 6.15).

Table 6.15: The number of COPD patients with each SNP genotype, separated by time-point and allocation

Gene	SNP	Genotype	Control 0 Month	Control 12 Month	Intervention 0 Month	Intervention 12 Month
VDR	rs4334089	GG	10	10	3	3
		AG	3	3	4	4
		AA	1	1	1	1
VDR	rs10783219	AA	6	6	4	4
		AT	5	5	3	3
		TT	3	3	0	0
VDR	rs11568820	CC	9	9	2	2
		CT	4	4	6	6
		TT	0	0	0	0
VDR	rs7976091	CC	10	10	2	2
		CT	4	4	6	6
		TT	0	0	0	0
VDR	rs7970314	AA	10	10	2	2
		AG	4	4	6	6
		GG	0	0	0	0
RXRA	rs7861779	GG	11	11	5	5
		AG	3	3	2	2
		AA	0	0	0	0

Abbreviations used: RXR: retinoid X receptor; SNP: single nucleotide polymorphism; VDR: vitamin D receptor

6.3 Discussion

It has been demonstrated here that supplementation with vitamin D resulted in a significant increase in serum 25(OH)D concentration throughout the course of the study for all four study populations. This was associated with a decrease in the number and percentage of B-cells in the peripheral whole blood of older adults with neither asthma nor COPD living in sheltered accommodation in a comparison between high-dose and low-dose vitamin D supplementation. However, no other differences in the cellular profile or the inflammatory response mounted to antigenic stimulation in the blood of asthmatics or COPD patients were observed.

B-cells are components of the adaptive immune system, which are able to secrete antibodies, and inflammatory mediators, as well as acting as antigen presenting cells in response to infection by viruses or bacteria. The B-cell population in this study was quantified by selecting lymphocytes

based in forward and side scatter properties, before defining B-cells as being $CD3^-CD19^+$. CD19 is a component of the B-cell co-receptor complex, which modulates signal transduction through the B-cell receptor (BCR) to affect tolerance, differentiation, proliferation, activation and antigen presentation. It is present on the surface of B-cells from the early pro-B-cell stage in the bone marrow, to mature B-cells in the periphery which are able to bind antigens and secrete antibodies, as well as on regulatory B-cells, which aid in maintaining tolerance [604]. Expression of CD19 is only lost upon terminal differentiation into plasma cells [605, 606]. Therefore, the B-cell population quantified here potentially includes immature, naive, activated and memory B-cells, but not plasma cells, with distinction between the different types impossible without further staining for cell surface markers such as the use of CD27 to distinguish memory B-cells. CD19 is also expressed on follicular dendritic cells, however, since they are only present in the follicles of lymphoid organs, CD19 can be used accurately as a marker B-cells in peripheral blood without detecting any other cell types [605, 607].

B-cells are known to express the vitamin D receptor and CYP27B1, and thus can be modulated by $1,25(OH)_2D$ [252, 255]. It has previously been demonstrated that $1,25(OH)_2D$ suppresses cellular proliferation and antibody production following activation of B-cells in PBMCs [608], while also increasing apoptosis and decreasing proliferation, plasma cell differentiation and memory cell differentiation in activated primary B-cells [255]. B-cells have been demonstrated to have a role in autoimmune diseases, by way of secretion of autoantibodies and presentation of autoantigens [398, 609, 610]. It has been shown that people with autoimmune diseases such as systemic lupus erythematous (SLE) often have low serum concentrations of 25(OH)D, with an inverse correlation with disease activity [611-613]. As such, vitamin D supplementation has been trialled in patients with SLE, demonstrating a decrease in memory B-cells in PBMCs [399]. However, no effect was seen when looking at circulating B-cell subsets and immunoglobulin production in the plasma of multiple sclerosis patients supplemented with 20,000IU /day of vitamin D_3 [614].

As previously mentioned, none of the intervention studies of vitamin D supplementation in acute respiratory tract infections took blood samples to investigate the effects of vitamin D on the cellular components and inflammatory mediators present in peripheral whole blood stimulated with TLR ligands or pathogens *ex vivo*. As such, the results of this study cannot be compared with others in the literature. While *in vitro* work has demonstrated an effect of $1,25(OH)_2D$ on B-cell proliferation, this was only shown in cells activated by molecules such as IL-21 and CD40 [255, 608]. However, the population in this study which demonstrated an effect of high-dose vitamin D supplementation in

decreasing the number of B-cells in peripheral whole blood was made up of older adults living in sheltered accommodation. It has been demonstrated that aging is associated with chronic inflammation and impaired immunity [615], with an increased serum leptin concentration one potential component of this [616, 617]. Leptin is an adipokine which can induce secretion of pro-inflammatory cytokines such as TNF-α and IL-6 [618], and has also been shown to induce activation of B-cells [619, 620]. As such, the B-cells of the aged ViDiFlu residents population in this study may have been chronically activated, and thus the decrease in $CD3^{-}CD19^{+}$ B-cells demonstrated here may corroborate the other studies on the effects of vitamin D on B-cells. Additional staining would have been needed to be carried out to determine the activation status of the B-cells present, and also if any sub-populations were specifically effected. Therefore, while a decrease in B-cell numbers may not be beneficial in protecting against acute respiratory tract infections, since B-cells are a vital part in adaptive immunity, it may be advantageous in reducing age-related chronic inflammation.

While a vitamin D-induced reduction in the proportion and absolute numbers of B-cells was observed in older adults living in sheltered accommodation, it was not shown in data pooled from asthma and COPD patients. Asthma and COPD are both diseases of chronic inflammation, and thus it might be expected that, if this effect of vitamin D was dependent on the activation state of the B-cells, that these patients would also have chronically activated B-cells and thus exhibit a reduction in numbers following vitamin D supplementation. Additionally, both asthma and COPD have been reported to be associated with increased concentrations of B-cell activating factor (BAFF) [621, 622]. Thus, this may contradict the hypothesis that the vitamin D reduction of B-cells was dependent on their activation state, suggesting that other factors present in the elderly but not in asthma and COPD patients may be behind this effect. As evaluated in Chapter 8 in a cross-sectional analysis of the inflammatory mediators in serum and in blood following antigenic stimulation, the inflammatory phenotype of COPD, asthma and older adults is markedly different. As such, while asthma and COPD patients do exhibit chronic inflammation, there are many differences compared to the elderly, which may be behind the discrepancies observed in this study. Finally, even though the p and q-values for this analysis were significant, the fact that only one outcome out of the vast number of analyses performed showed any effect of vitamin D supplementation means that type I error cannot be ruled out.

Somewhat surprisingly, vitamin D supplementation was not shown to have any other effects on the cellular profile of whole blood, or on the release of inflammatory mediators following stimulation with TLR ligands and pathogens. As reviewed in chapter 1, *in vitro* work has shown that vitamin D

has an extensive effect on the immune system, primarily by inhibition of DC differentiation and the antigen-presenting functions of APCs, increased IL-10 secretion, and skewing of the Th1/Th2 balance. However, the results from these immunological assays do largely corroborate the results from the primary outcomes of the clinical trials. Except for a modest improvement in quality of life in asthma patients (determined by St George's Respiratory Questionnaire (SGRQ) scores), no effects of vitamin D supplementation were observed on time to first exacerbation, or time to first URI in the ViDiAs and ViDiCO trials. In ViDiCO, when stratified by baseline vitamin D deficiency (defined as serum 25(OH)D concentration < 50nmol/L), vitamin D supplementation significantly reduced the risk of moderate or severe exacerbation (aHR 0.57, 95% CI 0.35 to 0.92, p = 0.021). An attempt was made to do similar stratification for the results of the immunological assays from pooled data from asthma and COPD patients and ViDiFlu staff members, with an increase in LPS-stimulated release of RANTES demonstrated, but with a q-value of greater than 0.1 a false positive could not be ruled out. Likewise, in the primary outcomes, the effect of vitamin D supplementation on time to URI was modified by genotype, but we were again underpowered to carry out similar stratification for the immunological assays presented in this chapter.

In the ViDiFlu trial, high-dose vitamin D supplementation was associated with an increased risk of URI (aHR 1.48, 95% CI 1.02 to 2.16, p = 0.04). While the majority of the results presented here demonstrated no effect following vitamin D supplementation, the reduction in B-cells could play a role in this increased risk of acute respiratory tract infection. Likewise, false positives could not be ruled out with q-values of greater than 0.1, although a trend towards a decreased pro-inflammatory response following polyI:C stimulation was observed when stratifying by baseline vitamin D deficiency, also potentially suggesting a mechanism by which administration of bolus vitamin D may increase the risk of infection compared to daily dosing.

The main limitation of this study was in being underpowered, especially when stratifying by baseline vitamin D deficiency or by genotype. Power calculations for the trials were calculated based on the primary outcomes of time to first exacerbation/URI, and with the delays in implementation of immunological assays and budgetary constraints on the magnitude of the analyses carried out, sufficient numbers may not have been gained to reach significance thresholds. To attempt to increase power, data from asthmatics, COPD patients and ViDiFlu staff members who all underwent similar vitamin D dosing regimens were pooled. This raises another possible limitation, since the inflammatory response for these three distinct populations was likely to differ markedly. To compensate for this, "study" was eliminated as a confounder during analysis, thus removing any

influence of having asthma or COPD on the outcome of the analysis. Power was also reduced for analytes such as IL-6, IP-10 and RANTES which had concentrations frequently exceeding the upper limit of detection, with KNN-imputation used during analysis to attempt to compensate for this. If samples had been diluted to allow such analytes to fall within the detectable range, many more analytes would have been at concentrations below the sensitivity threshold for the assay. Additionally, it would not have been feasible to run singleplex ELISAs with samples diluted accordingly for each individual assay for the number of analytes that we wished to quantify and the number of samples to be tested. While samples could be diluted and re-analysed in the future for the analytes which were consistently highly expressed, currently this is not possible due to budgetary constraints.

The use of peripheral blood is another limitation of this study. Asthma and COPD are both respiratory diseases, the pathogens used were all respiratory pathogens, and the main outcomes of the trial were associated with respiratory health. Thus investigations into the immune response mounted and the effects of vitamin D would have been more valuable at the site of infection. However, blood provides a readily available model for assessing the release of inflammatory mediators, and is much better tolerated than the use of techniques such as experimental rhinovirus challenge, sputum induction, and bronchoscopy, thus enabling greater numbers to be recruited.

Therefore, here it has been demonstrated that, while supplementation was successful in increasing the vitamin D status of trial participants, the only immunological effect observed was a decrease in peripheral blood B-cells in older adults without asthma or COPD. There may be additional effects of vitamin D supplementation in those with profound vitamin D deficiency at baseline or in people with certain genotypes, but we were not adequately powered to accurately assess these hypotheses in this study.

7. Comparison of cellular profiles and supernatant inflammatory mediators in induced sputum of patients with asthma or COPD

7.1 Introduction

In addition to the primary analyses evaluating the effect of vitamin D supplementation on the cellular profile and inflammatory mediators present in induced sputum, and the inflammatory response to antigenic stimulation of peripheral whole blood as presented in Chapters 5 and 6 respectively, clinical samples were further utilised to perform cross-sectional comparisons of the underlying phenotypes associated with asthma and COPD.

As previously described, induced sputum provides a useful tool for profiling the cellular components and inflammatory mediators present in the lower airways. Asthma and COPD are both diseases of chronic airway inflammation, and thus assessment of induced sputum is a useful tool to determine the pathophysiology of these diseases and associated risk of exacerbation. Both diseases have similar characteristics, such as mucus hypersecretion and airway remodelling and hyper-responsiveness, dyspnoea, cough, wheezing and chest tightness, as well as the intermittent occurrence of exacerbations. However, the underlying inflammatory profile of the two diseases is markedly different [140, 623, 624], and these differences may affect susceptibility to respiratory tract infections and response to treatments. It is also becoming increasingly clear that asthma is a heterogeneous disease, comprised of subgroups with different underlying inflammatory profiles [180, 181, 625-627] and the typical airway eosinophilia and TH2 phenotype not observed in all patients. [628]. Likewise, COPD is also emerging as a heterogeneous disease, with eosinophilic inflammation reported in sub-groups rather than the typical neutrophilic inflammation [598, 629]. A better understanding of the underlying inflammatory profile of both disease types has the potential to provide insight into pathogenesis and improve the management of these conditions.

Therefore, a cross-sectional analysis of the differences in lower airway inflammation between asthma and COPD was conducted by analysis of induced sputum samples in patients at enrolment to the ViDiAs and ViDiCO trials by differential cell count, flow cytometry and multiplex ELISA of induced sputum supernatants.

As with the prospective analyses presented in the two previous chapters, a 30-plex panel was chosen for analysis of induced sputum supernatants to include a range of inflammatory mediators to encompass any potential differences between asthma and COPD. The following analytes were thus included in the analysis: IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8 (CXCL8), IL-10, IL-12, IL-13, IL-15, IL-17, IL-1RA, IL-2R, IFN-α, IFN-γ, TNF-α, MCP-1 (CCL2), MIP-1α (CCL3), MIP-1β (CCL4), RANTES (CCL5), eotaxin (CCL11), MIG (CXCL9), IP-10 (CXCL10), EGF (epidermal growth factor), FGF-basic (basic fibroblast growth factor), HGF (hepatocyte growth factor), VEGF (vascular endothelial growth factor), G-CSF (granulocyte colony-stimulating factor) and GM-CSF (granulocyte macrophage colony-stimulating factor).

7.2 Results

7.2.1 Study recruitment and baseline characteristics of trial participants

As described in Chapter 2.1, a subset of participants recruited onto the ViDiAs and ViDiCO clinical trials were invited to give induced sputum samples. These samples were collected at randomisation (month 0), and at 2 and 12 month follow-up visits, to enable characterisation of baseline characteristics, and determination of any effect caused by supplementation with vitamin D_3. Figure 7.1 shows the recruitment profile for these two clinical trials and the number of participants who were able to provide baseline samples. In total, 88% of participants in both studies produced an induced sputum sample at baseline. Of those who did not produce a sample, 4 asthma and 1 COPD participant were unable to expectorate, 2 asthma participants had baseline lung function tests which rendered it unsafe to proceed with nebulisation, and 5 COPD participants withdrew consent. In participants with asthma, 20% produced samples with a high degree of squamous cell contamination, thus obscuring other cell types and rendering differential cell counts impossible, compared to 0% in participants with COPD. Flow cytometry was implemented 1 year after recruitment for the trials began, resulting in 10 asthma and 6 COPD participants having already provided baseline samples before this period. The procedure was only carried out in samples with more than 1 million cells remaining after sputum processing and cytospin preparation, ensuring enough cells for the staining to be successful and accurate results to be yielded. Additionally, a subset of COPD participants had their study visits at an alternative site. Due to the importance of sputum being processed within 2 hours to ensure optimum staining, as reported elsewhere [587,

630], transportation times did not allow for the additional steps involved in preparing the samples for flow cytometric analysis. Thus 34% of ViDiAs participants and 52% of ViDiCO participants who provided a sputum sample sufficient for a differential cell count to be carried out also had a sample which was sufficient to undergo additional analysis by flow cytometry.

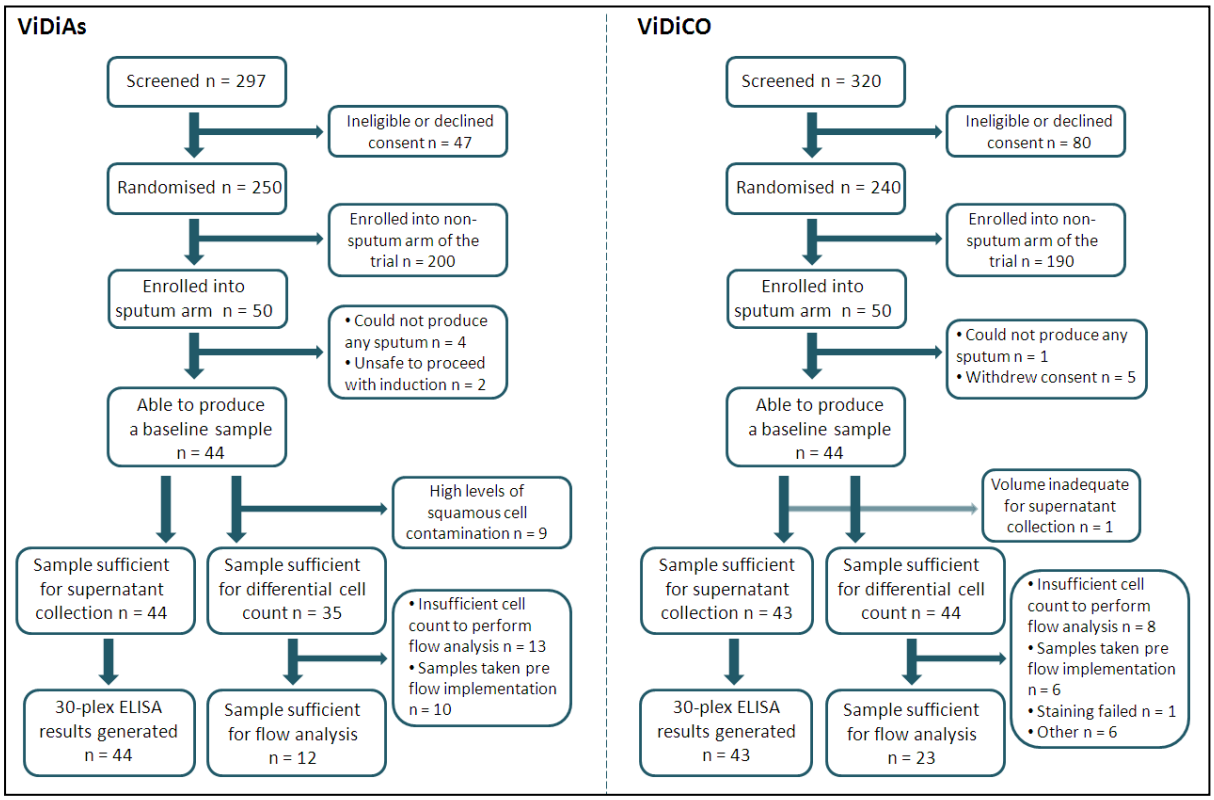


Figure 7.1: Recruitment profile for sputum sub-study at baseline.

The baseline characteristics of participants who were able to produce a sample sufficient for differential cell count are presented in Table 7.1. There was no significant difference between the sex, ethnic distribution, BMI, uptake of influenza vaccine, baseline 25(OH)D status and inhaled corticosteroid use in subjects with asthma and COPD. However, a difference in age ($p < 0.0001$), pack years ($p < 0.0001$) and pneumococcal vaccine uptake ($p = 0.006$) was observed. All can be attributed to the nature of the two disease states, with COPD usually caused by smoking, and the likelihood of it developing increasing with age and smoke exposure. While influenza vaccination is routinely given both to the elderly and those with chronic respiratory conditions such as asthma and COPD, pneumococcal vaccination is only routinely given to older adults. Thus the difference in the two groups observed here is likely due to the higher median age in the COPD study. These data also reflect the eligibility criteria for the studies, with participants excluded from ViDiAs if older than 80

or with a smoking history of greater than 15 pack years, while ViDiCO participants were ineligible if they were younger than 40 or had a smoking history of less than 15 pack years.

Table 7.1: Baseline characteristics of participants with asthma and COPD

	Asthma (n = 35)	**COPD (n = 44)**	**P-Value**
Median Age (IQR)	54 (38 to 63)	66 (62 to 71)	< 0.0001
Sex			0.27
Male, n (%)	18 (51)	28 (64)	
Female, n (%)	17 (49)	16 (36)	
Ethnic Group			0.26
White, n (%)	31 (88)	42 (95)	
Black, n (%)	2 (6)	2 (5)	
Other, n (%)	2 (6)	0 (0)	
Median Pack Years (IQR)[1]	0.0 (0.0 to 5.0)	41.3 (30.3 to 60.0)	< 0.0001
Median BMI (IQR)	26.6 (24.3 to 30.1)	28.4 (25.7 to 33.0)	0.22
Pneumococcal Vaccine			0.006
Received, n (%)	13 (37)	30 (68)	
Did not receive, n (%)	22 (63)	14 (32)	
Influenza Vaccine			0.1
Received, n (%)	31 (89)	43 (98)	
Did not receive, n (%)	4 (11)	1 (2)	
Median 25(OH)D status in nmol/L (IQR)	45 (35 to 69)	39 (30 to 55)	0.2
Median inhaled corticosteroid (ICS) use (IQR) [2] (µg)	400 (200 to 1000)	400 (0 to 1000)	0.2

[1] *Pack years calculated by number of cigarettes smoked per day divided by 20 and multiplied by the number of years smoked for*

[2] *ICS dose given as betamethasone equivalents: 1 microgram betamethasone assumed equivalent to 1 µg budesonide, 0.5 µg fluticasone dipropionate and 0.75 µg ciclesonide.*

Contingency tables were used to calculate p-values for sex, ethnicity, pneumococcal vaccine uptake and influenza vaccine uptake; unpaired T-tests were used to calculate p-values for age, BMI and 25(OH)D status (due to these data being normally distributed); Mann-Whitney tests were used to calculate p-values for pack years and ICS use (due to these data being non-normally distributed).

Abbreviations used: IQR: interquartile range; BMI: body mass index (individuals body mass divided by the square of their height); ICS: inhaled corticosteroid; COPD: chronic obstructive pulmonary disease; 25(OH)D: 25-hydroxy vitamin D.

7.2.2 Analysis of induced sputum by differential cell counts shows a difference in the percentage of macrophages and neutrophils between asthma and COPD

Induced sputum samples were collected and processed as described in Chapter 2.3. Following cytospin preparation, differential cell counts were carried out. Averages were taken from counts of 400 non-squamous cells from 3 cytospins for each participant. Results were calculated as the percentage of each cell type counted and also as absolute cell counts. Qlucore Omics Explorer 2.3 was used to analyse the data, modelling using a two-group analysis (i.e. Student's T-Test) on study (i.e. whether participants had asthma or COPD) with log-transformed data, and including adjustment for age, sex, baseline 25(OH)D concentration, ethnicity, smoking status, vaccination record and inhaled corticosteroid use. All results are presented in Table 7.2, and those with a significant difference between participants with asthma and COPD are presented in Figure 7.2. There was a higher percentage of macrophages in people with asthma compared to COPD (p = 0.04), while people with COPD had a greater number of neutrophils, as assessed both by percentage and absolute cell count (p = 0.03 and 0.04 respectively). No significant difference was found in eosinophil, lymphocyte or epithelial cell counts.

Table 7.2: Comparison of sputum differential cell counts in people with asthma or COPD

Cell Type	Asthma		COPD		Statistics		
	n	median (IQR)	n	median (IQR)	T-Statistic	P-Value	Q-Value
% Neutrophils	35	54.080 (41 to 70)	44	73.045 (66 to 81)	2.213	0.030	0.054
Absolute Neutrophils	35	632316.000 (178332 to 1950000)	44	2230420.375 (903582 to 3858000)	2.084	0.041	0.066
% Macrophages	35	38.920 (22 to 51)	44	19.125 (15 to 27)	-2.059	0.043	0.096
% Lymphocytes	35	0.670	44	0.580	-1.552	0.125	0.259
% Epithelial Cells	35	1.000	44	0.915	-1.019	0.312	0.526
% Eosinophils	35	1.500	44	1.540	-0.975	0.333	0.544
Absolute Macrophages	35	513251.646	44	564606.250	0.714	0.477	0.675
Absolute Epithelial Cells	35	19192.800	44	17880.788	-0.611	0.543	0.733
Absolute Lymphocytes	35	8679.684	44	15375.046	0.316	0.753	0.830
Absolute Eosinophils	35	22610.250	44	32259.128	0.282	0.779	0.841

The interquartile range (IQR) is only stated where statistical significant difference was evident

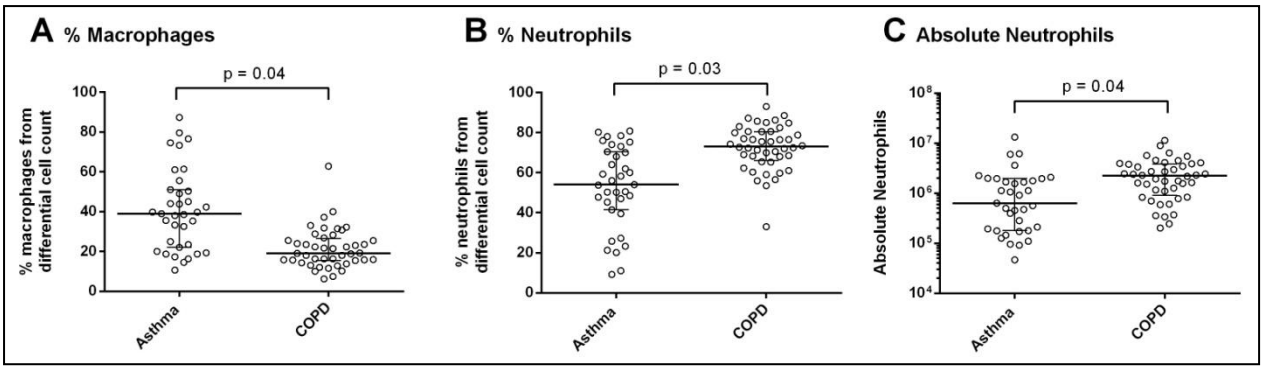


Figure 7.2: Cross-sectional comparison of differential cell counts in people with asthma or COPD. Induced sputum samples were processed and differential cell counts carried out on n = 35 for asthma and n = 44 for COPD. Each count was done in triplicate with an average value calculated. Data are represented as scatter plots with median + IQR, with p-values calculated using a Two-Group Comparison (i.e. an unpaired Student's T-Test) with the model including adjustment for age, sex, baseline 25(OH)D status, ethnicity, smoking status, vaccination record and inhaled corticosteroid use, using the program Qlucore Omics Explorer 2.3. P-values were deemed significant when less than 0.05, with a corresponding q-value of less than 0.1.

Since differential cell counting is subject to human error due to the subjective nature of identifying different cell types, validation of cell count data was carried out. A subset of 10 asthma and 10 COPD cytospins were chosen at random, and cells were counted by another assessor (Will Monteiro, Leicester Respiratory Biomedical Research Unit, Glenfield Hospital, UK) following the same protocol, with results presented in Figure 7.3. The correlation between the original counts carried out and the counts calculated during validation was strong for neutrophils, macrophages, eosinophils and epithelial cells ($r > 0.9$ and $p<0.0001$ for all 4). However, there was no correlation in the percentage of lymphocytes ($r = 0.1$, $p = 0.7$)

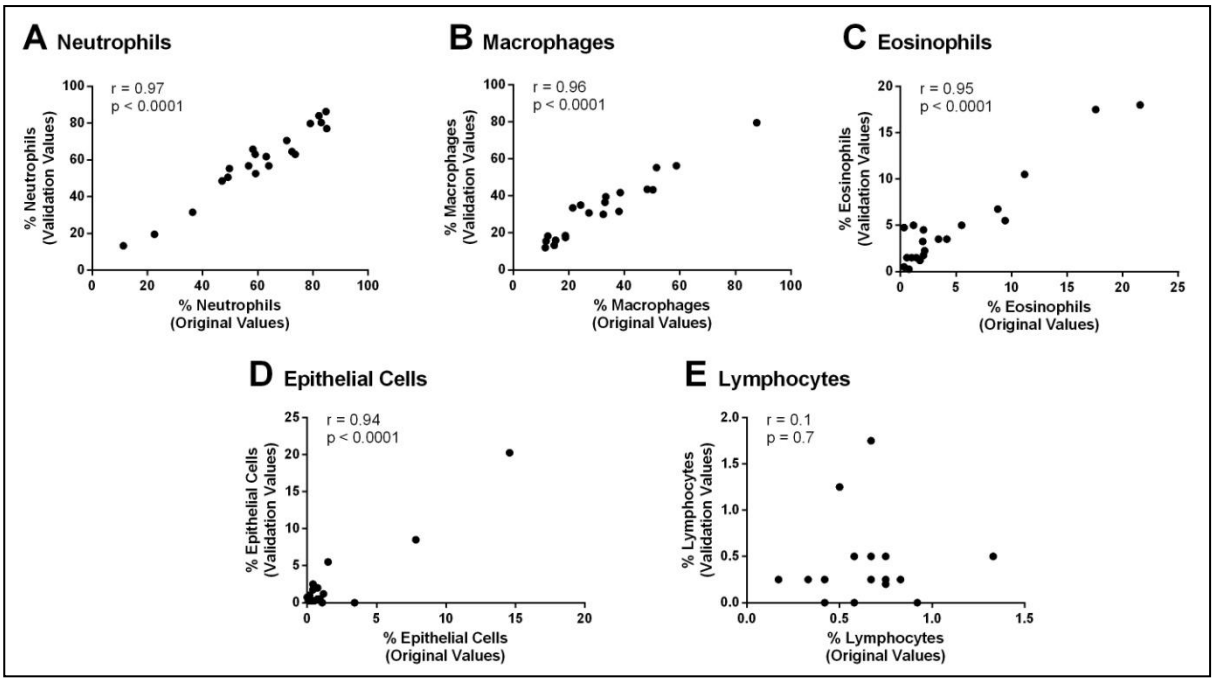


Figure 7.3: Validation of differential cell counts. A subset of n = 20 had differential cell counts carried out by two different people. Counts were compared using Pearson's R Correlation Test.

A Bland-Altman plot can also be used to assess the agreement between two methods, with a high correlation, as seen above, not automatically implying good agreement between the two. As such, Bland-Altman analysis was carried out on the two sets of cell counts, with plots displaying the mean of each pair of values, and the difference between each pair of values (Figure 7.4). The bias value calculated represents the mean difference between the two data sets, with a smaller bias indicating similar results, and thus validating the data. The bias values for macrophages (0.96), eosinophils (0.05), epithelial cells (0.54) and lymphocytes (-0.21) were all between -1 and 1, with neutrophils marginally outside this range (-1.34), thus showing a good agreement between the two sets of cell counts. Additionally, the 95% limits of agreement (calculated as the mean bias ± 1.96 * SD, and represented as dotted lines on the plots below) were narrow for each cell type, also demonstrating a good agreement between the two sets of cell counts. Therefore, using a sub-set of n=20, we have successfully validated the differential cell counts carried out on sputum sample slides.

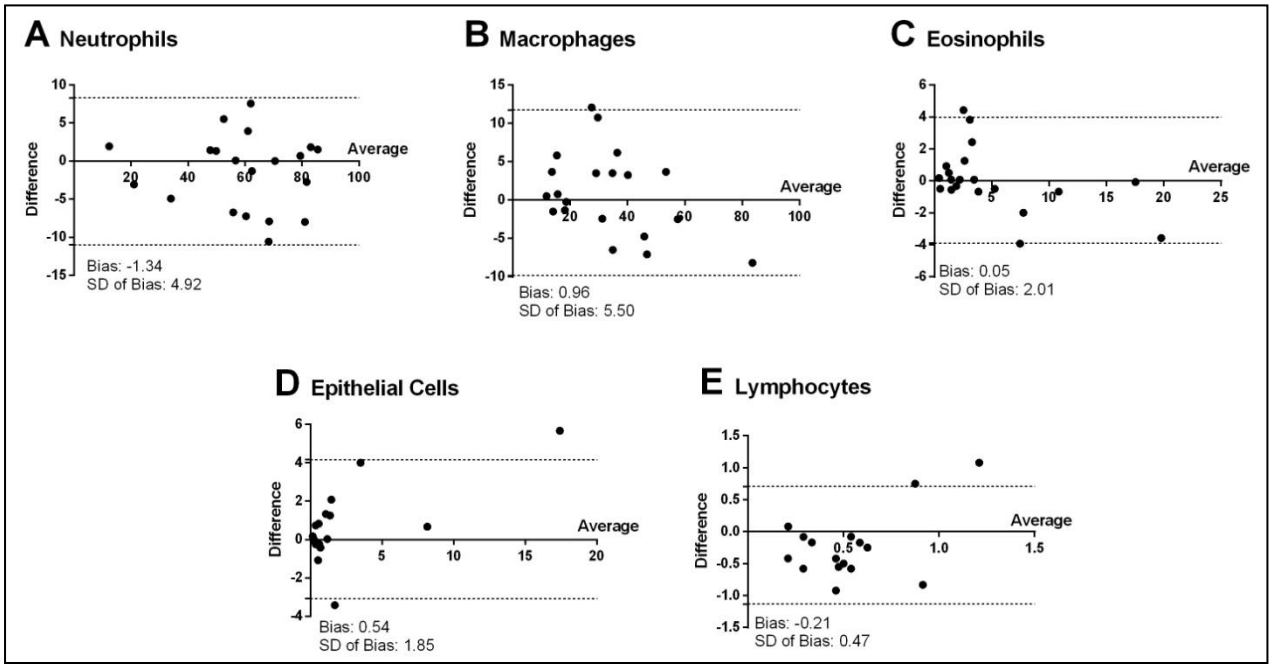


Figure 7.4: Validation of differential cell counts. A subset of n = 20 had differential cell counts carried out by two different people. Counts were compared using Bland-Altman analysis, with dotted lines representing the upper and lower 95% limits of agreement.

7.2.3 Analysis of induced sputum by flow cytometry shows no differences in absolute numbers or proportions of any cell type between asthma and COPD

Induced sputum samples were collected and processed as described in Chapter 2.3. In a subset of samples (n = 12 for asthma and n = 23 for COPD) additional analysis was carried out by flow cytometry. This was to enable further determination of the cellular profiles of asthma and COPD, in addition to the differential cell counts. The gating strategies described in Chapter 2 were used to establish the proportion of B-cells, $CD4^+$ T-cells, $CD8^+$ T-cells, myeloid dendritic cells (mDCs), plasmacytoid dendritic cells (pDCs) and regulatory T-cells (Tregs) present in the sputum samples. Since all of them were based on a lymphocyte population, lymphocyte counts from the differential cell counts were used to calculate absolute cell counts for each cell type. A comparison between the baseline cellular profile of asthma and COPD patients was then carried out (Table 7.3). Qlucore Omics Explorer 2.3 was used to analyse the data, modelling using a two-group analysis (i.e. Student's T-Test) on study (i.e. whether participants had asthma or COPD) with log-transformed data, and including adjustment for age, sex, baseline 25(OH)D concentration, ethnicity, smoking status,

vaccination record and inhaled corticosteroid use. No statistically significant difference was seen between asthma and COPD patients for any of the cell types looked at.

Table 7.3: Cross-sectional comparison of cellular profiles of asthma and COPD as determined by flow cytometry

Cell Type	Asthma		COPD		Statistics		
	n	median	n	median	T-Statistic	P-Value	Q-Value
% mDC	12	50.900	23	45.600	1.431	0.157	0.309
% CD4 T-cells	12	69.200	23	66.600	1.128	0.263	0.458
Absolute Tregs	12	248.448	23	417.638	0.748	0.457	0.666
% B-cells	12	3.095	23	3.480	0.562	0.576	0.757
Absolute CD4 T-cells	12	1702.186	23	3160.333	0.502	0.617	0.757
Absolute pDC	12	41.593	23	42.137	-0.447	0.656	0.787
%pDC	12	6.685	23	7.590	-0.417	0.678	0.795
% CD8 T-cells	12	15.600	23	19.400	-0.335	0.739	0.830
% Tregs	12	12.050	23	11.900	0.331	0.741	0.830
Absolute B-cells	12	546.017	23	531.222	-0.238	0.812	0.860
Absolute mDC	12	330.031	23	311.510	-0.133	0.895	0.911
Absolute CD8 T-cells	12	697.745	23	719.114	0.038	0.970	0.970

7.2.4 Validation of methodology used for flow cytometric analysis of induced sputum

The addition of dithiothreitol (DTT) is vital during the sputum processing procedure, since it facilitates the dissociation of disulphide bonds present in mucin molecules, thus allowing the complete homogenisation of the sample. This results in cells being released from the mucus present in the sample, and enables a clearer distinction between different cell types during cell counts, therefore facilitating more accurate results [630-633]. However, other studies have demonstrated detrimental effect of using DTT, with a reduction in viable cells [588], altered cell function [634], and diminished fluorescence intensity for some leukocyte surface markers [635]. It was also observed in the results generated from carrying out flow cytometry on sputum samples that CD127 APC staining was visibly diminished. Therefore, the effect of DTT on staining and the subsequent characterisation of cell populations was determined. Peripheral whole blood was used for this analysis, since sputum samples would be hard to process and stain without the use of DTT for homogenisation, and blood provided higher cell counts to work with. Blood samples were incubated with an equal volume of 0.1% DTT for 15 minutes, as per the sputum processing protocol, before undergoing staining for flow cytometry, as described in Chapter 2.4. The results from this analysis are displayed in Figure 7.5.

Populations of lymphocyte subsets, dendritic cells and regulatory T-cells were comparable between normal samples and those treated with DTT. As with the sputum samples, diminished staining with APC CD127 was observed following DTT treatment, with the $CD127^{+}CD4^{+}$ population reduced 3-fold from 45% to 15% following incubation with DTT (Figure 7.5 C). However, this effect was shown to be CD127 specific and did not affect other antigens conjugated with the APC fluorophore, with, for example, comparable percentages of $CD4^{+}$ T-cells (42% and 46%, Figure 7.5 A), and pDCs (28% and 25%, Figure 7.5 B) which also used the APC fluorophore. Since only $CD127^{-}$ cells were of importance for this analysis, this effect on APC CD127 staining was deemed to be unimportant in this context, and analysis of sputum samples was continued with the use of DTT.

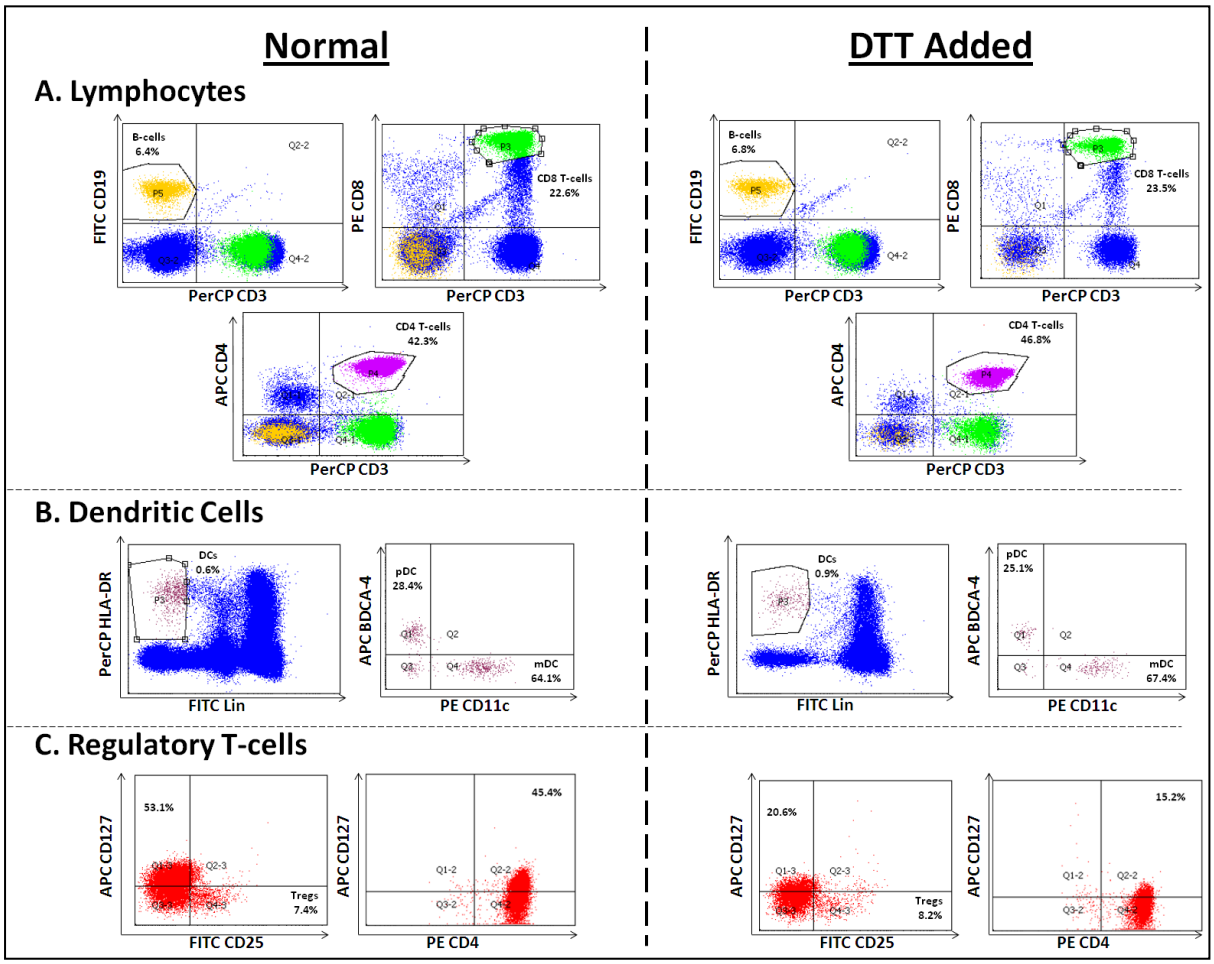


Figure 7.5: Effect of DTT on staining for flow cytometry in peripheral whole blood. Lymphocyte subsets (A), dendritic cells (B) and regulatory T-cells (C) were stained following the normal protocol (left panel) and following incubation with DTT (right panel) to compare fluorescence intensities and the cell percentages observed between the two protocols.

The discovery of the transcription factor FoxP3 (forkhead box P3) has enabled the accurate detection of regulatory T-cells by flow cytometry, with $CD4^+CD25^+FoxP3^+$ cells representing this population. However, due to the time constraints and low cell numbers encountered when performing flow cytometry on sputum samples, the intracellular staining protocol involved in FoxP3 detection becomes unfeasible. As such, CD127 was used instead, due to its strong inverse correlation with FoxP3 expression [636, 637]. Since the use of FoxP3 is the gold standard for Treg detection, Foxp3 staining was carried out in a subset of n = 11 blood and sputum samples to compare the proportion of Tregs counted by each method. Regulatory T-cells were distinguished as being $CD4^+CD25^+CD127^-$ or as being $CD4^+CD25^+FoxP3^+$ as described in Chapter 2.4, and isotype controls were used to help determine what constituted the $CD25^+$ population. A strong correlation was seen between the amount of Tregs calculated from the two methods (r=0.99, p<0.0001, Figure 7.6).

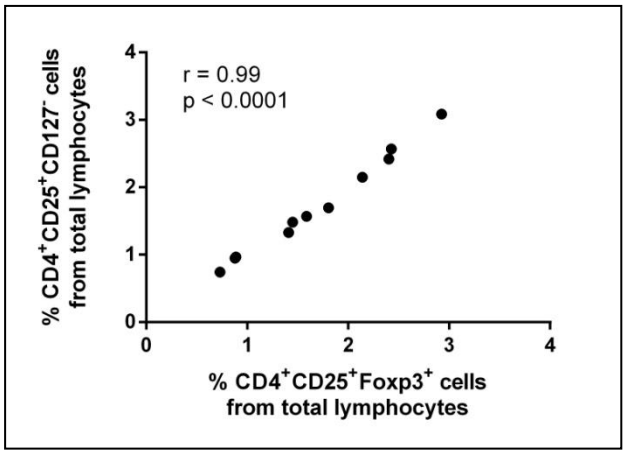


Figure 7.6: Validation of the use of CD127 in place of Foxp3 when defining regulatory T-cell populations. A subset of n = 11 blood and sputum samples underwent staining for $CD4^+CD25^+CD127^-$ and $CD4^+CD25^+FoxP3^+$ cells to compare the number of Tregs calculated using each method. Counts were compared using Pearson's R Correlation Test.

Again, a Bland-Altman analysis was also carried out in order to further assess the agreement between the two methods used for calculating the Treg population. A small bias value was calculated (0.03) with narrow 95% limits of agreement (Figure 7.7), thus demonstrating a strong agreement between the two methods used, and corroborating the use of CD127 instead of FoxP3 in this study.

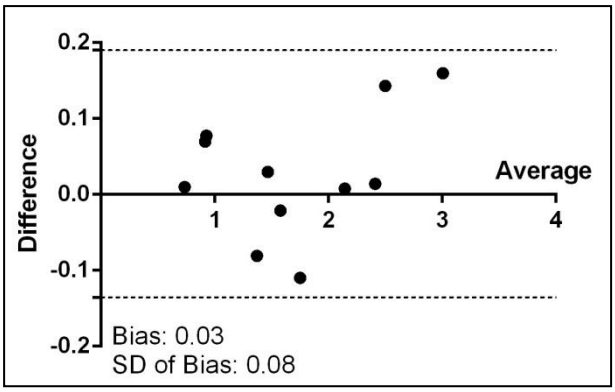


Figure 7.7: Validation of the use of CD127 in place of Foxp3 when defining regulatory T-cell populations. A subset of n = 11 blood and sputum samples underwent staining for $CD4^+CD25^+CD127^-$ and $CD4^+CD25^+FoxP3^+$ cells to compare the number of Tregs calculated using each method. Counts were compared using Bland-Altman analysis, with dotted lines representing the upper and lower 95% limits of agreement.

7.2.5 Analysis of induced sputum supernatant inflammatory mediators shows differences between asthma and COPD patients

Cytokines and chemokines play an important role in orchestrating the recruitment, activation and regulation of inflammatory cells in the respiratory tract of people with asthma and COPD. While asthma and COPD are both characterised by airway obstruction and have many similar clinical features, there is a marked difference in their underlying inflammatory phenotype [140, 623, 624]. However, very few studies have investigated the protein constituents of sputum from asthma and COPD patients during their steady state. Those that have, have focused mainly on proteins such as eosinophilic cationic protein and IL-8, which are associated with the eosinophils and neutrophils which are major components of airway inflammation in these patient groups, have assessed levels during exacerbation, or have focused solely on one of the disease types [455, 625, 638-640]. As such, it was desirable to use a multiplex approach to determine the profiles of a wide range of cytokines and chemokines to compare between these two disease states.

Induced sputum samples were collected and processed as described in Chapter 2.3. Supernatants were aspirated and stored at -80^{o}C, before being analysed by multiplex ELISA. Qlucore Omics Explorer 2.3 was used to analyse the data, modelling using a two-group analysis (i.e. Student's T-Test) on study with log-transformed data, and including adjustment for age, sex, baseline 25(OH)D concentration, ethnicity, smoking status, vaccination record and inhaled corticosteroid use. All results are presented in Table 7.4, and significant results are presented in Figure 7.8. Concentrations of IL-2 ($p = 0.0006$), IL-4 ($p = 0.002$), IL-13 ($p = 0.04$) and GM-CSF ($p = 0.04$) were all significantly

higher in asthma samples compared to COPD (Figure 7.8 A – D). Concentrations of IFN-α ($p<0.0001$), MIP-1α ($p = 0.0003$), IL-8 ($p = 0.0003$), IL-6 ($p = 0.0004$), G-CSF ($p = 0.0006$), IL-1RA ($p = 0.001$), eotaxin ($p = 0.005$), MCP-1 ($p = 0.02$), HGF ($p = 0.02$) and IL-1β ($p = 0.04$) were all significantly higher in COPD compared to asthma (Figure 7.8 E – N). The remaining inflammatory mediators assayed (FGF Basic, IL-10, IL-12, RANTES, IL-17, MIP-1β, IL-15, EGF, IL-5, VEGF, IFN-γ, TNF-α, IL-7, IP-10, IL-2R and MIG) displayed no difference between participants with asthma and those with COPD (Table 7.4).

Table 7.4: Comparison of induced sputum supernatant inflammatory mediators in people with asthma or COPD

Analyte	Asthma		COPD		Statistics		
	n	median (IQR)	n	median (IQR)	T-Statistic	P-Value	Q-Value
IFN-α	44	0.00 (0 to 0)	43	0.00 (0 to 17)	4.185	0.00007	0.004
MIP-1 alpha	44	0.00 (0 to 0)	43	11.39 (6 to 20)	3.837	0.0003	0.005
IL-8	44	122.20 (21 to 543)	43	1461.06 (373 to 5615)	3.777	0.0003	0.005
IL-6	44	10.24 (2 to 31)	43	46.55 (19 to 75)	3.702	0.0004	0.005
IL-2	44	0.51 (0 to 1)	43	0.00 (0 to 0)	-3.576	0.0006	0.006
G-CSF	44	20.30 (0 to 43)	43	100.99 (39 to 188)	3.567	0.0006	0.006
IL-1RA	44	95.17 (76 to 162)	43	317.79 (147 to 754)	3.381	0.001	0.009
IL-4	44	7.26 (2 to 9)	43	0.78 (0 to 5)	-3.183	0.002	0.014
Eotaxin	44	0.00 (0 to 0)	43	0.81 (0 to 1.4)	2.920	0.005	0.028
MCP-1	44	13.48 (0 to 45)	43	39.99 (10 to 116)	2.487	0.015	0.081
HGF	44	0.00 (0 to 36)	43	63.88 (15 to 107)	2.432	0.017	0.085
IL-13	44	8.07 (6 to 12)	43	5.24 (2 to 7)	-2.112	0.038	0.086
GM-CSF	44	3.93 (3 to 4)	43	2.46 (2 to 3)	-2.065	0.042	0.096
IL-1β	44	0.00 (0 to 0)	43	0.00 (0 to 0)	2.064	0.042	0.096
IL-2R	44	13.25	43	22.56	1.849	0.068	0.196
MIG	44	0.00	43	17.23	1.810	0.074	0.196
VEGF	44	8.52	43	14.23	1.798	0.076	0.196
IL-7	44	0.00	43	14.55	1.797	0.076	0.196
TNF-α	44	0.00	43	0.00	1.765	0.081	0.196
MIP-1β	44	5.79	43	20.33	1.752	0.084	0.196
IL-5	44	0.00	43	0.68	1.731	0.088	0.196
IL-12	44	0.00	43	7.58	1.713	0.091	0.196
IP-10	44	7.07	43	38.79	1.343	0.183	0.341
IL-10	44	0.93	43	1.28	0.812	0.419	0.653
IL-15	44	5.98	43	6.82	0.805	0.424	0.653
IL-17	44	0.00	43	0.00	0.778	0.439	0.658
IFN-γ	44	0.61	43	0.00	-0.698	0.487	0.675
RANTES	44	0.00	43	0.00	-0.537	0.593	0.757
FGF-Basic	44	0.00	43	0.00	0.510	0.611	0.757
EGF	44	11.05	43	17.74	0.167	0.868	0.901

All medians are given in pg/ml

The interquartile range (IQR) is only stated where statistical significant difference was evident

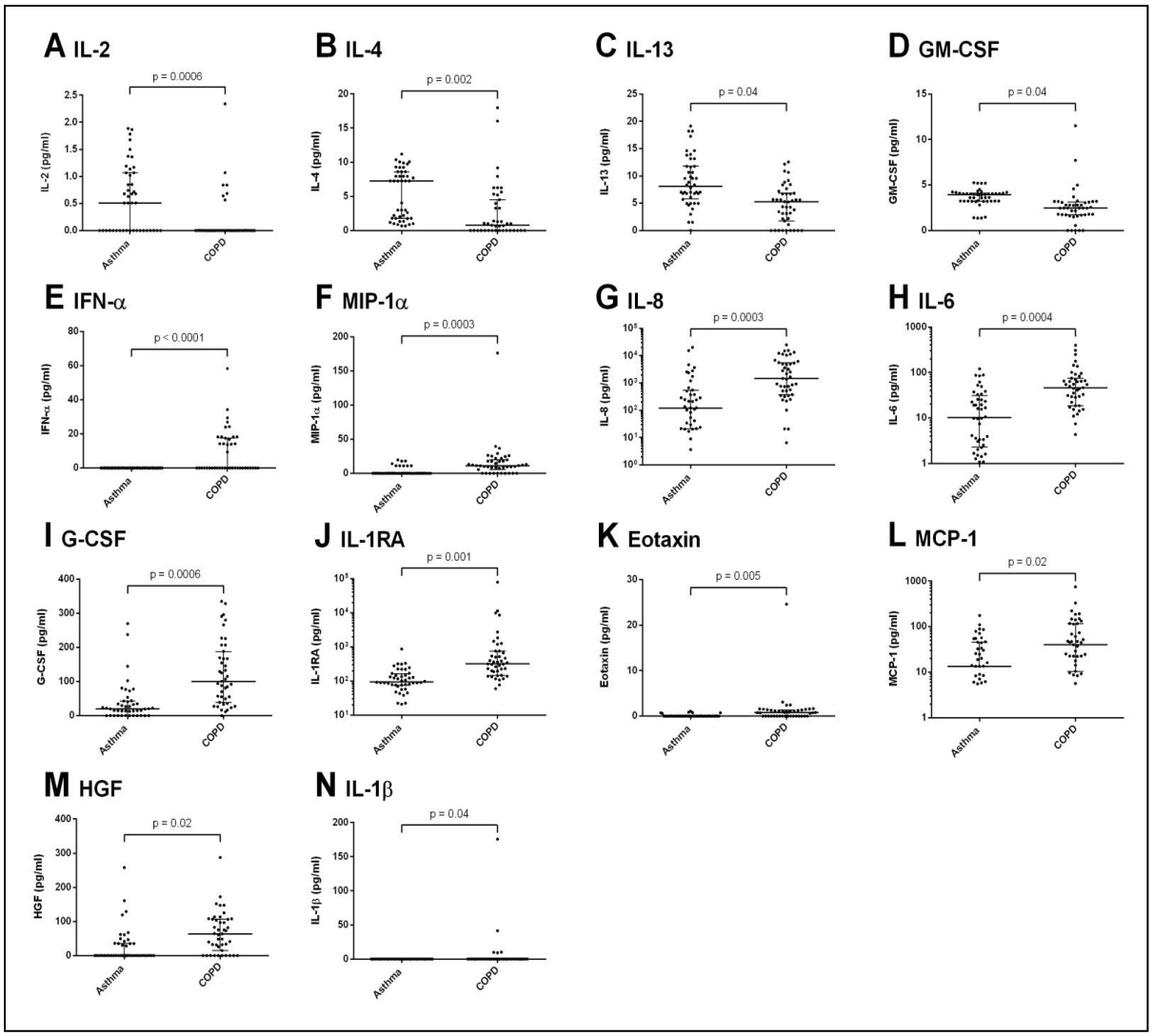


Figure 7.8: Cross-sectional comparison of inflammatory mediators in people with asthma or COPD. Induced sputum samples were collected and supernatants from n = 44 asthma and n = 43 COPD patients were used in a multiplex ELISA. Data are represented as scatter plots with median + IQR, with p-values calculated using a Two-Group Comparison (i.e. unpaired T-Test) with the model including adjustment for age, sex, baseline 25(OH)D status, ethnicity, smoking status, vaccination record and inhaled corticosteroid use, using the program Qlucore Omics Explorer 2.3. P-values were deemed significant when less than 0.05, with a corresponding q-value of less than 0.1. Any values under the limit of detection according to the product data sheet for each analyte were given a value of 0.

7.3 Discussion

It has been demonstrated here that there is a significant difference in the underlying cellular and inflammatory profile of asthma and COPD. Asthmatic subjects had an increased proportion of macrophages, with higher concentrations of IL-2, IL-4, IL-13 and GM-CSF present in their induced sputum samples, while COPD patients demonstrated greater numbers of neutrophils, and increased production of IFN-α, MIP-1α, IL-8, IL-6, G-CSF, IL-1RA, eotaxin, MCP-1, HGF and IL-1β.

Asthma is typically classified as a disease of eosinophilic airway inflammation, with an increased number of $CD4^+$ T-cells found in the airways and an associated Th2 phenotype [140]. In this study, asthma was not found to be associated with an increased proportion of eosinophils or $CD4^+$ T-cells when compared to people with COPD. When defining eosinophilic airway disease as having a sputum eosinophil percentage of >2% (as defined in other studies [625, 641, 642]), in this study at baseline 32 people had eosinophilic airway disease, 13 of whom had asthma and 19 who had COPD. This equates to 37% of asthma participants and 43% of COPD participants. As such, it is clear that not all asthmatics have underlying eosinophilic airway disease, and that COPD patients can also have an eosinophilic phenotype. Other studies have also demonstrated the importance of eosinophils in COPD, with eosinophilic inflammation reported to be present in 20 – 40% of cases [597, 598], and a reduction in sputum eosinophilia associated with a reduction in COPD exacerbations [629]. In addition to this, it has been recognised that only 50% of asthmatics have airway eosinophilia [598, 599]. Thus with the frequency of non-eosinophilic asthma and eosinophilic COPD, it is unsurprising that no difference in eosinophil count was observed between the two study groups.

$CD4^+$ T-cells are classically described as helper T-cells, which function by producing pro-inflammatory Th1 cytokines, or allergy-related Th2 cytokines. While asthma was not associated with an increased population of $CD4^+$ T-cells in this study, there was a Th2 phenotype compared to COPD patients. IL-4 is produced by Th2 T-cells, mast cells, basophils and eosinophils. It drives further Th2 T-cell differentiation and inhibits Th1 production, as well as stimulating B-cell proliferation, driving B-cell production of IgE, stimulating dendritic cell maturation and increasing MHC class II expression [643, 644]. Its over-production is also associated with allergy [645]. IL-4 shares sequence homology, receptors and functional effects with IL-13 [646-648], so it is unsurprising that both cytokines were shown to be increased in asthma when compared to COPD. GM-CSF was also upregulated in asthmatics in this study. This cytokine induces proliferation and differentiation of neutrophils, eosinophils and macrophages, is important in dendritic cell development, and has been associated

with atopy, with a role in allergic sensitisation [649-653]. Therefore, an allergic phenotype has been demonstrated in asthmatic subjects in this study, despite no increase in eosinophils or $CD4^+$ T-cells. The lack of difference in $CD4^+$ T-cells may be due to lack of power, with sufficient sputum samples for flow cytometry hard to obtain in people with mild asthma who could not easily expectorate a sample. Additionally, invariant T cell receptor-positive natural killer T (*i*NKT) cells have been demonstrated to play a role similar to $CD4^+$ T-cells in asthma, including the production of Th2 cytokines, and have been demonstrated to be required for airway inflammation induced by environmental antigens [654, 655]. Therefore, they could represent an important subpopulation to look at in any subsequent studies, and could be behind the increased production of Th2 cytokines.

IL-2 is a growth and activating factor for both Th1 and Th2 cells [656, 657]. It has been demonstrated to be produced by Th2 cells in response to allergen stimulation, and its production has been shown to be increased in BAL of asthmatics [658, 659]. Additionally, use of inhaled IL-2 in the treatment of metastasising renal cell carcinoma has been demonstrated to cause temporary symptomatic, functional and inflammatory alterations similar to those typical of bronchial asthma, thus further demonstrating its importance in asthma [660]. As such, our finding that IL-2 concentration was increased in asthma compared to COPD corroborates previously demonstrated associations.

Finally, the percentage of macrophages was demonstrated to be increased in asthma. Since absolute numbers of macrophages were not increased, this difference is likely due to the nature of differential cell counts and the use of percentages rather than whole numbers, rather than any functional explanation. The majority of cells counted were either macrophages or neutrophils, so in the absence of neutrophilic disease, as seen in the asthma participants compared to COPD, more macrophages were counted to fulfil the requirement of counting 400 non-squamous cells.

COPD is typically classified as a disease of neutrophilic airway inflammation, with an increased number of $CD8^+$ T-cells found in the airway wall [140]. In this study, COPD was found to be strongly associated with the percentage and absolute number of neutrophils found in the sputum. Defining neutrophilia as a sputum neutrophil content greater than 60%, while a subset of 40% of asthmatics did exhibit a neutrophilic phenotype, 86% of COPD patients were classified as having sputum neutrophilia. Profound neutrophilia has been demonstrated numerous times in COPD patients , and is thought to contribute to the pathophysiology of COPD by stimulating EGFR-dependent mucus hypersecretion, releasing elastase which causes goblet cell degranulation, secreting proteases which

mediate tissue damage, and releasing reactive oxygen species which damage the epithelium, reduce ciliary function and increase mucosal permeability [175, 661, 662]. Neutrophil migration into the respiratory tract has been demonstrated to be orchestrated by IL-8 and TNF-α, with IL-8 mobilising the MAC-1 (macrophage-1) antigen to the neutrophil surface to facilitate adhesion to the endothelium and subsequent extravasation into the airways [175, 663, 664]. Neutrophils are also able to produce IL-8 themselves, and thus it is unsurprising that, as in other studies [665, 666], we found a significantly greater concentration of IL-8 in the sputum of COPD patients compared to asthma. While we didn't find a difference in TNF-α expression, this was also observed in other studies [665]. Additionally G-CSF, which is involved in neutrophil proliferation and maturation [667, 668], was also demonstrated to be up-regulated in the sputum of COPD patients in this study.

The pro-inflammatory cytokines IL-1β and IL-6 were shown to up-regulated in people with COPD in this study. IL-1β has been implicated in the development of emphysema and induces leukocytosis and fibroblast proliferation, while IL-6 is a marker of inflammation, and both have been shown elsewhere to be up-regulated in COPD [665, 669-671]. Additionally, the chemokines MCP-1 and MIP-1α which attract macrophages and T-cells to exacerbate to the inflammation of the airways were shown to be increased in the sputum of COPD patients when compared to asthma in this study and elsewhere [671-673]. The chemokine eotaxin, which attracts eosinophils, was also demonstrated to be at an increased concentration in COPD sputum compared to asthma. While COPD has been linked to increased eotaxin production compared to healthy subjects [671, 674], its increase compared to asthma is surprising, but may be associated with the lack of eosinophilia observed in asthmatics in this study, and has also been reported in serum in another study [675].

Less easy to explain in the context of COPD, are the up-regulation of IFN-α, HGF and IL-1RA. IFN-α is typically associated with anti-viral immunity, with functions such as stimulation of NK cells, increased MHC class I expression, and induction of apoptotic cell death of infected cells [43]. HGF is a growth factor, which has not been linked to COPD, but has been demonstrated to prevent neutrophil extravasation in the kidneys of mice to inhibit endothelial injury [676], thus contrasting to the neutrophilic pathophysiology of COPD. Finally, IL-1RA is an anti-inflammatory agent, which blocks the actions of IL-1, again leading to questions surrounding its role in the inflammatory airways of COPD.

Cross sectional studies such as this are important in defining the underlying inflammatory phenotypes associated with diseases such as asthma and COPD. The increasingly clear view that

asthma is a heterogeneous condition comprised of subgroups with different underlying inflammatory profiles makes such studies vital, with different inflammatory phenotypes potentially requiring different treatment strategies [180, 625, 626, 628]. Non-allergic forms of asthma, triggered by stimuli such as air pollution, stress, obesity and viral infection, respond poorly to corticosteroids, which are the most common therapy for asthma [677]. Likewise, COPD patients can have underlying eosinophilic airway inflammation rather than the classical neutrophilia [597, 598], again having implications on how it should be treated, as it has been demonstrated that corticosteroids are only effective against eosinophilic inflammation, and not neutrophilic [179, 180, 597, 678-680].

Other studies have carried out similar comparisons between asthma and COPD in induced sputum samples, although none have used such a large panel of inflammatory mediators and cell populations to allow a definitive comparison between the two disease types. Such studies have also seen no difference in sputum eosinophils in asthma and COPD [681, 682], again demonstrating that eosinophilia is not just a feature of asthma, but is in fact observed in both diseases. No differences were observed between asthma and COPD in one study, looking at IL-4, IL-5, Il-6, Il-8, Il-10, LTB-4, TNF-α, IFN-γ and TGF-β in the induced sputum samples of 37 asthma and 36 COPD patients, although levels of TGF-β, IL-8 and LTB-4 were elevated in COPD patients compared with healthy subjects, and differences were seen when stratifying by smoking status or presence of eosinophilic airway disease [623]. They concluded that there is ambiguity between the two diseases, with overlapping phenotypes making it difficult to distinguish between the two, thus requiring stratification by the underlying phenotype of the inflammation instead.

Where this study is limited in comparison to the other similar studies is in the lack of a healthy control to compare asthma and COPD phenotypes to. More differences may have been observed in asthma and COPD if they had been able to be compared to healthy subjects, with, for example, an increased likelihood of observing eosinophilia in asthmatic patients. The ViDiFlu study could potentially have been used to provide a healthy (i.e. no asthma or COPD) population to study, but due to the nature of the visits involving travelling to sheltered accommodation residences, including inducing sputum to the protocol was not feasible. Additionally, people without airway inflammation are likely to find expectoration difficult, even following nebulisation, thus resulting in samples with a high squamous content which renders differential cell counts less accurate. The majority of this population were also older adults, thus potentially exhibiting an altered cellular and inflammatory phenotype compared to healthy younger adults. Finally, these participants did not undergo

spirometry, and thus undiagnosed asthma or COPD could not be ruled out, and other co-morbidities may also have been present, thus preventing the use of ViDiFlu patients as healthy controls.

The study is also limited due to being underpowered, especially for the flow cytometric analysis. Due to the nature of requiring high cell numbers to be able to perform flow cytometry in sputum as a result of the large amount of mucus and debris present in the samples, only a sub-set of those undergoing sputum induction provided samples adequate for this additional procedure. Therefore, any differences in numbers of $CD4^+$ or $CD8^+$ T-cells, for example, between the asthma and COPD populations may have been missed. Larger numbers would need to be recruited to ensure sufficient power to reach accurate conclusions.

An interesting sub-analysis would have been to investigate differences due to disease severity, or by comparing eosinophilic to non-eosinophilic, and neutrophilic to non-neutrophilic disease. However, the nature of the recruitment protocol meant that only people with mild asthma or COPD were randomised to the sputum arm, due to the potential dangers of nebulisation in people with severe disease. Additionally, more people would have needed to be recruited to have the power to stratify results by inflammatory phenotype for analysis.

Therefore, here it has been demonstrated that induced sputum is a useful tool in assessing airway inflammation, and that there is a significant difference underlying the inflammatory profiles of the airways of people with asthma and COPD. Asthmatics were shown to have a Th2 cytokine profile, while COPD patients had an increased number of neutrophils in their airways and a variety of other pro-inflammatory cytokines and chemokines. However, it should also be taken into account that asthma and COPD are heterogeneous diseases, and that phenotypes between the two may overlap, thus obscuring a clear distinction between them.

8. Comparison of TLR ligand- and pathogen-stimulated whole blood inflammatory responses in patients with asthma vs. COPD vs. controls with neither condition

8.1 Introduction

As previously described, blood is a suspension of cellular components and inflammatory mediators, and as such provides a useful and readily available tool in assessing the immune response to pathogens. Asthma and COPD are both diseases of chronic airway inflammation, with acute respiratory infections more frequent and resulting in more severe disease and longer-lasting symptoms compared to healthy people [158-163]. In asthma, this has been associated with a deficient innate immune response to rhinovirus infection [172], increased infiltration of neutrophils resulting in tissue damage [169], and a Th2-skewed adaptive immune response inhibiting the Th1 response required for viral clearance [170, 683]. In COPD, this is associated with an impaired innate immune response in alveolar macrophages to respiratory pathogens mediated by diminished TLR responses [684], and, conversely, a disproportionate increase in cytokine expression following viral infection both locally and systemically [163, 685]. However, studies looking at whole blood inflammatory responses to a broad range of stimuli have not been carried out, and investigation into the differences in the peripheral blood inflammatory response between asthma and COPD patients is lacking, with previous studies using induced sputum instead [665].

Therefore, a cross-sectional analysis of the differences in whole blood inflammatory responses between asthma, COPD and people with neither condition was conducted, using the whole blood assay and multiplex ELISA, with baseline samples from patients enrolled in the three clinical trials ViDiAs, ViDiCO and ViDiFlu.

As previously discussed, a multiplex ELISA platform was used to quantify the following 30 inflammatory mediators: IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8 (CXCL8), IL-10, IL-12, IL-13, IL-15, IL-17, IL-1RA, IL-2R, IFN-α, IFN-γ, TNF-α, MCP-1 (CCL2), MIP-1α (CCL3), MIP-1β (CCL4), RANTES (CCL5), eotaxin (CCL11), MIG (CXCL9), IP-10 (CXCL10), EGF (epidermal growth factor), FGF-basic (basic fibroblast growth factor), HGF (hepatocyte growth factor), VEGF (vascular endothelial growth factor), G-CSF (granulocyte colony-stimulating factor) and GM-CSF (granulocyte macrophage colony-stimulating factor).

8.2 Results

8.2.1 Study recruitment and baseline characteristics of trial participants

As described in chapter 2.1, participants from all three trials provided blood samples at baseline, 2 month and 12 month follow-up. A subset of these samples were used in a whole blood assay to determine the immune response mounted in peripheral blood to a range of TLR ligands and pathogens. Figures 8.1, 8.2 and 8.3 shows the recruitment profiles for all three trials, and the number of samples included in the whole blood assays at baseline. The implementation of the whole blood assay was not achieved until part-way through the trials, resulting in only a subset of participants providing baseline sodium heparin blood samples to undergo this analysis (n = 119 for ViDiAs; n = 99 for ViDiCO; n = 88 for ViDiFlu). From the offset of the whole blood assay, all samples were stimulated with the panel of TLR ligands (LPS, Pam2CSK4, Pam3CSK4, PolyI:C and R848), as well as providing unstimulated blood and serum samples. At a later date, following procurement of the whole pathogens from collaborators at Imperial College London and optimization of the conditions needed (as described in chapter 3), the pathogens RV-16, RV-1B, RSV, *Haemophilus influenzae* and *Streptococcus pneumoniae* were added as stimuli in the whole blood assay. Due to budgetary limitations, only a subset of samples that had undergone baseline whole blood assays were analysed by multiplex ELISA. The decision on which samples to run was based on having undergone the whole blood assay with the whole range of stimuli, and having provided both baseline and 12 month samples, in order to allow prospective analysis of the effects of vitamin D on the immune response to stimulation and adjustment for baseline values. As such, n = 45 participants samples were analysed for ViDiAs, and n = 22 for ViDiCO. Of these, the whole panel of stimuli, including serum and unstimulated blood, were incorporated into statistical analysis for all ViDiCO samples, while, due to the plate-reader failing to generate results for one column of one plate, 8 variables had 44 rather than 45 samples for ViDiAs. For ViDiFlu, residents and staff members were pooled to provide a control population of participants with neither asthma nor COPD. As with the prospective analysis, all samples with both a baseline and 12 month sample were analysed by multiplex ELISA, regardless of whether the whole panel of stimuli were used. This was due to the fact that the primary results of the ViDiFlu clinical trial indicated an effect of vitamin D supplementation on risk of URTI, whereas no such effect was observed in ViDiAs and ViDiCO. As such, the samples analysed consisted of n= 24 participants who had blood samples stimulated by the whole range of whole blood assay stimuli, n = 14 with samples stimulated by TLR ligands and viral pathogens, and n = 36 with samples stimulated

just by TLR ligands. Each participant also provided a serum sample for analysis (n = 73), with n = 1 having insufficient volume to be included.

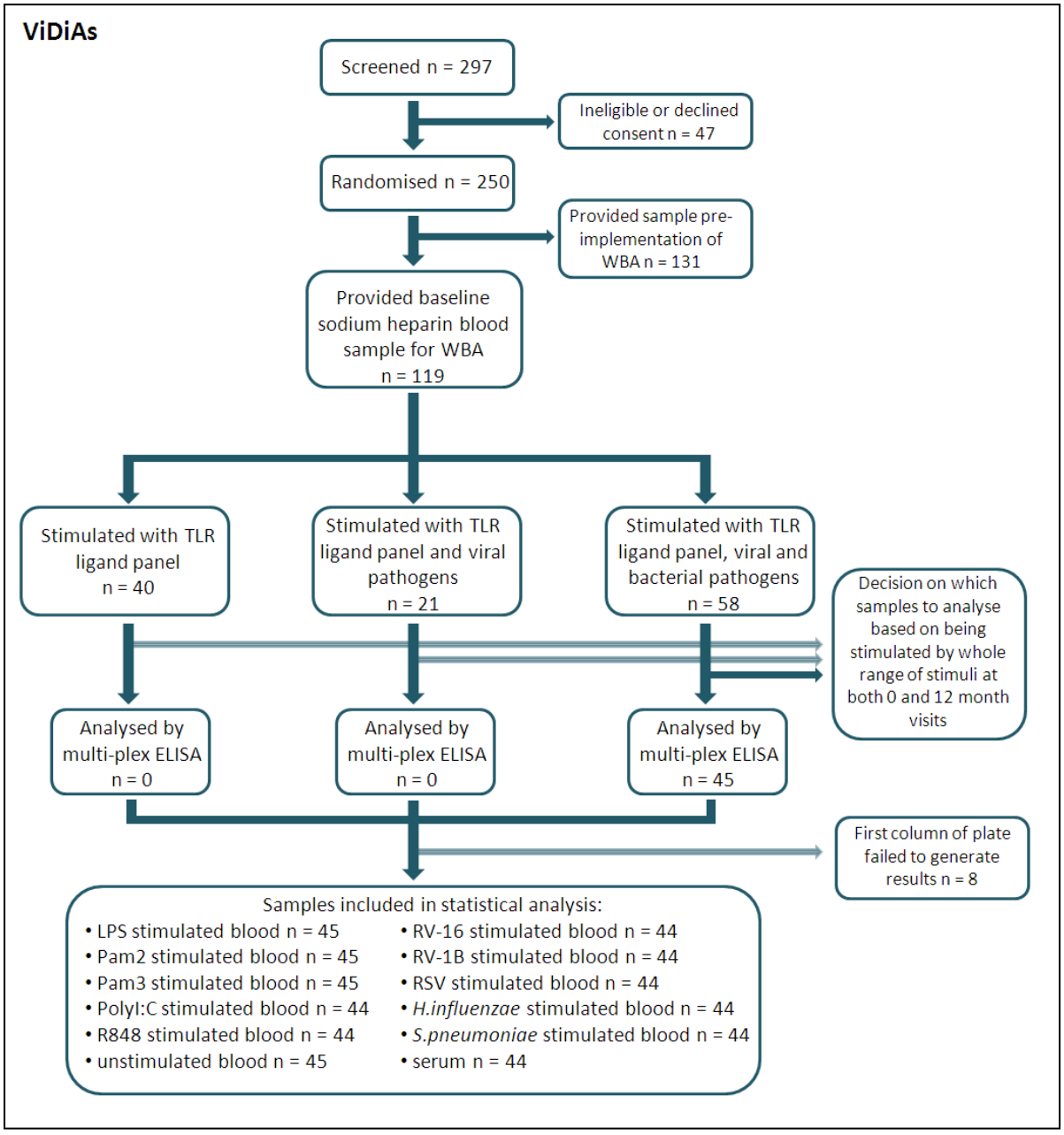


Figure 8.1: Recruitment profile for the whole blood assay at baseline in the ViDiAs trial.

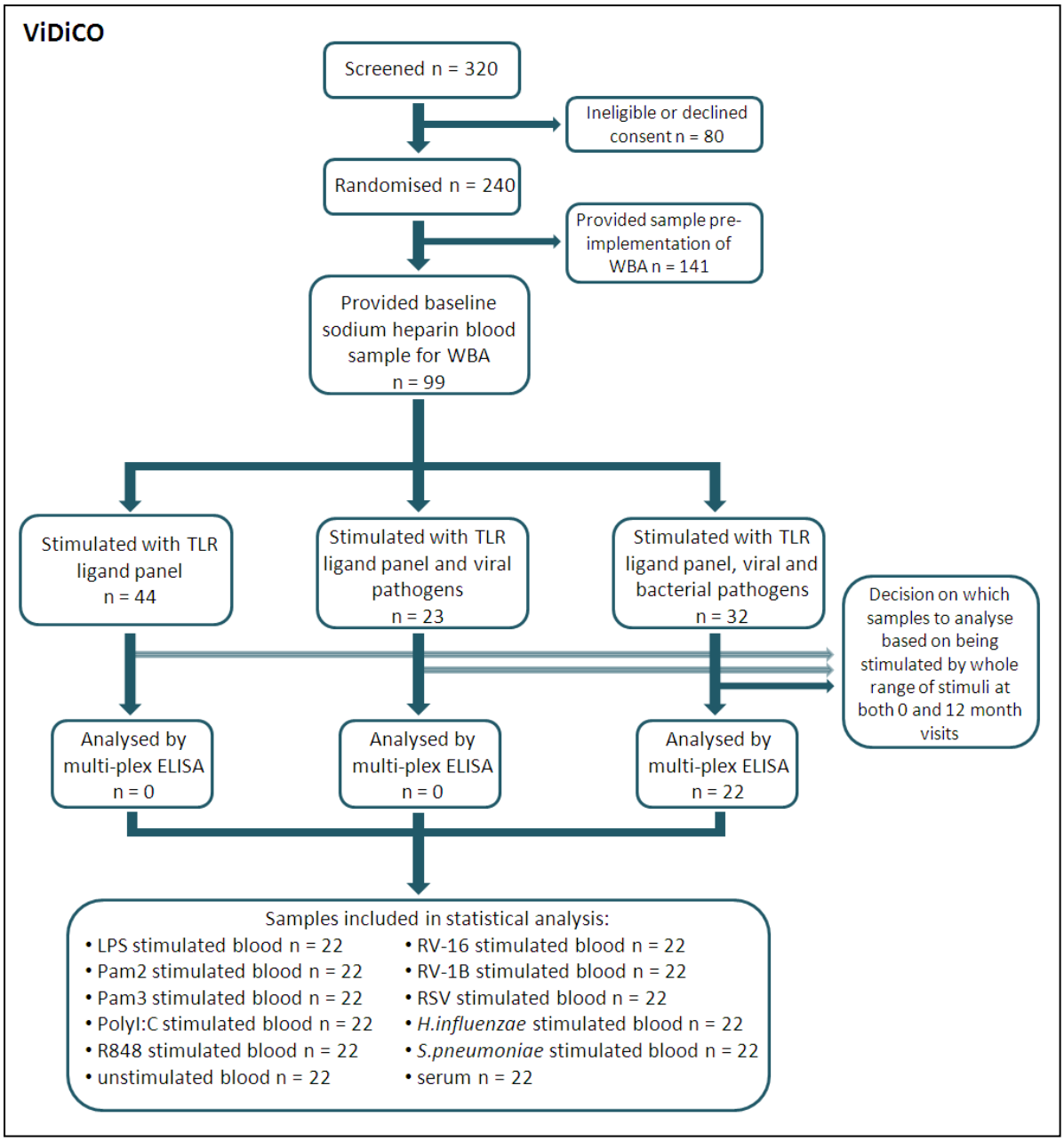


Figure 8.2: Recruitment profile for the whole blood assay at baseline in the ViDiCO trial.

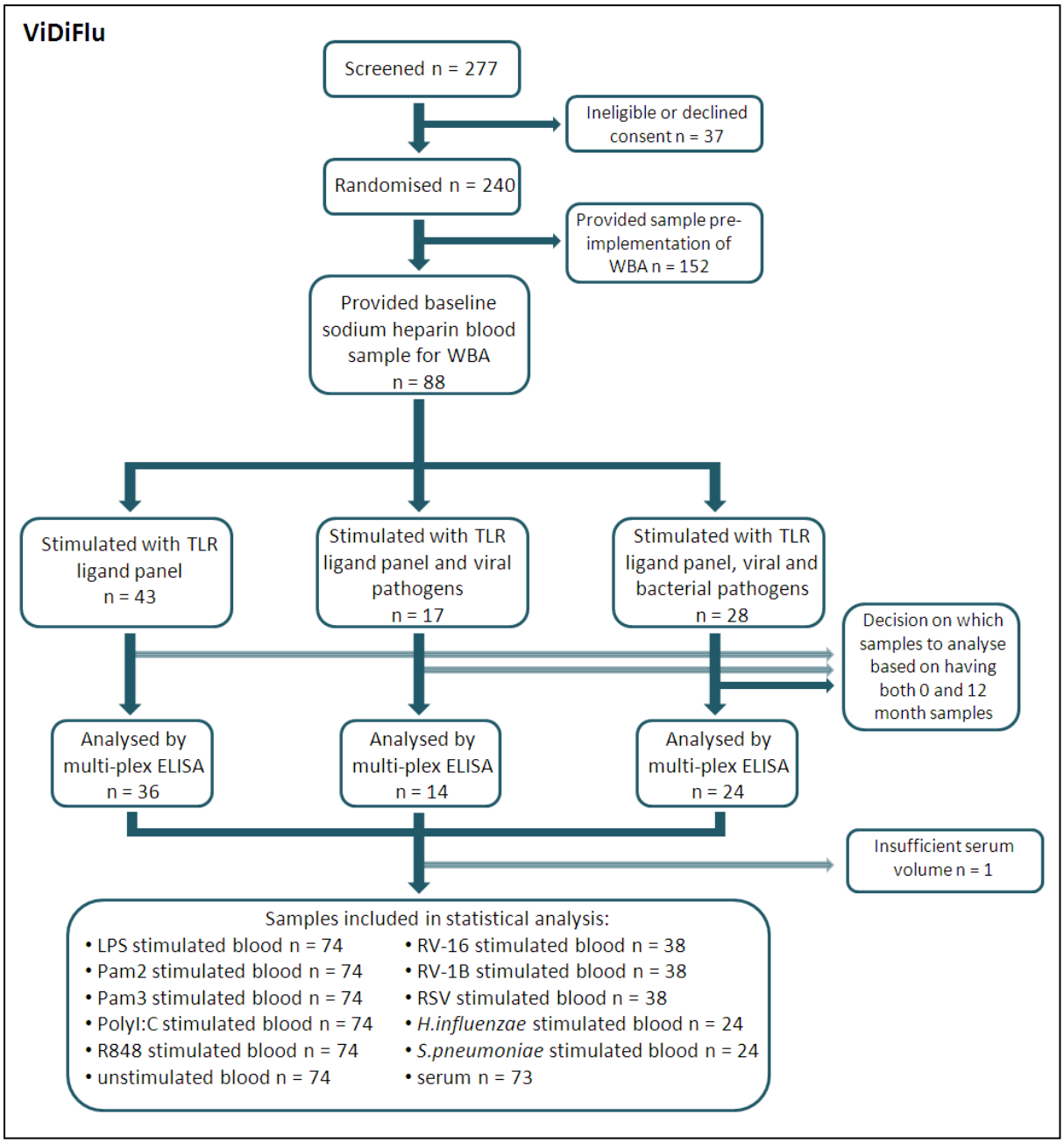


Figure 8.3: Recruitment profile for the whole blood assay at baseline in the ViDiFlu trial.

The baseline characteristics of participants who produced samples for the whole blood assay at baseline are presented in Table 8.1. There was no difference in the ethnic composition, BMI and pneumococcal vaccine uptake between the three groups of participants. However, there was a difference in the age ($p < 0.0001$), sex ($p = 0.003$), smoking status ($p = 0.02$), influenza vaccine uptake ($p = 0.01$) and 25(OH)D status ($p = 0.002$) between the three trials, with an additional difference seen in inhaled corticosteroid use between asthma and CODP patients ($p = 0.02$). While ViDiFlu participants are considered controls for this cross-sectional analysis, due to absence of asthma or COPD, the populations making up this study were either adults living in sheltered accommodation, or staff members at participating units. In the subset studied in the whole blood assay, 20% were staff, with the remaining 80%, mostly older adults, and as such, the median age was higher in this control population than in asthma patients. Additionally, with the likelihood of developing COPD increasing with age and smoke exposure, and the eligibility criteria excluding those who were younger than 40, the median age in COPD patients was higher than in asthmatics. Also due to the nature of the disease and the eligibility criteria of having a smoking history of at least 15 pack years, a higher proportion of COPD patients were current smokers compared to asthmatics. ViDiAs participants also displayed a lower proportion of current smokers compared to ViDiFlu controls, potentially refraining from smoking in response to it exacerbating their asthma. Influenza vaccination is routinely offered to older adults, and those with chronic respiratory conditions such as asthma and COPD. Vaccine uptake was high in both asthma and COPD patients, however the presence of younger care staff in the ViDiFlu population may be the cause of the difference in vaccine uptake in this group. Median 25(OH)D concentration indicated deficiency in all three study populations, but was significantly lower in COPD patients compared to controls, potentially due to decreased mobility and thus limited time outside with associated lack of sun exposure in these participants. By chance, the subset of whole blood assay samples analysed had differing proportions of males and females within the three studies, with the ratio in asthma fairly even, but more males in the COPD study, and more females amongst the control ViDiFlu participants. Finally, inhaled corticosteroid (ICS) use was higher in asthmatics compared to COPD patients, with ICS use an inclusion criterion for the ViDiAs trial, but not for the ViDiCO trial.

Table 8.1: Baseline characteristics of participants undergoing whole blood assay at baseline from all three trials

	Asthma (n = 45)	**COPD (n = 22)**	**Control (n = 74)**	**P-Value**
Median Age (IQR)	50 (38 to 63)	67 (63 to 73)	67 (60 to 74)	< 0.0001
Sex				0.003
Male, n (%)	26 (58)	17 (77)	28 (38)	
Female, n (%)	19 (42)	5 (23)	46 (62)	
Ethnic Group				0.65
White, n (%)	38 (84)	21 (95)	62 (84)	
Black, n (%)	3 (7)	1 (5)	5 (7)	
Other, n (%)	4 (9)	0 (0)	7 (9)	
Current Smoker				0.02
Yes, n (%)	2 (4)	6 (27)	17 (23)	
No, n (%)	43 (96)	16 (73)	57 (77)	
Median BMI (IQR)	26.5 (23.8 to 29.5)	25.7 (21.3 to 31.6)	27.51 (24.5 to 32.2)	0.05
Pneumococcal Vaccine				0.17
Received, n (%)	12 (27)	11 (50)	26 (35)	
Did not receive, n (%)	33 (73)	11 (50)	48 (65)	
Influenza Vaccine				0.01
Received, n (%)	39 (87)	21 (95)	52 (70)	
Did not receive, n (%)	6 (13)	1 (5)	22 (30)	
Median 25(OH)D Concentration in nmol/L (IQR)	37 (26 to 54)	31 (15 to 39)	50 (31 to 67)	0.002
Median Inhaled Corticosteroid Use in µg (IQR)[1]	600 (250 to 1000)	200 (0 to 850)	n/a	0.02

[1] *Inhaled corticosteroid dose given as betamethasone equivalents: 1 microgram betamethasone assumed equivalent to 1 µg budesonide, 0.5 µg fluticasone dipropionate and 0.75 µg ciclesonide.*

Ordinary one-way ANOVA tests were used to calculate p-values for age and 25(OH)D status (due to these data being normally distributed); a Kruskal-Wallis tests was used to calculate the p-value for BMI (due to data being non-normally distributed); a Mann-Whitney test was used to calculate the p-value for ICS use (due to data being non-normally distributed); Chi-Squared tests were used to calculate p-values for sex, ethnicity, smoking status, pneumococcal vaccine uptake and influenza vaccine uptake.

Abbreviations used: IQR: interquartile range; BMI: body mass index (individuals body mass divided by the square of their height); 25(OH)D: 25-hydroxy vitamin D.

8.2.2 Serum concentrations of inflammatory mediators and leukocyte numbers in peripheral blood differ between asthma, COPD and controls

Peripheral blood samples were collected from participants at baseline into both sodium heparin tubes and serum separating tubes (SST). Sodium heparin blood samples were used in the whole blood assay (as described in Chapter 2.2), with samples incubated with PBS for 24 hours as a negative control. SST tubes were used to obtain serum samples, which underwent analysis by multiplex ELISA to determine the concentrations of inflammatory mediators in the serum of controls, and people with asthma or COPD. Qlucore Omics Explorer 2.3 was used to analyse the data, modelling using a two-group comparison (i.e. Student's T-Test) between each study (i.e. participants with asthma, COPD, or neither condition) with log-transformed data, and including adjustment for age, sex, baseline 25(OH)D concentration, ethnicity, smoking status and vaccination record. All results are presented in Table 8.2, and concentrations of inflammatory mediators that were significantly different between groups are presented in Figure 8.4. When compared to controls, the concentrations of EGF ($p < 0.0001$ for asthma; $p = 0.0005$ for COPD) and MIP-1β ($p = 0.003$ for asthma; $p = 0.01$ for COPD) were lower in both asthma and COPD patients, while the concentration of TNF-α was lower in asthmatics compared to controls ($p = 0.003$). The concentrations of IL-6 ($p = 0.01$) and IL-8 ($p = 0.02$) were both lower in COPD patients compared to control participants, while the concentrations of IL-1RA ($p = 0.002$), IL-17 ($p = 0.01$) and IL-2R ($p = 0.02$) were all higher. In a comparison of the inflammatory mediators present in the serum of people with asthma or COPD, the concentration of EGF was higher in COPD patients ($p < 0.0001$). The remaining inflammatory mediators assayed displayed no differences between control participants, asthmatics, and COPD patients (Table 8.2).

Table 8.2: Comparison of serum inflammatory mediator concentrations in controls, and patients with asthma or COPD

Analytes	Asthma		COPD		Controls		Statistics: Asthma vs. COPD			Statistics: Asthma vs. Controls			Statistics: COPD vs. Controls		
	n	median (IQR)	n	median (IQR)	n	median (IQR)	T-Statistic	P-Value	Q-Value	T-Statistic	P-Value	Q-Value	T-Statistic	P-Value	Q-Value
EGF	44	12.21 (0 to 26)	22	32.39 (12 to 46)	73	59.94 (40 to 73)	-4.220	< 0.0001	0.003	-9.228	< 0.0001	< 0.0001	-3.643	0.0005	0.014
Eotaxin	44	36.63	22	65.76	73	53.70	-1.357	0.180	0.772	-1.356	0.178	0.379	1.626	0.108	0.242
FGF-Basic	44	16.25	22	0.00	73	12.63	0.852	0.398	0.905	0.242	0.809	0.934	0.030	0.976	0.996
G-CSF	44	43.49	22	50.30	73	36.09	-0.236	0.815	0.945	0.139	0.889	0.962	0.460	0.647	0.843
GM-CSF	44	2.46	22	2.71	73	3.32	-0.288	0.775	0.945	-0.129	0.898	0.962	1.347	0.181	0.320
HGF	44	162.55	22	180.36	73	157.60	0.495	0.622	0.909	-0.404	0.687	0.859	0.403	0.688	0.843
IFN-α	44	34.98	22	45.07	73	29.31	-1.913	0.061	0.456	-1.286	0.201	0.379	-0.377	0.707	0.843
IFN-γ	44	0.00	22	0.28	73	0.00	1.006	0.319	0.857	-2.456	0.016	0.117	-1.912	0.059	0.194
IL-10	44	2.04	22	4.72	73	3.27	0.090	0.929	0.967	0.461	0.646	0.859	1.504	0.136	0.256
IL-12	44	81.66	22	85.67	73	75.44	0.035	0.972	0.972	1.071	0.287	0.455	2.054	0.043	0.161
IL-13	44	12.74	22	17.26	73	10.62	-0.476	0.636	0.909	-0.771	0.443	0.664	0.522	0.603	0.843
IL-15	44	38.07	22	40.21	73	21.58	0.487	0.628	0.909	-1.067	0.288	0.455	-1.282	0.203	0.339
IL-17	44	0.00 (0 to 3)	22	2.79 (0.4 to 3)	73	0.97 (0 to 2)	-1.615	0.112	0.559	-0.055	0.956	0.970	2.577	0.012	0.084
IL-1β	44	0.00	22	0.00	73	0.00	1.064	0.292	0.857	1.188	0.237	0.419	0.928	0.356	0.562
IL-1RA	44	812.28 (538 to 992)	22	981.53 (766 to 1046)	73	525.24 (174 to 807)	-2.241	0.029	0.289	1.284	0.202	0.379	3.231	0.002	0.026
IL-2	44	0.00	22	1.07	73	0.57	0.740	0.462	0.905	-0.408	0.684	0.859	-0.446	0.657	0.843
IL-2R	44	159.32 (95 to 204)	22	197.68 (171 to 250)	73	155.18 (102 to 183)	-2.336	0.023	0.289	-0.719	0.473	0.676	2.411	0.018	0.084
IL-4	44	5.78	22	9.18	73	6.79	-0.082	0.935	0.967	-2.120	0.036	0.181	-0.345	0.731	0.843
IL-5	44	0.57	22	1.53	73	0.67	-0.483	0.631	0.909	-1.360	0.177	0.379	-0.005	0.996	0.996
IL-6	44	2.30 (0 to 4)	22	3.25 (2 to 7)	73	2.27 (1 to 7)	0.775	0.442	0.905	-1.992	0.049	0.183	-2.507	0.014	0.084
IL-7	44	5.26	22	15.78	73	0.00	0.189	0.851	0.945	-0.359	0.720	0.864	-0.235	0.815	0.905
IL-8	44	9.12 (0 to 21)	22	8.03 (0 to 15)	73	21.23 (11 to 91)	1.738	0.088	0.525	-1.567	0.120	0.360	-2.381	0.019	0.084
IP-10 [2]	44	21.33	22	24.47	72	31.19	1.068	0.290	0.857	-1.313	0.192	0.379	-1.871	0.065	0.194
MCP-1	44	230.38	22	379.53	73	338.20	-0.956	0.343	0.857	-2.211	0.029	0.175	-1.686	0.095	0.238
MIG	44	39.07	22	35.90	73	32.38	1.064	0.292	0.857	1.858	0.066	0.220	0.710	0.479	0.719
MIP-1α	44	36.27	22	36.75	73	34.68	0.206	0.838	0.945	-2.022	0.046	0.183	-1.601	0.113	0.242
MIP-1β	44	39.26 (25 to 54)	22	50.31 (38 to 62)	73	51.54 (33 to 107)	-0.706	0.483	0.905	-3.011	0.003	0.033	-2.525	0.013	0.084
RANTES [2]	31	4684.76	12	6980.14	51	5199.32	-0.356	0.723	0.945	-0.037	0.970	0.970	1.561	0.122	0.244
TNF-α	44	3.85 (0 to 6)	22	5.44 (0 to 6)	73	4.47 (0 to 15)	-0.333	0.740	0.945	-3.009	0.003	0.033	-1.727	0.088	0.238
VEGF	44	5.39	22	11.48	73	8.47	-0.646	0.521	0.909	-1.449	0.150	0.379	-0.008	0.993	0.996

Medians are given in pg/ml.

[2] For some analytes numbers were smaller due to mean fluorescence index (MFI) values exceeding the upper limit of detection, resulting in concentrations being unable to be calculated

The interquartile range (IQR) is only stated where statistical significant difference was evident ($p < 0.05$, $q < 0.1$)

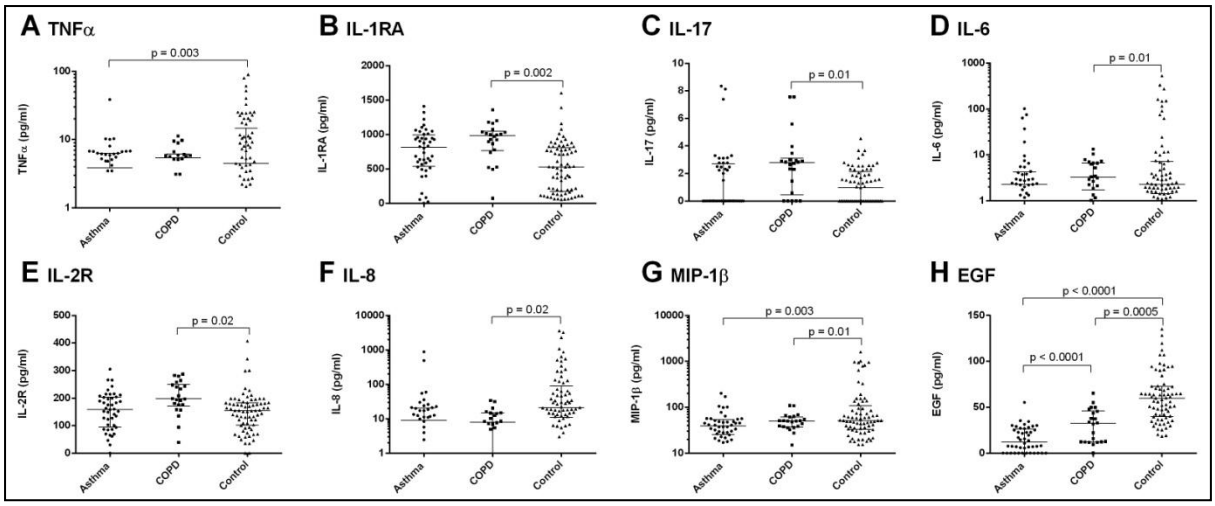


Figure 8.4: Cross-sectional comparison of inflammatory mediators present in the serum of controls, and those with asthma and COPD. Peripheral whole blood was collected and centrifuged before aspiration of serum and analysis by multiplex ELISA (n = 44 for asthma, n = 22 for COPD and n = 73 for controls). Data are represented as scatter plots with median + IQR, with p-values calculated using two-group comparisons between each patient group, with the model including adjustment for age, sex, baseline 25(OH)D status, ethnicity, smoking status and vaccination record, using the programme Qlucore Omics Explorer 2.3. P-values were deemed significant when less than 0.05, with a corresponding q-value of less than 0.1. Any values under the limit of detection (as specified within the product data sheet for each analyte) were given a value of 0.

Finally, coulter counts were carried out on unstimulated peripheral whole blood samples, to determine any differences in cell types between controls and those with asthma and COPD. Qlucore Omics Explorer 2.3 was used to analyse the data, modelling using a two-group comparison (i.e. Student's T-Test) between each study with log-transformed data, and including adjustment for age, sex, baseline 25(OH)D concentration, ethnicity, smoking status and vaccination record. All results are presented in Table 8.3, and significant results are presented in Figure 8.5. The number of eosinophils was significantly higher in both asthma (p = 0.002) and COPD (p = 0.008) compared to controls, while COPD patients also displayed increased lymphocyte (p = 0.0003), neutrophil (p = 0.002) and monocyte (p = 0.003) counts compared to controls. In a comparison of asthma versus COPD, COPD patients were shown to have a greater number of neutrophils (p = 0.003) and lymphocytes (p = 0.01) compared to asthmatics.

Table 8.3: Comparison of peripheral whole blood cell populations in controls, and patients with asthma or COPD

Cell Counts	Asthma		COPD		Controls		Statistics: Asthma vs. COPD			Statistics: Asthma vs. Controls			Statistics: COPD vs. Controls		
	n	median (IQR)	n	median (IQR)	n	median (IQR)	T-Statistic	P-Value	Q-Value	T-Statistic	P-Value	Q-Value	T-Statistic	P-Value	Q-Value
Basophils	45	0.10 (0 to 0.1)	22	0.10 (0 to 0.1)	74	0.00 (0 to 0.1)	0.412	0.682	0.691	3.158	0.002	0.005	0.805	0.423	0.423
Eosinophils	45	0.20 (0.2 to 0.4)	22	0.20 (0.1 to 0.3)	74	0.20 (0.1 to 0.2)	0.400	0.691	0.691	3.149	0.002	0.005	2.730	0.008	0.010
Lymphocytes	45	2.00 (1.6 to 2.4)	22	2.15 (1.9 to 2.8)	74	2.00 (1.7 to 2.4)	-2.660	0.010	0.025	0.431	0.667	0.667	3.726	0.000	0.002
Monocytes	45	0.50 (0.4 to 0.6)	22	0.70 (0.5 to 0.9)	74	0.40 (0.4 to 0.6)	-1.026	0.309	0.516	1.634	0.105	0.175	3.045	0.003	0.005
Neutrophils	45	3.60 (2.9 to 4.3)	22	4.85 (3.8 to 5.7)	74	3.40 (2.8 to 4.3)	-3.080	0.003	0.016	-0.567	0.572	0.667	3.228	0.002	0.004

Absolute cell counts are given as x 10^9 cells/L

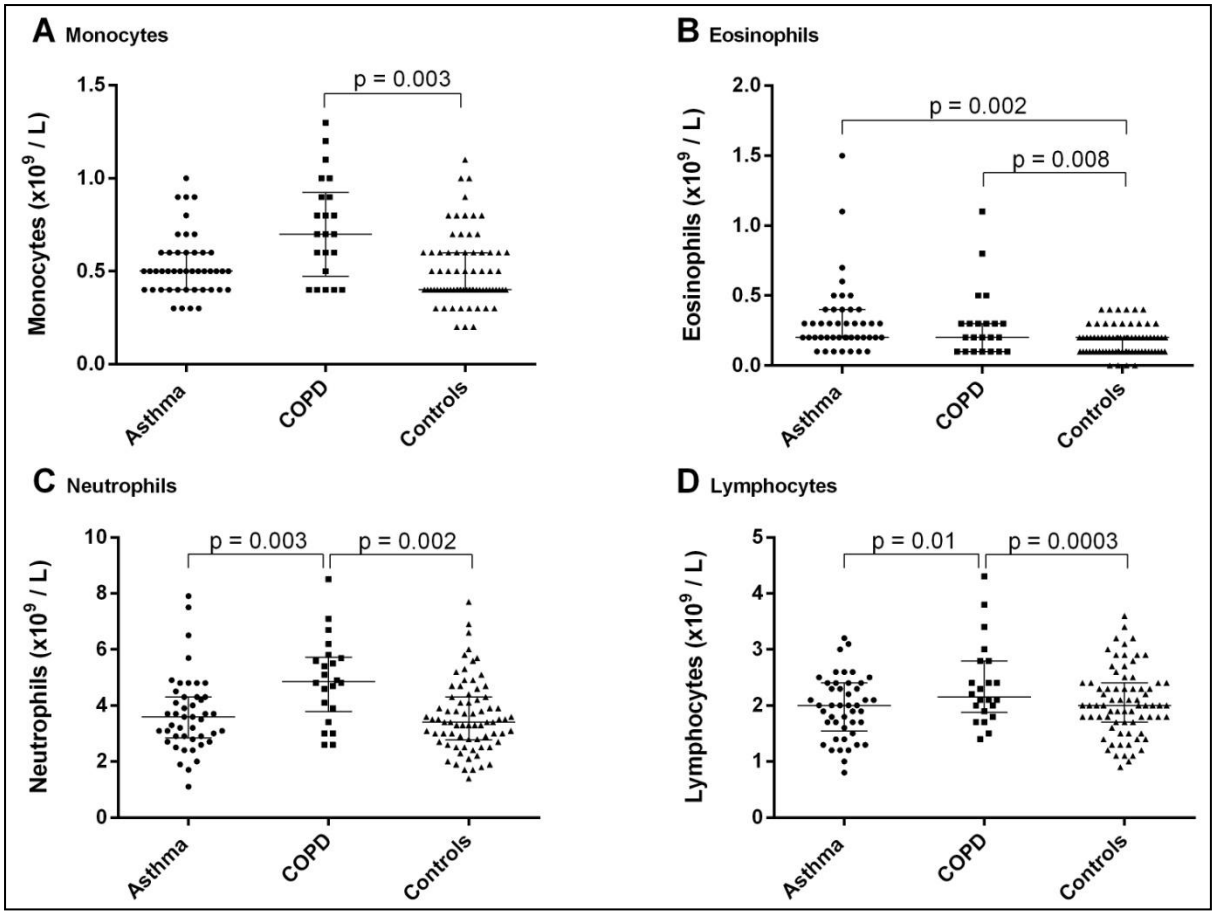


Figure 8.5: Cross-sectional comparison of cells present in peripheral blood of controls, and those with asthma and COPD. Peripheral whole blood was collected and cells were counted by coulter counter (n = 45 for asthma, n = 22 for COPD and n = 74 for controls). Data are represented as scatter plots with median + IQR, with p-values calculated using a two-group comparisons between each patient group, with the model including adjustment for age, sex, baseline 25(OH)D status, ethnicity, smoking status and vaccination record, using the programme Qlucore Omics Explorer 2.3. P-values were deemed significant when less than 0.05, with a corresponding q-value of less than 0.1. Any values under the limit of detection (as specified within the product data sheet for each analyte) were given a value of 0.

8.2.3 Analysis of the secretion of inflammatory mediators in the blood following LPS stimulation shows differences between asthma, COPD and controls

Peripheral blood samples were stimulated with LPS, a ligand for TLR 4, which is typically found on the outer membrane of Gram-negative bacteria such as *Haemophilus influenza*. Qlucore Omics Explorer 2.3 was used to carry out comparisons between the three clinical groups, modelling using a two-group comparison (i.e. Student's T-Test) between each group with log-transformed data, and including adjustment for age, sex, baseline 25(OH)D concentration, ethnicity, smoking status and vaccination record. All results obtained from 30-plex ELISA analysis of supernatants from stimulated whole blood are presented in Table 8.4, and significant results are presented in Figure 8.6. When compared to controls, the supernatant concentration of LPS-stimulated eotaxin was lower in asthma and COPD patients ($p < 0.0001$ and $p = 0.001$ respectively), while the concentrations of LPS-stimulated IL-4 ($p = 0.0007$), IL-15 ($p = 0.001$), IL-12 ($p = 0.01$) and IFN-α ($p = 0.01$) were all higher in COPD patients. In a comparison of the inflammatory response to LPS in patients with asthma or COPD, concentrations of IL-2R ($p = 0.0006$), FGF-Basic ($p = 0.001$), IL-12 ($p = 0.003$), IL-15 ($p = 0.008$), IL-6 ($p = 0.009$), EGF ($p = 0.01$) and IL-1RA ($p = 0.02$) were all higher in COPD. The remaining inflammatory mediators assayed in the supernatants of LPS-stimulated whole blood displayed no statistically significant differences between controls, asthmatics, and COPD patients (Table 8.4).

Table 8.4: Comparison of inflammatory mediators released following whole blood stimulation by LPS in controls and patients with asthma or COPD

Analytes	Asthma		COPD		Controls		Statistics: Asthma vs. COPD			Statistics: Asthma vs. Controls			Statistics: COPD vs. Controls		
	n	median (IQR)	n	median (IQR)	n	median (IQR)	T-Statistic	P-Value	Q-Value	T-Statistic	P-Value	Q-Value	T-Statistic	P-Value	Q-Value
EGF	45	58.71 (40 to 76)	22	68.49 (48 to 84)	74	42.78 (31 to 56)	-2.561	0.013	0.066	-1.266	0.208	0.664	1.926	0.057	0.162
Eotaxin	45	62.47 (41 to 85)	22	54.44 (41 to 95)	74	61.35 (40 to 89)	0.543	0.590	0.885	-4.060	< 0.0001	0.003	-3.312	0.001	0.014
FGF-Basic	45	55.95 (42 to 72)	22	68.33 (52 to 107)	74	53.80 (38 to 68)	-3.433	0.001	0.017	-1.480	0.142	0.664	2.001	0.049	0.162
G-CSF	45	188.77	22	181.76	74	180.95	-0.293	0.771	0.889	-1.230	0.221	0.664	-0.536	0.593	0.635
GM-CSF	45	3.49	22	4.18	74	3.57	0.458	0.649	0.885	0.722	0.472	0.845	0.644	0.521	0.617
HGF	45	239.72	22	247.05	74	202.17	-0.415	0.679	0.885	0.093	0.926	0.926	1.417	0.160	0.250
IFN-α	45	182.13 (151 to 220)	22	190.82 (154 to 224)	74	153.95 (119 to 189)	-1.594	0.116	0.318	0.248	0.805	0.893	2.542	0.013	0.077
IFN-γ	45	4.58	22	6.51	74	6.76	-2.235	0.029	0.110	-0.694	0.489	0.845	1.765	0.081	0.162
IL-10	45	336.83	22	406.06	74	284.31	0.548	0.586	0.885	0.622	0.535	0.845	0.704	0.484	0.604
IL-12	45	306.45 (224 to 416)	22	996.94 (376 to 1453)	74	333.94 (202 to 714)	-3.098	0.003	0.030	-0.824	0.412	0.824	2.622	0.010	0.077
IL-13	45	28.40	22	29.89	74	24.74	-0.772	0.443	0.831	0.402	0.688	0.875	2.172	0.033	0.131
IL-15	45	303.97 (208 to 370)	22	441.62 (343 to 592)	74	262.83 (196 to 362)	-2.771	0.008	0.056	0.456	0.649	0.875	3.378	0.001	0.014
IL-17	45	7.46	22	7.73	74	6.16	0.431	0.668	0.885	1.638	0.104	0.626	0.884	0.379	0.517
IL-1β	45	230.48	22	341.57	74	225.41	-1.289	0.203	0.434	0.210	0.834	0.893	1.535	0.128	0.227
IL-1RA	45	2290.25 (1952 to 3058)	22	3610.10 (2871 to 5136)	74	2284.77 (1862 to 3247)	-2.346	0.022	0.096	-1.252	0.213	0.664	2.144	0.035	0.131
IL-2	45	5.95	22	5.41	74	5.18	-0.026	0.980	0.980	1.151	0.252	0.688	1.544	0.126	0.227
IL-2R	45	349.04 (298 to 393)	22	413.21 (362 to 468)	74	310.89 (241 to 390)	-3.611	0.0006	0.017	-0.630	0.530	0.845	1.791	0.077	0.162
IL-4	45	20.53 (16 to 31)	22	28.01 (25 to 31)	74	16.54 (11 to 24)	-1.180	0.243	0.486	2.400	0.018	0.271	3.526	0.0007	0.014
IL-5	45	3.15	22	5.75	74	2.13	-2.164	0.035	0.116	0.827	0.410	0.824	1.899	0.061	0.162
IL-6 [2]	21	8476.38 (4091 to 12106)	7	15220.74 (10301 to 17443)	40	9720.79 (3046 to 12391)	-2.686	0.009	0.057	-0.973	0.333	0.824	1.394	0.167	0.250
IL-7	45	85.45	22	74.72	74	63.77	0.398	0.692	0.885	0.308	0.759	0.875	1.857	0.067	0.162
IL-8 [2]	36	14920.09	21	12964.19	64	17204.30	-0.316	0.753	0.889	-0.331	0.741	0.875	-0.707	0.482	0.604
IP-10 [2]	44	155.94	16	358.90	72	235.40	-1.986	0.052	0.156	-0.359	0.720	0.875	1.783	0.078	0.162
MCP-1 [2]	45	11937.90	21	12913.79	68	14514.30	0.142	0.887	0.926	-1.939	0.055	0.413	-2.181	0.032	0.131
MIG	45	68.86	22	67.03	74	65.44	-0.181	0.857	0.926	-2.086	0.039	0.393	-0.935	0.353	0.504
MIP-1α [2]	45	6155.29	22	5212.55	73	5178.53	0.377	0.708	0.885	0.410	0.683	0.875	-0.587	0.559	0.621
MIP-1β [2]	30	15571.69	12	12137.35	51	12895.12	1.540	0.129	0.323	1.370	0.174	0.664	-0.247	0.806	0.806
RANTES [2]	24	7324.95	13	6166.61	59	3979.78	1.369	0.176	0.407	0.346	0.730	0.875	-0.623	0.535	0.617
TNF-α	45	122.35	22	126.26	74	130.75	-0.132	0.895	0.926	0.161	0.872	0.902	0.405	0.686	0.710
VEGF	45	182.26	22	184.98	74	157.62	-0.669	0.506	0.885	-0.836	0.405	0.824	1.396	0.166	0.250

Medians are given in pg/ml.

[2] For some analytes numbers were smaller due to mean fluorescence index (MFI) values exceeding the upper limit of detection, resulting in concentrations being unable to be calculated

The interquartile range (IQR) is only stated where statistical significant difference was evident ($p < 0.05$, $q < 0.1$)

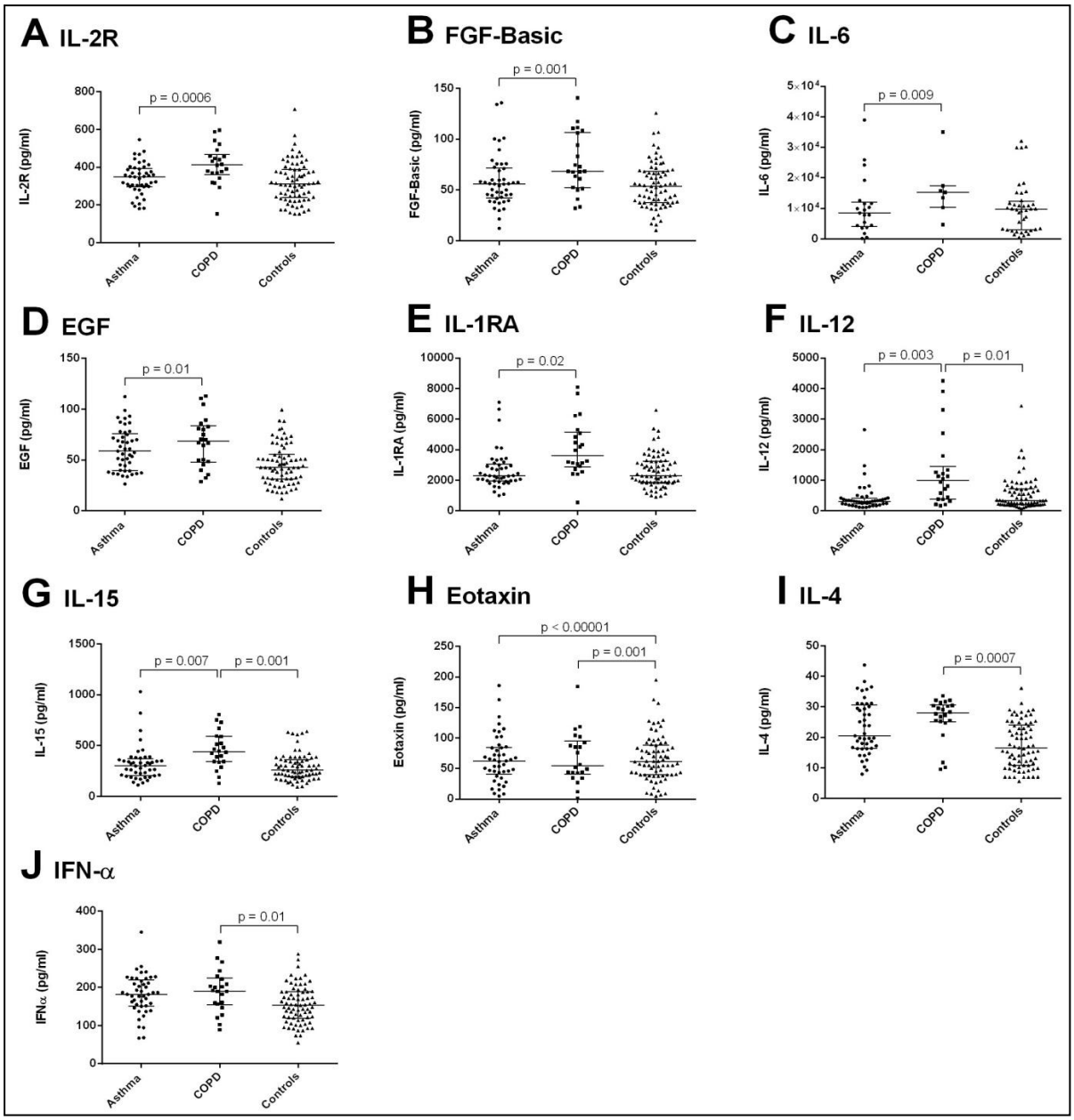


Figure 8.6: Cross-sectional comparison of inflammatory mediators released in response to LPS stimulation in the blood of controls, and those with asthma and COPD. Peripheral whole blood was collected and stimulated with LPS for 24 hours, before supernatant aspiration and analysis by multiplex ELISA (n = 45 for asthma, n = 22 for COPD and n = 74 for controls). Data are represented as scatter plots with median + IQR, with p-values calculated using two-group comparisons between each clinical group, with the model including adjustment for age, sex, baseline 25(OH)D status, ethnicity, smoking status and vaccination record, using the programme Qlucore Omics Explorer 2.3. P-values were deemed significant when less than 0.05, with a corresponding q-value of less than 0.1. Any values under the limit of detection (as specified within the product data sheet for each analyte) were given a value of 0.

8.2.4 Analysis of the secretion of inflammatory mediators in the blood following Pam2CSK4 stimulation shows differences between asthma, COPD and controls

Peripheral blood samples were stimulated with Pam2CSK4 (Pam2), a synthetic diacylated bacterial lipopeptide which is a ligand for TLR 2/6 heterodimers, and associated with infections caused by bacteria such as *Streptococcus pneumoniae.* Qlucore Omics Explorer 2.3 was used to carry out comparisons between the three clinical groups, modelling using a two-group comparison (i.e. Student's T-Test) between each group with log-transformed data, and including adjustment for age, sex, baseline 25(OH)D concentration, ethnicity, smoking status and vaccination record. All results obtained. All results obtained from 30-plex ELISA analysis of supernatants from stimulated whole blood are presented in Table 8.5, and significant results are presented in Figure 8.7. When compared to controls, the concentration of eotaxin was lower in supernatants of Pam2-stimulated whole blood from asthma and COPD patients ($p < 0.0001$ and $p = 0.008$ respectively), while the concentrations of MCP-1 ($p = 0.0005$), and FGF-Basic ($p = 0.001$) were higher in COPD patients. However, the magnitude of all differences was minimal. In a comparison of the immune response to Pam2 in patients with asthma or COPD, no differences were observed. The remaining inflammatory mediators assayed displayed no statistically significant differences between controls, asthmatics, and COPD patients (Table 8.5).

Table 8.5: Comparison of inflammatory mediators released following whole blood stimulation by Pam2CSK4 in controls and patients with asthma or COPD

Analytes	Asthma		COPD		Controls		Statistics: Asthma vs. COPD			Statistics: Asthma vs. Controls			Statistics: COPD vs. Controls		
	n	median (IQR)	n	median (IQR)	n	median (IQR)	T-Statistic	P-Value	Q-Value	T-Statistic	P-Value	Q-Value	T-Statistic	P-Value	Q-Value
EGF	45	44.47	22	35.53	74	31.50	-0.329	0.743	0.967	-2.116	0.037	0.233	-2.406	0.018	0.137
Eotaxin	45	54.17 (31 to 71)	22	51.96 (33 to 87)	74	56.09 (42 to 77)	-0.209	0.836	0.967	-4.467	< 0.0001	0.001	-2.720	0.008	0.079
FGF-Basic	45	27.17 (23 to 34)	22	26.79 (16 to 31)	74	28.04 (22 to 34)	-0.063	0.950	0.967	-1.189	0.237	0.508	-3.335	0.001	0.019
G-CSF	45	91.98	22	72.27	74	77.33	1.232	0.223	0.967	-2.091	0.039	0.233	-1.407	0.163	0.408
GM-CSF	45	2.60	22	3.04	74	2.90	0.209	0.835	0.967	0.864	0.390	0.557	0.892	0.375	0.590
HGF	45	145.89	22	135.06	74	121.69	0.502	0.618	0.967	-0.707	0.481	0.602	-0.978	0.331	0.590
IFN-α	45	147.94	22	130.08	74	125.62	0.185	0.854	0.967	-0.797	0.427	0.582	0.691	0.491	0.590
IFN-γ	45	0.00	22	0.99	74	1.37	0.710	0.481	0.967	1.063	0.290	0.544	0.681	0.497	0.590
IL-10	45	19.23	22	20.67	74	22.38	0.161	0.873	0.967	0.000	1.000	1.000	-0.649	0.518	0.590
IL-12	45	72.02	22	83.57	74	78.67	0.397	0.693	0.967	1.190	0.237	0.508	0.669	0.505	0.590
IL-13	45	13.07	22	10.70	74	12.60	0.296	0.768	0.967	0.577	0.565	0.669	0.629	0.531	0.590
IL-15	45	120.22	22	138.60	74	120.26	0.460	0.647	0.967	0.254	0.800	0.874	-0.955	0.342	0.590
IL-17	45	3.28	22	3.92	74	0.00	-0.087	0.931	0.967	1.524	0.130	0.391	1.884	0.063	0.264
IL-1β	45	21.01	22	23.49	74	15.95	1.134	0.262	0.967	0.763	0.447	0.583	-0.031	0.975	0.975
IL-1RA	45	1005.30	22	1117.79	74	904.29	0.292	0.771	0.967	-0.945	0.347	0.548	-1.176	0.243	0.560
IL-2	45	2.49	22	1.41	74	0.00	-0.089	0.930	0.967	0.918	0.361	0.548	1.537	0.128	0.384
IL-2R	45	210.47	22	248.28	74	203.72	-0.322	0.749	0.967	-1.863	0.065	0.326	-1.622	0.108	0.361
IL-4	45	13.02	22	11.51	74	8.56	0.581	0.563	0.967	1.445	0.151	0.413	1.085	0.281	0.590
IL-5	45	1.19	22	2.06	74	0.93	1.302	0.198	0.967	0.956	0.341	0.548	0.692	0.491	0.590
IL-6	45	341.41	22	336.75	74	392.43	0.042	0.967	0.967	-1.583	0.116	0.388	-1.484	0.141	0.386
IL-7	45	28.92	22	24.52	74	23.26	0.635	0.528	0.967	-1.112	0.268	0.537	-0.083	0.934	0.966
IL-8 [2]	44	1815.31	22	1379.58	74	2051.58	0.335	0.739	0.967	-1.248	0.215	0.508	-2.006	0.048	0.264
IP-10	45	50.44	22	63.91	74	55.68	0.822	0.415	0.967	-0.004	0.997	1.000	0.655	0.514	0.590
MCP-1 [2]	45	10385.05 (6591 to 14980)	22	6490.81 (4325 to 10917)	72	12298.91 (8597 to 19029)	1.595	0.116	0.967	-2.614	0.010	0.153	-3.624	0.0005	0.015
MIG	45	49.45	22	36.14	74	44.09	-0.423	0.674	0.967	-1.688	0.094	0.353	-0.756	0.452	0.590
MIP-1α	45	91.02	22	72.92	74	97.32	0.583	0.562	0.967	-0.909	0.365	0.548	-0.741	0.461	0.590
MIP-1β	45	952.21	22	773.51	74	1295.79	0.264	0.793	0.967	-2.185	0.031	0.233	-1.874	0.064	0.264
RANTES [2]	33	7926.79	18	3154.92	67	2785.76	1.612	0.112	0.967	0.233	0.816	0.874	-0.996	0.322	0.590
TNF-α	45	7.88	22	9.17	74	9.59	1.546	0.128	0.967	0.555	0.580	0.669	0.549	0.584	0.626
VEGF	45	47.51	22	41.77	74	45.38	1.063	0.292	0.967	-1.750	0.083	0.353	-1.832	0.070	0.264

Medians are given in pg/ml.

[2] For some analytes numbers were smaller due to mean fluorescence index (MFI) values exceeding the upper limit of detection, resulting in concentrations being unable to be calculated

The interquartile range (IQR) is only stated where statistical significant difference was evident ($p < 0.05$, $q < 0.1$)

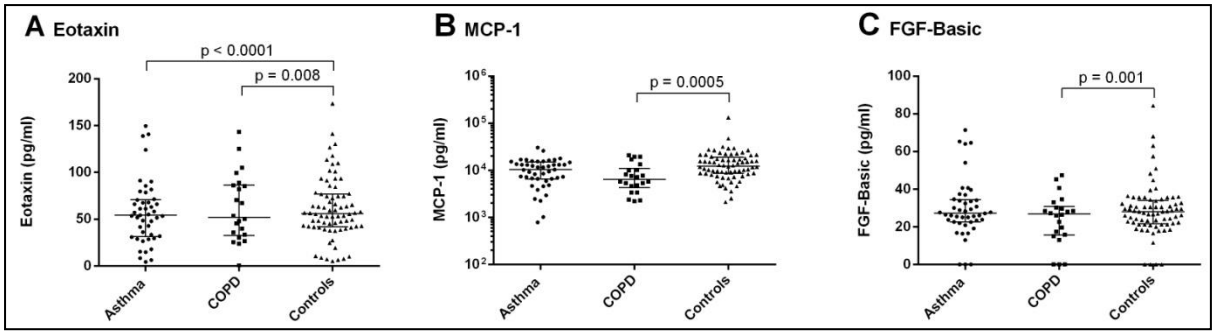


Figure 8.7: Cross-sectional comparison of inflammatory mediators released in response to Pam2CSK4 stimulation in the blood of controls, and those with asthma and COPD. Peripheral whole blood was collected and stimulated with Pam2CSK4 for 24 hours, before supernatant aspiration and analysis by multiplex ELISA (n = 45 for asthma, n = 22 for COPD and n = 74 for controls). Data are represented as scatter plots with median + IQR, with p-values calculated using two-group comparisons between each clinical group, with the model including adjustment for age, sex, baseline 25(OH)D status, ethnicity, smoking status and vaccination record, using the programme Qlucore Omics Explorer 2.3. P-values were deemed significant when less than 0.05, with a corresponding q-value of less than 0.1. Any values under the limit of detection (as specified within the product data sheet for each analyte) were given a value of 0.

8.2.5 Analysis of the secretion of inflammatory mediators in the blood following Pam3CSK4 stimulation shows differences between asthma, COPD and controls

Peripheral blood samples were stimulated with Pam3CSK4 (Pam3), a synthetic triacylated bacterial lipopeptide, which is a ligand for TLR 2/1 heterodimers, and associated with infections caused by bacteria such as *Mycoplasma pneumoniae*. Qlucore Omics Explorer 2.3 was used to carry out comparisons between the three clinical groups, modelling using a two-group comparison (i.e. Student's T-Test) between each group with log-transformed data, and including adjustment for age, sex, baseline 25(OH)D concentration, ethnicity, smoking status and vaccination record. All results obtained from 30-plex ELISA analysis of supernatants from stimulated whole blood are presented in Table 8.6, and significant results are presented in Figure 8.8. The only statistical significant difference seen was when comparing COPD patients to controls, with higher levels of Pam3-stimulated IFN-α produced by COPD patients ($p < 0.0001$). The remaining inflammatory mediators assayed displayed no statistically significant differences between controls, asthmatics, and COPD patients (Table 8.6).

Table 8.6: Comparison of inflammatory mediators released following whole blood stimulation by Pam3CSK4 in controls and patients with asthma or COPD

Analytes	Asthma		COPD		Controls		Statistics: Asthma vs. COPD			Statistics: Asthma vs. Controls			Statistics: COPD vs. Controls		
	n	median (IQR)	n	median (IQR)	n	median (IQR)	T-Statistic	P-Value	Q-Value	T-Statistic	P-Value	Q-Value	T-Statistic	P-Value	Q-Value
EGF	45	52.11	22	35.72	74	32.89	0.341	0.734	0.905	-0.075	0.940	0.940	-1.434	0.155	0.423
Eotaxin	45	57.58	22	59.93	74	58.03	0.816	0.418	0.871	-2.817	0.006	0.168	-2.127	0.036	0.183
FGF-Basic	45	36.12	22	35.69	74	31.05	-0.798	0.428	0.871	-1.244	0.216	0.453	-0.214	0.831	0.895
G-CSF	45	107.30	22	92.22	74	90.33	0.803	0.425	0.871	-1.017	0.311	0.519	-0.320	0.750	0.886
GM-CSF	45	3.12	22	3.44	74	3.02	0.756	0.453	0.871	1.227	0.222	0.453	0.134	0.894	0.895
HGF	45	204.56	22	195.83	74	144.36	0.563	0.576	0.871	1.168	0.245	0.460	1.093	0.278	0.542
IFN-α	45	195.20 (159 to 223)	22	187.26 (170 to 215)	74	147.19 (118 to 175)	-1.160	0.251	0.871	2.164	0.033	0.220	4.108	< 0.0001	0.003
IFN-γ	45	1.27	22	1.79	74	2.03	-0.120	0.905	0.905	1.074	0.285	0.503	1.138	0.258	0.542
IL-10	45	60.54	22	109.09	74	49.85	0.200	0.842	0.905	0.700	0.486	0.658	1.602	0.113	0.338
IL-12	45	103.46	22	118.02	74	99.75	-0.463	0.645	0.871	1.511	0.134	0.386	2.415	0.018	0.183
IL-13	45	18.76	22	16.78	74	14.37	0.923	0.360	0.871	1.866	0.065	0.253	1.636	0.105	0.338
IL-15	45	153.39	22	179.80	74	141.34	0.571	0.570	0.871	0.588	0.557	0.697	0.155	0.877	0.895
IL-17	45	4.85	22	4.47	74	0.00	-0.432	0.667	0.871	0.745	0.458	0.658	2.265	0.026	0.183
IL-1β	45	31.66	22	34.53	74	22.51	0.747	0.266	0.871	1.865	0.065	0.253	1.018	0.311	0.542
IL-1RA	45	1230.51	22	1363.59	74	1027.10	1.122	0.458	0.871	0.503	0.616	0.711	0.956	0.342	0.542
IL-2	45	3.88	22	2.96	74	0.00	0.198	0.844	0.905	1.370	0.173	0.433	2.124	0.037	0.183
IL-2R	45	248.18	22	262.19	74	213.99	-1.318	0.193	0.871	-0.350	0.727	0.779	0.546	0.586	0.765
IL-4	45	16.25	22	18.30	74	10.45	0.471	0.639	0.871	2.581	0.011	0.168	2.283	0.025	0.183
IL-5	45	2.12	22	3.67	74	1.29	-0.310	0.757	0.905	2.115	0.037	0.220	1.780	0.079	0.295
IL-6	45	892.34	22	1038.67	74	837.29	0.129	0.898	0.905	1.216	0.226	0.453	1.241	0.218	0.542
IL-7	45	46.30	22	41.97	74	31.81	0.495	0.622	0.871	0.739	0.462	0.658	1.869	0.065	0.279
IL-8 [2]	43	5601.22	22	5404.29	73	5005.64	0.720	0.475	0.871	0.695	0.488	0.658	0.132	0.895	0.895
IP-10	45	58.49	22	79.45	74	57.32	-0.141	0.888	0.905	0.251	0.802	0.830	0.953	0.343	0.542
MCP-1 [2]	43	16793.83	21	14590.31	66	15785.06	0.708	0.482	0.871	0.430	0.668	0.742	-0.364	0.717	0.886
MIG	45	52.50	22	43.42	74	49.43	-0.666	0.508	0.871	-1.848	0.067	0.253	-0.761	0.449	0.673
MIP-1α	45	316.83	22	182.38	74	231.05	0.876	0.384	0.871	1.480	0.142	0.386	0.724	0.471	0.673
MIP-1β	45	2304.20	22	2224.54	74	2139.83	0.578	0.566	0.871	1.666	0.099	0.329	0.992	0.324	0.542
RANTES [2]	29	6500.41	13	3828.21	65	2946.25	1.477	0.145	0.871	-0.543	0.588	0.705	-0.296	0.768	0.886
TNF-α	45	16.95	22	17.65	74	14.28	2.318	0.024	0.722	2.178	0.032	0.220	1.037	0.303	0.542
VEGF	45	80.93	22	68.94	74	63.48	1.104	0.274	0.871	0.669	0.505	0.658	0.679	0.499	0.680

Medians are given in pg/ml.

[2] For some analytes numbers were smaller due to mean fluorescence index (MFI) values exceeding the upper limit of detection, resulting in concentrations being unable to be calculated

The interquartile range (IQR) is only stated where statistical significant difference was evident ($p < 0.05$, $q < 0.1$)

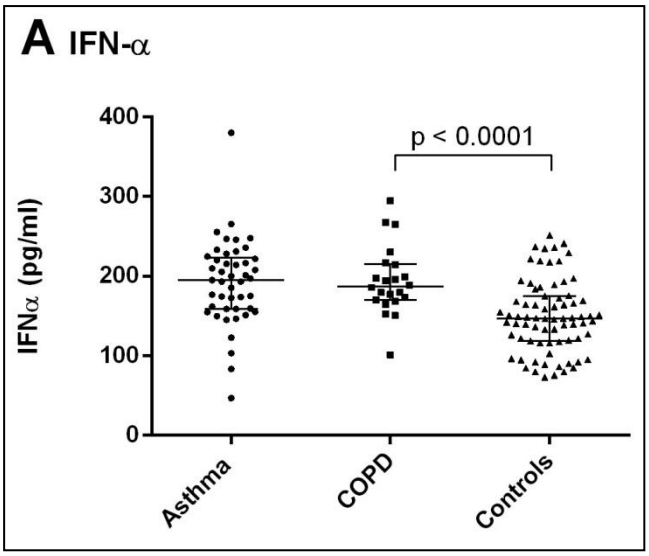


Figure 8.8: Cross-sectional comparison of inflammatory mediators released in response to Pam3CSK4 stimulation in the blood of controls, and those with asthma and COPD. Peripheral whole blood was collected and stimulated with Pam3CSK4 for 24 hours, before supernatant aspiration and analysis by multiplex ELISA (n = 45 for asthma, n = 22 for COPD and n = 74 for controls). Data are represented as scatter plots with median + IQR, with p-values calculated using two-group comparisons between each clinical group, with the model including adjustment for age, sex, baseline 25(OH)D status, ethnicity, smoking status and vaccination record, using the programme Qlucore Omics Explorer 2.3. P-values were deemed significant when less than 0.05, with a corresponding q-value of less than 0.1. Any values under the limit of detection (as specified within the product data sheet for each analyte) were given a value of 0.

8.2.6 Analysis of the secretion of inflammatory mediators in the blood following PolyI:C stimulation shows differences between asthma, COPD and controls

Peripheral blood samples were stimulated with PolyI:C, a synthetic analogue of dsRNA which is a ligand for TLR 3, and typically associated with viruses such as influenza and rhinovirus. Qlucore Omics Explorer 2.3 was used to carry out comparisons between the three clinical groups, modelling using a two-group comparison (i.e. Student's T-Test) between each group with log-transformed data, and including adjustment for age, sex, baseline 25(OH)D concentration, ethnicity, smoking status and vaccination record. All results obtained from 30-plex ELISA analysis of supernatants from stimulated whole blood are presented in Table 8.7, and significant results are presented in Figure 8.9. When compared to controls, the concentrations of PolyI:C-stimulated TNF-α (asthma p <

0.0001; COPD p = 0.005), MIP-1β (asthma p = 0.004; COPD p = 0.003), IL-6 (asthma p = 0.007; COPD p = 0.002), VEGF (asthma p = 0.02; COPD p = 0.005), MIP-1α (asthma p = 0.02; COPD p = 0.003) and IL-15 (asthma p = 0.04; COPD p < 0.0001) were higher in both asthma and COPD patients respectively, while the concentration of eotaxin (asthma p = 0.0009; COPD p = 0.03) was lower in both. The concentrations of PolyI:C-stimulated G-CSF (p = 0.003), IL-2 (p = 0.01), HGF (p = 0.02), IL-1β (p = 0.03), IL-7 (p = 0.03) and FGF-Basic (p = 0.04) were all higher in asthma patients compared to controls, while the concentrations of IL-17 (p = 0.0006), IP-10 (p = 0.001), IL-1RA (p = 0.004), IFN-γ (p = 0.007) and MIG (p = 0.03) were higher in COPD patients compared to controls. There was no difference in the inflammatory response to PolyI:C when comparing patients with asthma and COPD, with all of the remaining inflammatory mediators assayed displaying no statistically significant differences between controls, asthmatics, and COPD patients (Table 8.7).

Table 8.7: Comparison of inflammatory mediators released following whole blood stimulation by PolyI:C in controls and patients with asthma or COPD

Analytes	Asthma		COPD		Controls		Statistics: Asthma vs. COPD			Statistics: Asthma vs. Controls			Statistics: COPD vs. Controls		
	n	median (IQR)	n	median (IQR)	n	median (IQR)	T-Statistic	P-Value	Q-Value	T-Statistic	P-Value	Q-Value	T-Statistic	P-Value	Q-Value
EGF	44	44.03	22	47.01	74	25.14	-1.565	0.123	0.605	-1.866	0.065	0.112	0.031	0.976	0.976
Eotaxin	44	20.09 (11 to 34)	22	21.80 (14 to 36)	74	19.10 (14 to 26)	-1.327	0.190	0.605	-3.411	0.0009	0.014	-2.248	0.027	0.074
FGF-Basic	44	40.00 (21 to 86)	22	22.24 (17 to 34)	74	23.59 (15 to 36)	1.113	0.270	0.676	2.093	0.039	0.091	0.837	0.405	0.552
G-CSF	44	98.25 (76 to 152)	22	70.15 (58 to 88)	74	57.02 (43 to 74)	0.966	0.338	0.781	3.015	0.003	0.030	0.715	0.476	0.596
GM-CSF	44	2.64	22	2.77	74	3.45	-0.159	0.874	0.904	0.460	0.646	0.746	1.077	0.285	0.451
HGF	44	163.50 (140 to 207)	22	151.38 (113 to 186)	74	105.96 (76 to 142)	0.480	0.633	0.829	2.391	0.019	0.062	1.676	0.097	0.172
IFN-α	44	170.76	22	158.30	74	110.45	-1.291	0.202	0.605	1.194	0.235	0.353	1.913	0.059	0.127
IFN-γ	44	2.30 (0 to 9)	22	5.89 (2 to 7)	74	1.59 (0 to 4)	0.013	0.990	0.990	1.849	0.067	0.112	2.758	0.007	0.021
IL-10	44	5.54	22	6.31	74	4.60	0.496	0.622	0.829	0.340	0.735	0.816	0.342	0.733	0.785
IL-12	44	80.70	22	86.99	74	71.21	0.502	0.617	0.829	1.902	0.060	0.112	0.981	0.330	0.494
IL-13	44	10.67	22	7.93	74	7.96	0.743	0.460	0.829	0.065	0.948	0.948	-0.881	0.381	0.544
IL-15	44	272.01 (183 to 472)	22	471.04 (270 to 644)	74	209.40 (128 to 326)	-2.347	0.022	0.224	2.087	0.039	0.091	4.568	< 0.0001	0.000
IL-17	44	2.87 (0 to 5)	22	2.69 (2 to 5)	74	0.99 (0 to 2)	-1.856	0.069	0.515	-0.870	0.386	0.552	3.546	0.0006	0.010
IL-1β	44	14.78 (8 to 23)	22	12.44 (8 to 16)	74	5.36 (0 to 11)	0.679	0.500	0.829	2.198	0.030	0.089	1.972	0.052	0.120
IL-1RA	44	1379.58 (872 to 1978)	22	1787.92 (1103 to 2645)	74	859.67 (550 to 1290)	-1.334	0.187	0.605	1.886	0.062	0.112	2.978	0.004	0.016
IL-2	44	3.23 (0 to 6)	22	2.15 (0 to 3)	74	1.10 (0 to 2)	1.167	0.248	0.676	2.474	0.015	0.062	1.836	0.070	0.140
IL-2R	44	216.70	22	232.05	74	184.72	0.377	0.707	0.849	-0.177	0.860	0.890	0.434	0.665	0.745
IL-4	44	11.89	22	10.79	74	8.58	-0.319	0.751	0.855	1.391	0.167	0.264	1.765	0.081	0.152
IL-5	44	1.10	22	1.80	74	0.86	-0.294	0.769	0.855	-0.484	0.629	0.746	-0.427	0.670	0.745
IL-6	44	77.93 (39 to 191)	22	71.55 (32 to 102)	74	29.68 (16 to 56)	0.656	0.514	0.829	2.729	0.007	0.044	3.260	0.002	0.012
IL-7	44	23.56 (15 to 40)	22	12.29 (0 to 26)	74	12.77 (0 to 17)	1.715	0.092	0.550	2.164	0.033	0.089	1.075	0.286	0.451
IL-8	44	316.38	22	273.47	74	329.32	-0.438	0.663	0.829	-0.527	0.599	0.746	-0.653	0.516	0.619
IP-10 [2]	38	510.71 (326 to 943)	16	1358.87 (393 to 2253)	70	303.07 (148 to 720)	-2.504	0.015	0.224	0.741	0.461	0.601	3.407	0.001	0.010
MCP-1 [2]	43	11602.44	21	10567.75	72	9940.73	0.749	0.457	0.829	0.801	0.425	0.579	0.171	0.864	0.894
MIG	44	56.42 (45 to 81)	22	55.94 (44 to 87)	74	45.52 (32 to 66)	0.475	0.637	0.829	2.005	0.047	0.102	2.155	0.034	0.085
MIP-1α	44	80.45 (58 to 194)	22	67.01 (50 to 79)	74	46.99 (39 to 61)	0.498	0.620	0.829	2.402	0.018	0.062	3.017	0.003	0.016
MIP-1β [2]	44	838.91 (517 to 2032)	22	718.37 (311 to 1139)	73	438.11 (259 to 664)	0.592	0.556	0.829	2.944	0.004	0.030	3.076	0.003	0.016
RANTES [2]	42	2658.54	22	2177.06	70	1346.68	1.467	0.148	0.605	0.272	0.786	0.842	-0.760	0.449	0.586
TNF-α	44	11.05 (8 to 16)	22	8.06 (7 to 12)	74	5.17 (0 to 8)	2.765	0.008	0.224	4.120	< 0.0001	0.002	2.885	0.005	0.016
VEGF	44	41.17 (30 to 55)	22	34.83 (28 to 44)	74	24.99 (19 to 31)	0.241	0.810	0.868	2.413	0.017	0.062	2.892	0.005	0.016

Medians are given in pg/ml.

[2] For some analytes numbers were smaller due to mean fluorescence index (MFI) values exceeding the upper limit of detection, resulting in concentrations being unable to be calculated

The interquartile range (IQR) is only stated where statistical significant difference was evident ($p < 0.05$, $q < 0.1$)

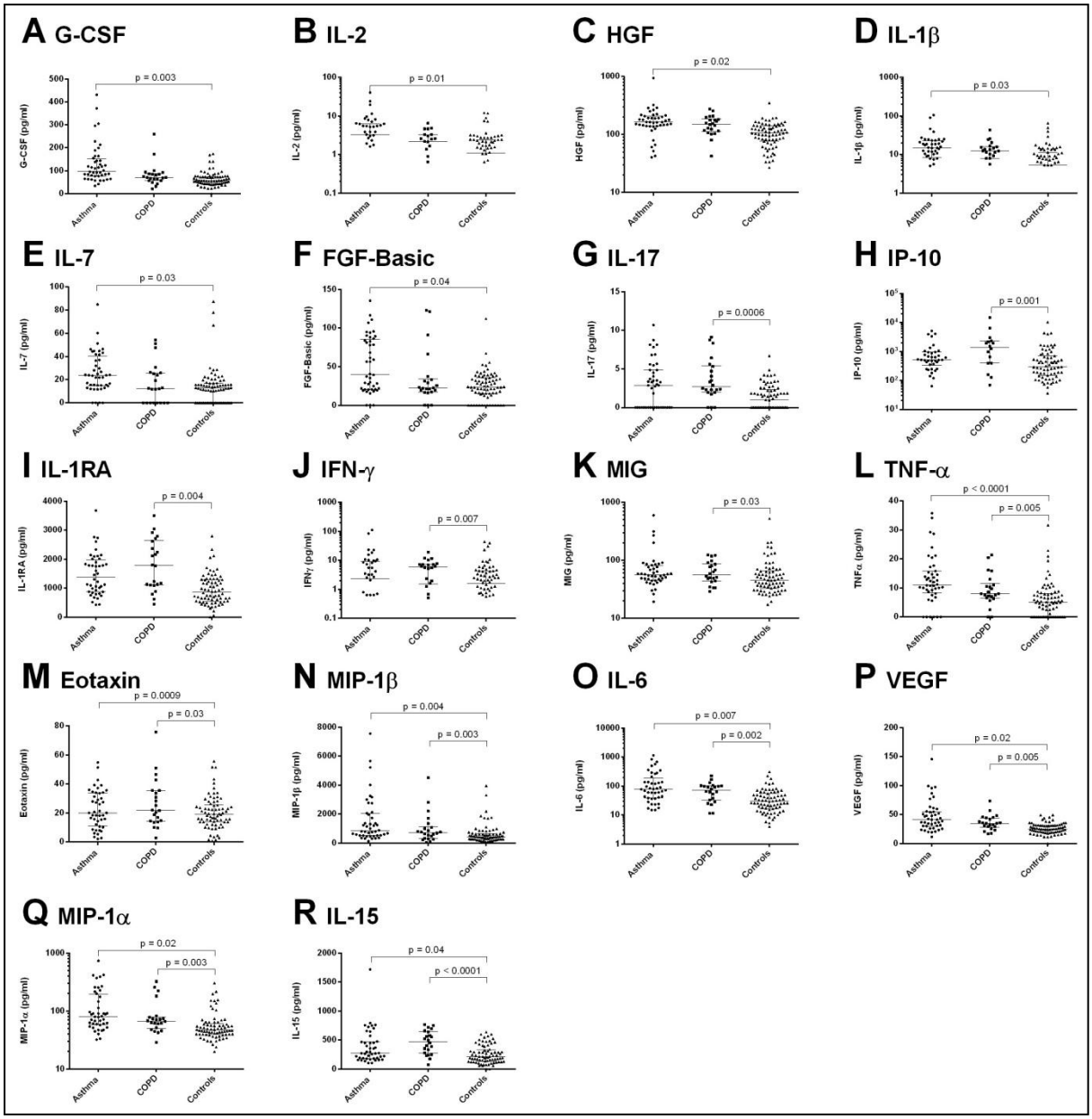


Figure 8.9: Cross-sectional comparison of inflammatory mediators released in response to PolyI:C stimulation in the blood of controls, and those with asthma and COPD. Peripheral whole blood was collected and stimulated with PolyI:C for 24 hours, before supernatant aspiration and analysis by multiplex ELISA (n = 44 for asthma, n = 22 for COPD and n = 74 for controls). Data are represented as scatter plots with median + IQR, with p-values calculated using two-group comparisons between each clinical group, with the model including adjustment for age, sex, baseline 25(OH)D status, ethnicity, smoking status and vaccination record, using the programme Qlucore Omics Explorer 2.3. P-values were deemed significant when less than 0.05, with a corresponding q-value of less than 0.1. Any values under the limit of detection (as specified within the product data sheet for each analyte) were given a value of 0.

8.2.7 Analysis of the secretion of inflammatory mediators in the blood following R848 stimulation shows differences between asthma, COPD and controls

Peripheral blood samples were stimulated with R848, an imidazoquinoline compound which mimics the ssRNA activation of TLR 7/8, and is typically associated with viruses such as RSV and rhinovirus. Qlucore Omics Explorer 2.3 was used to carry out comparisons between the three clinical groups, modelling using a two-group comparison (i.e. Student's T-Test) between each group with log-transformed data, and including adjustment for age, sex, baseline 25(OH)D concentration, ethnicity, smoking status and vaccination record. All results from 30-plex ELISA analysis of supernatants from stimulated whole blood are presented in Table 8.8, and significant results are presented in Figure 8.10. When compared to controls, the concentration of R848-stimulated IFN-α (asthma $p = 0.0004$; COPD $p = 0.0002$) was higher in both asthma and COPD patients, while the concentrations of R848-stimulated eotaxin (asthma $p < 0.0001$; COPD $p = 0.0007$) and IL-8 (asthma $p = 0.004$; COPD $p = 0.002$,) were lower in both. The concentrations of R848-stimulated IL-4 ($p < 0.0001$), IL-7 ($p = 0.0001$), IL-10 ($p = 0.0007$), HGF ($p = 0.009$), VEGF ($p = 0.01$), IL-15 ($p = 0.01$), IL-2R ($p = 0.01$), IL-1RA ($p = 0.01$), FGF-Basic ($p = 0.02$), IL-6 ($p= 0.02$), IL-17 ($p = 0.02$), IL-12 ($p = 0.03$), IL-2 ($p = 0.03$) and MIG ($p = 0.04$) were all higher in COPD patients compared to controls. In a comparison of the immune response to R848 in patients with asthma or COPD, concentrations of FGF-Basic ($p = 0.0007$), IL-12 ($p = 0.001$), IL-2R ($p = 0.002$), IL-1RA ($p = 0.005$), EGF ($p = 0.01$) and IL-15 ($p = 0.02$) were all higher in COPD. The remaining inflammatory mediators assayed displayed no statistically significant differences between controls, asthmatics, and COPD patients (Table 8.8).

Table 8.8: Comparison of inflammatory mediators released following whole blood stimulation by R848 in controls and patients with asthma or COPD

Analytes	Asthma		COPD		Controls		Statistics: Asthma vs. COPD			Statistics: Asthma vs. Controls			Statistics: COPD vs. Controls		
	n	median (IQR)	n	median (IQR)	n	median (IQR)	T-Statistic	P-Value	Q-Value	T-Statistic	P-Value	Q-Value	T-Statistic	P-Value	Q-Value
EGF	44	73.86 (47 to 99)	22	92.54 (69 to 101)	74	69.61 (50 to 82)	-2.666	0.010	0.060	-2.105	0.038	0.226	0.834	0.407	0.488
Eotaxin	44	55.32 (37 to 87)	22	56.35 (37 to 86)	74	57.50 (41 to 73)	0.344	0.732	0.842	-5.443	< 0.0001	< 0.0001	-3.508	0.0007	0.004
FGF-Basic	44	101.15 (73 to 135)	22	144.98 (95 to 172)	74	100.70 (58 to 129)	-3.568	0.0007	0.016	-1.189	0.237	0.599	2.437	0.017	0.038
G-CSF	44	257.10	22	224.18	74	208.00	-0.935	0.353	0.707	-0.476	0.635	0.866	0.722	0.472	0.525
GM-CSF	44	4.81	22	4.91	74	5.62	0.754	0.454	0.800	-0.900	0.370	0.653	-1.053	0.295	0.422
HGF	44	279.09 (230 to 336)	22	310.85 (239 to 339)	74	238.32 (165 to 277)	-1.522	0.134	0.343	0.194	0.846	0.919	2.662	0.009	0.035
IFN-α	44	340.28 (269 to 517)	22	404.20 (220 to 741)	74	224.87 (173 to 302)	-0.477	0.635	0.805	3.632	0.0004	0.006	3.945	0.0002	0.002
IFN-γ	44	158.71	22	244.87	74	245.02	-1.507	0.137	0.343	-0.359	0.721	0.919	1.390	0.168	0.265
IL-10	44	901.80 (710 to 1297)	22	1251.87 (975 to 1781)	74	860.48 (598 to 1171)	-1.612	0.113	0.338	0.251	0.802	0.919	3.539	0.0007	0.004
IL-12 [2]	44	2935.77 (2307 to 4500)	20	5151.80 (2997 to 7582)	73	3223.77 (1883 to 5185)	-3.366	0.001	0.016	-0.608	0.544	0.791	2.262	0.026	0.052
IL-13	44	33.31	22	34.26	74	31.67	-0.604	0.548	0.805	-1.120	0.265	0.599	0.069	0.945	0.945
IL-15	44	510.36 (405 to 764)	22	713.17 (537 to 865)	74	508.58 (366 to 662)	-2.484	0.016	0.080	-0.265	0.792	0.919	2.620	0.010	0.035
IL-17	44	8.77 (7 to 12)	22	10.88 (8 to 14)	74	7.53 (6 to 8)	-0.548	0.586	0.805	1.033	0.304	0.599	2.413	0.018	0.038
IL-1β	44	872.09	22	981.54	74	1083.61	-0.273	0.786	0.842	-1.133	0.260	0.599	-1.015	0.313	0.427
IL-1RA	44	8630.51 (7020 to 10586)	22	14060.36 (8775 to 19386)	74	7457.50 (4491 to 11475)	-2.894	0.005	0.040	-0.130	0.896	0.919	2.527	0.013	0.036
IL-2	44	7.94 (6 to 12)	22	6.83 (6 to 10)	74	6.50 (5 to 8)	-0.486	0.629	0.805	1.506	0.135	0.568	2.162	0.033	0.063
IL-2R	44	385.60 (327 to 454)	22	463.98 (415 to 562)	74	379.60 (308 to 444)	-3.325	0.002	0.016	-2.260	0.026	0.194	2.580	0.012	0.035
IL-4	44	25.65 (17 to 33)	22	31.02 (27 to 37)	74	20.96 (15 to 28)	-1.893	0.063	0.232	1.353	0.179	0.568	4.085	< 0.0001	0.002
IL-5	44	4.12	22	6.85	74	3.48	-1.850	0.069	0.232	0.102	0.919	0.919	1.230	0.222	0.333
IL-6 [2]	22	11806.63 (9276 to 25692)	5	14982.45 (10480 to 16302)	18	9698.23 (2527 to 11637)	-1.306	0.197	0.454	1.321	0.189	0.568	2.421	0.018	0.038
IL-7	44	101.02 (75 to 138)	22	99.18 (70 to 127)	74	70.03 (54 to 93)	-0.402	0.689	0.827	1.956	0.053	0.265	4.063	0.0001	0.002
IL-8 [2]	44	6786.75 (4562 to 10186)	22	6603.52 (3423 to 8473)	69	11492.30 (5299 to 15175)	-0.127	0.900	0.900	-2.920	0.004	0.043	-3.184	0.002	0.010
IP-10 [2]	22	1872.59	4	1426.50	40	1601.28	-1.248	0.217	0.465	0.251	0.803	0.919	0.919	0.361	0.451
MCP-1 [2]	43	15501.50	21	16122.43	60	18560.14	0.711	0.480	0.800	-0.713	0.477	0.791	-1.507	0.136	0.226
MIG	44	98.30 (74 to 124)	22	122.58 (63 to 172)	74	77.20 (64 to 167)	-0.793	0.431	0.800	0.675	0.501	0.791	2.017	0.047	0.083
MIP-1α [2]	43	11001.39	21	11368.48	72	10102.12	0.181	0.857	0.887	0.594	0.554	0.791	-0.767	0.445	0.514
MIP-1β [2]	27	16185.49	13	10837.46	42	15251.93	0.465	0.644	0.805	1.412	0.161	0.568	-0.159	0.874	0.904
RANTES [2]	27	7205.98	15	4648.39	54	3350.26	0.274	0.785	0.842	1.033	0.304	0.599	0.934	0.353	0.451
TNF-α [2]	44	1736.70	22	2165.90	68	2421.03	-0.515	0.609	0.805	-1.000	0.319	0.599	-0.293	0.770	0.825
VEGF	44	318.25 (236 to 396)	22	382.63 (277 to 525)	74	285.04 (206 to 366)	-1.893	0.063	0.232	0.173	0.863	0.919	2.634	0.010	0.035

Medians are given in pg/ml.

[2] For some analytes numbers were smaller due to mean fluorescence index (MFI) values exceeding the upper limit of detection, resulting in concentrations being unable to be calculated

The interquartile range (IQR) is only stated where statistical significant difference was evident ($p < 0.05$, $q < 0.1$)

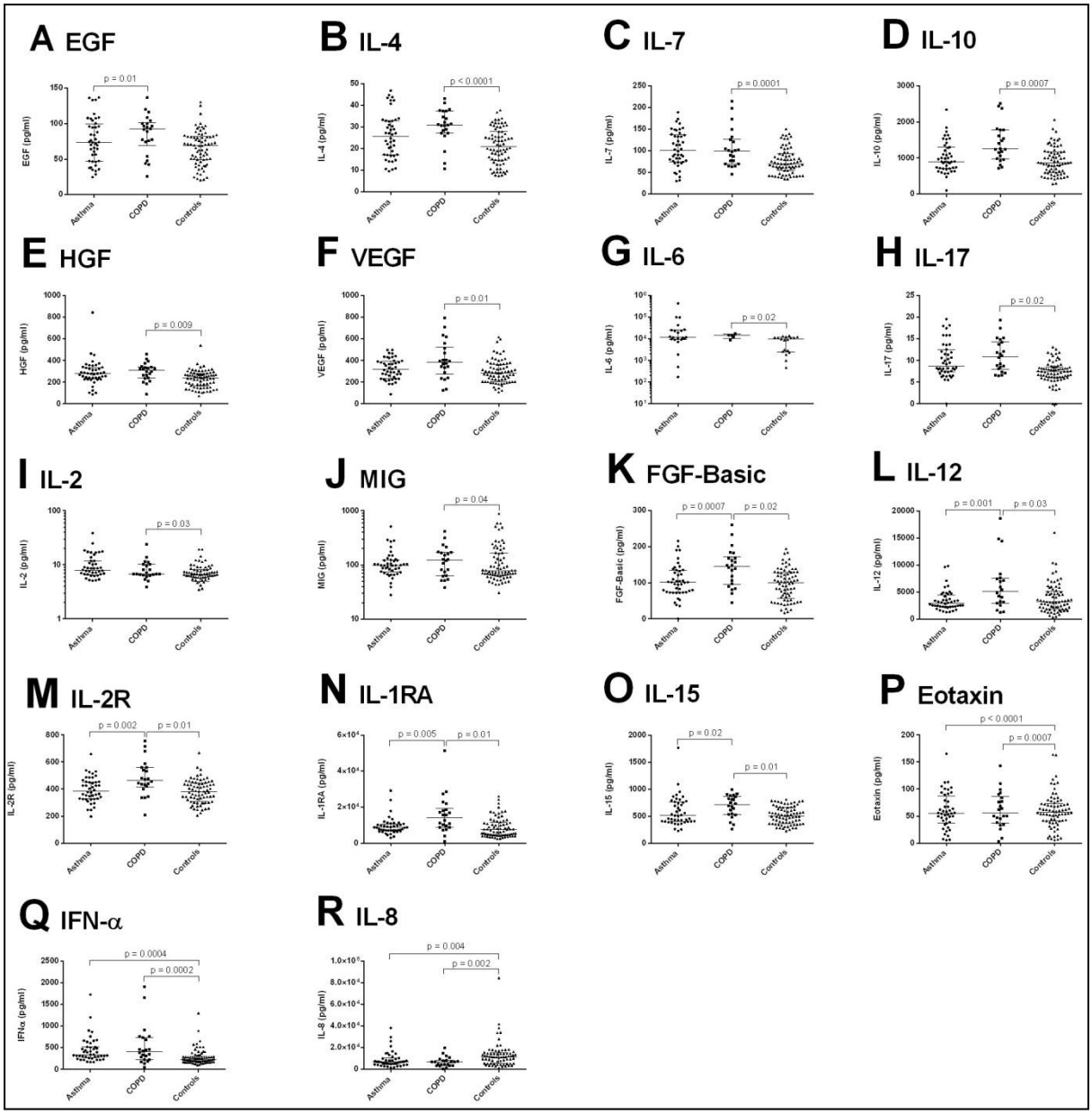


Figure 8.10: Cross-sectional comparison of inflammatory mediators released in response to R848 stimulation in the blood of controls, and those with asthma and COPD. Peripheral whole blood was collected and stimulated with R848 for 24 hours, before supernatant aspiration and analysis by multiplex ELISA (n = 44 for asthma, n = 22 for COPD and n = 74 for controls). Data are represented as scatter plots with median + IQR, with p-values calculated using two-group comparisons between each clinical group, with the model including adjustment for age, sex, baseline 25(OH)D status, ethnicity, smoking status and vaccination record, using the programme Qlucore Omics Explorer 2.3. P-values were deemed significant when less than 0.05, with a corresponding q-value of less than 0.1. Any values under the limit of detection (as specified within the product data sheet for each analyte) were given a value of 0.

8.2.8 Analysis of the secretion of inflammatory mediators in the blood following RV-16 stimulation shows differences between asthma, COPD and controls

Peripheral blood samples were stimulated with RV-16, a major type rhinovirus, with infection causing diseases such as the common cold. Qlucore Omics Explorer 2.3 was used to carry out comparisons between the three clinical groups, modelling using a two-group comparison (i.e. Student's T-Test) between each group with log-transformed data, and including adjustment for age, sex, baseline 25(OH)D concentration, ethnicity, smoking status and vaccination record. All results obtained from 30-plex ELISA analysis of supernatants from stimulated whole blood are presented in Table 8.9, and significant results are presented in Figure 8.11. When compared to controls, the concentrations of RV16-stimulated G-CSF (asthma $p < 0.0001$; COPD $p = 0.0003$) and IFN-α (asthma $p < 0.0001$; COPD $p = 0.0002$) were higher in both asthma and COPD patients, while the concentrations of RV16-stimulated IL-2R (asthma $p = 0.002$; COPD $p = 0.01$) and VEGF (asthma $p = 0.01$; COPD $p < 0.0001$) were lower in both. The concentration of RV16-stimulated EGF was lower in asthmatics compared to controls ($p = 0.005$), while concentrations of RV16-stimulated MIG ($p < 0.0001$), IL-17 ($p < 0.0001$), IFN-γ ($p < 0.0001$), IL-1RA ($p < 0.0001$), IL-7 ($p = 0.0002$), IL-4 ($p = 0.0004$), IL-6 ($p = 0.002$), FGF-basic ($p = 0.006$), MIP-1β ($p = 0.006$), HGF ($p = 0.01$), IL-2 ($p = 0.02$) and IL-15 ($p = 0.03$) were all higher in COPD patients compared to controls. RV16-stimulated eotaxin concentration was lower in COPD patients compared to controls. In a comparison of the immune response to RV-16 in patients with asthma or COPD, concentrations of IL-2R ($p = 0.002$), MIG ($p = 0.002$), IL-1RA ($p = 0.003$), IL-15 ($p = 0.004$), IFN-γ ($p = 0.004$) and FGF-Basic ($p = 0.01$) were all lower in asthma compared to COPD. The remaining inflammatory mediators assayed displayed no statistically significant differences between controls, asthmatics, and COPD patients (Table 8.9).

Table 8.9: Comparison of inflammatory mediators released following whole blood stimulation by RV-16 in controls and patients with asthma or COPD

Analytes	Asthma		COPD		Controls		Statistics: Asthma vs. COPD			Statistics: Asthma vs. Controls			Statistics: COPD vs. Controls		
	n	median (IQR)	n	median (IQR)	n	median (IQR)	T-Statistic	P-Value	Q-Value	T-Statistic	P-Value	Q-Value	T-Statistic	P-Value	Q-Value
EGF	44	68.01 (37 to 89)	22	74.08 (47 to 87)	38	68.59 (61 to 79)	-1.669	0.101	0.336	-2.866	0.005	0.037	-1.178	0.242	0.382
Eotaxin	44	54.03 (37 to 75)	22	53.27 (36 to 65)	38	42.25 (27 to 65)	1.295	0.200	0.501	-1.714	0.089	0.304	-2.932	0.004	0.012
FGF-Basic	44	54.00 (45 to 66)	22	61.81 (45 to 72)	38	48.21 (42 to 58)	-2.566	0.013	0.065	-1.470	0.144	0.343	2.809	0.006	0.015
G-CSF	44	161.88 (124 to 185)	22	122.85 (106 to 186)	38	91.68 (77 to 110)	-0.255	0.800	0.849	4.616	< 0.0001	0.0002	3.803	0.0003	0.001
GM-CSF	44	3.71	22	4.07	38	4.51	0.446	0.657	0.789	0.625	0.533	0.692	-0.473	0.637	0.797
HGF	44	236.76 (200 to 292)	22	240.04 (191 to 305)	38	194.04 (174 to 232)	-0.942	0.350	0.567	1.064	0.290	0.511	2.558	0.012	0.026
IFN-α [2]	44	952.33 (567 to 1288)	19	750.82 (355 to 1489)	37	404.26 (282 to 693)	-1.103	0.275	0.567	4.614	< 0.0001	0.0002	3.838	0.0002	0.001
IFN-γ	44	81.39 (51 to 177)	22	159.48 (39 to 358)	38	35.13 (11 to 77)	-2.964	0.004	0.027	1.378	0.171	0.366	4.850	< 0.0001	< 0.0001
IL-10	44	113.35	22	132.10	38	69.34	-0.240	0.811	0.849	-1.455	0.148	0.343	-0.054	0.957	0.957
IL-12	44	232.61	22	202.87	38	186.07	-0.978	0.332	0.567	-0.560	0.577	0.692	0.980	0.330	0.430
IL-13	44	24.31	22	24.27	38	24.63	-0.871	0.388	0.581	-0.563	0.575	0.692	0.202	0.840	0.938
IL-15	44	463.80 (365 to 783)	22	682.63 (514 to 823)	38	640.29 (578 to 698)	-3.007	0.004	0.027	-1.986	0.050	0.212	2.146	0.035	0.061
IL-17	44	6.59 (5 to 9)	22	7.01 (6 to 9)	38	4.80 (4 to 6)	-0.945	0.349	0.567	1.220	0.225	0.450	5.512	< 0.0001	< 0.0001
IL-1β	44	68.35	22	71.67	38	44.58	-0.756	0.453	0.647	-0.724	0.471	0.692	1.066	0.289	0.395
IL-1RA	44	3574.65 (2557 to 4269)	22	4691.30 (3650 to 5651)	38	3263.91 (2539 to 3639)	-3.156	0.003	0.026	-0.156	0.876	0.906	4.847	< 0.0001	< 0.0001
IL-2	44	15.64 (9 to 26)	22	13.13 (11 to 21)	38	10.83 (7 to 16)	-0.694	0.490	0.651	1.518	0.132	0.343	2.416	0.018	0.033
IL-2R	44	302.60 (247 to 388)	22	356.90 (313 to 480)	38	321.23 (272 to 372)	-3.273	0.002	0.026	-3.112	0.002	0.024	2.527	0.013	0.027
IL-4	44	21.44 (15 to 28)	22	26.52 (20 to 29)	38	23.11 (17 to 24)	-2.006	0.050	0.213	1.170	0.245	0.459	3.665	0.0004	0.001
IL-5	44	2.98	22	5.10	38	3.44	-1.478	0.145	0.435	-0.889	0.376	0.593	0.205	0.838	0.938
IL-6 [2]	42	2760.60 (1243 to 3613)	14	2558.57 (1327 to 3113)	28	1169.46 (612 to 1901)	-1.238	0.221	0.510	0.675	0.501	0.692	3.096	0.003	0.008
IL-7	44	68.39 (49 to 100)	22	64.23 (53 to 96)	38	51.53 (40 to 57)	-0.346	0.731	0.843	2.239	0.027	0.136	3.936	0.0002	0.0008
IL-8 [2]	41	5980.31	22	6407.76	33	6698.36	-0.680	0.499	0.651	-1.012	0.314	0.523	0.085	0.932	0.957
IP-10 [2]	12	2261.87	2	1091.43	8	2294.33	-1.344	0.184	0.501	-0.254	0.800	0.889	0.189	0.850	0.938
MCP-1 [2]	42	16679.35	21	17317.05	30	19861.51	0.227	0.821	0.849	-0.110	0.912	0.912	-0.157	0.876	0.938
MIG	44	93.66 (78 to 170)	22	148.94 (87 to 240)	38	60.55 (42 to 82)	-3.222	0.002	0.026	1.552	0.124	0.343	5.787	< 0.0001	< 0.0001
MIP-1α	44	1203.61	22	1952.74	38	823.33	-0.956	0.343	0.567	-0.396	0.693	0.800	1.143	0.256	0.384
MIP-1β [2]	41	3839.47 (2645 to 5817)	22	6533.25 (2900 to 14386)	36	2931.03 (1803 to 4941)	-1.729	0.089	0.335	0.636	0.526	0.692	2.790	0.006	0.015
RANTES [2]	28	10846.59	12	6339.76	17	5536.69	-0.077	0.939	0.939	1.704	0.091	0.304	1.111	0.270	0.385
TNF-α	44	61.98	22	77.79	38	36.53	-0.925	0.359	0.567	-0.174	0.862	0.906	1.857	0.067	0.111
VEGF	44	150.32 (104 to 179)	22	141.59 (99 to 197)	38	86.85 (72 to 109)	-0.613	0.543	0.678	2.584	0.011	0.066	4.534	< 0.0001	0.0001

Medians are given in pg/ml.

[2] For some analytes numbers were smaller due to mean fluorescence index (MFI) values exceeding the upper limit of detection, resulting in concentrations being unable to be calculated

The interquartile range (IQR) is only stated where statistical significant difference was evident ($p < 0.05$, $q < 0.1$)

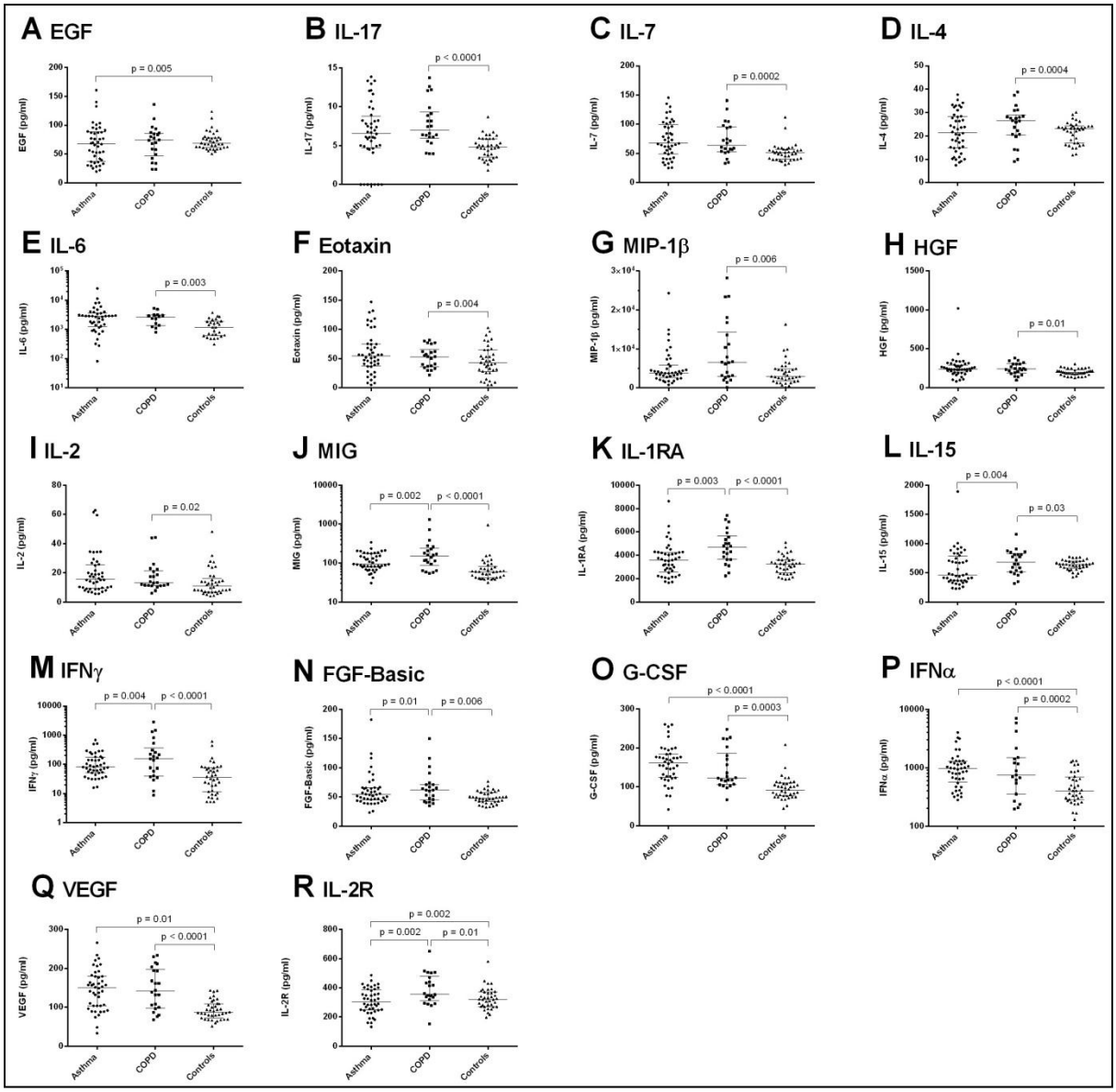


Figure 8.11: Cross-sectional comparison of inflammatory mediators released in response to RV-16 infection in the blood of controls, and those with asthma and COPD. Peripheral whole blood was collected and stimulated with RV-16 for 24 hours, before supernatant aspiration and analysis by multiplex ELISA (n = 44 for asthma, n = 22 for COPD and n = 38 for controls). Data are represented as median + IQR, with p-values calculated using two-group comparisons between each clinical group, with the model including adjustment for age, sex, baseline 25(OH)D status, ethnicity, smoking status and vaccination record, using the programme Qlucore Omics Explorer 2.3. P-values were deemed significant when less than 0.05, with a corresponding q-value of less than 0.1. Any values under the limit of detection (as specified within the product data sheet for each analyte) were given a value of 0.

8.2.9 Analysis of the secretion of inflammatory mediators in the blood following RV-1B stimulation shows differences between asthma, COPD and controls

Peripheral blood samples were stimulated with RV-1B, a minor type rhinovirus, with infection causing diseases such as the common cold. Qlucore Omics Explorer 2.3 was used to carry out comparisons between the three clinical groups, modelling using a two-group comparison (i.e. Student's T-Test) between each group with log-transformed data, and including adjustment for age, sex, baseline 25(OH)D concentration, ethnicity, smoking status and vaccination record. All results obtained from 30-plex ELISA analysis of supernatants from stimulated whole blood are presented in Table 8.10, and significant results are presented in Figure 8.12. When compared to controls, the concentration of RV1B-stimulated G-CSF (asthma $p = 0.0006$; COPD $p < 0.0001$) was higher in both asthma and COPD patients, while the concentration of RV1B-stimulated eotaxin (asthma $p = 0.0002$; COPD $p = 0.001$) was lower in both. The concentrations of RV1B-stimuated EGF ($p = 0.002$) and IL-2R ($p = 0.002$) were lower in asthmatics compared to controls, while concentrations of RV1B-stimulated MIG ($p < 0.0001$), IL-1RA ($p < 0.0001$), IL-17 ($p < 0.0001$), IL-4 ($p < 0.0001$), IFN-γ ($p < 0.0001$), IL-7 ($p < 0.0001$), VEGF ($p < 0.0001$), HGF ($p = 0.002$), IL-15 ($p = 0.006$), MIP-1β ($p = 0.02$), FGF-Basic ($p = 0.04$), IL-12 ($p = 0.04$), IFN-α ($p = 0.04$) were all higher in COPD patients compared to controls. In a comparison of the immune response to RV-1B in patients with asthma or COPD, concentrations of IL-2R ($p = 0.0008$), IL-15 ($p = 0.001$), MIG ($p = 0.002$), IL-1RA ($p = 0.003$), IFN-γ ($p = 0.008$), FGF-Basic ($p = 0.009$) and IL-4 ($p = 0.02$) were all lower in asthma compared to COPD. The remaining inflammatory mediators assayed displayed no statistically significant differences between controls, asthmatics, and COPD patients (Table 8.10).

Table 8.10: Comparison of inflammatory mediators released following whole blood stimulation by RV-1B in controls and patients with asthma or COPD

Analytes	Asthma		COPD		Controls		Statistics: Asthma vs. COPD			Statistics: Asthma vs. Controls			Statistics: COPD vs. Controls		
	n	median (IQR)	n	median (IQR)	n	median (IQR)	T-Statistic	P-Value	Q-Value	T-Statistic	P-Value	Q-Value	T-Statistic	P-Value	Q-Value
EGF	44	61.69 (41 to 82)	22	70.80 (45 to 89)	38	70.83 (62 to 79)	-1.820	0.074	0.203	-3.209	0.002	0.016	-0.931	0.355	0.507
Eotaxin	44	54.14 (31 to 79)	22	54.61 (37 to 68)	38	45.01 (29 to 61)	0.516	0.608	0.730	-3.799	0.0002	0.007	-3.293	0.001	0.005
FGF-Basic	44	55.67 (47 to 74)	22	60.96 (49 to 100)	38	53.59 (45 to 63)	-2.726	0.009	0.043	-2.168	0.032	0.121	2.112	0.038	0.081
G-CSF	44	160.52 (130 to 200)	22	139.79 (118 to 188)	38	94.40 (80 to 120)	-0.973	0.335	0.502	3.524	0.0006	0.009	4.401	< 0.0001	0.0001
GM-CSF	44	4.14	22	4.65	38	5.18	-0.396	0.694	0.800	-0.474	0.637	0.955	0.604	0.547	0.657
HGF	44	231.20 (197 to 288)	22	249.98 (200 to 334)	38	204.15 (163 to 243)	-1.539	0.129	0.298	0.722	0.472	0.787	3.262	0.002	0.005
IFN-α [2]	44	952.76 (469 to 1449)	21	817.18 (353 to 2993)	38	516.87 (309 to 761)	-0.811	0.421	0.574	2.181	0.031	0.121	2.029	0.046	0.085
IFN-γ	44	126.71 (61 to 562)	22	211.51 (106 to 768)	38	49.79 (29 to 125)	-2.758	0.008	0.043	0.954	0.342	0.642	4.624	< 0.0001	< 0.0001
IL-10	44	176.30	22	117.78	38	117.52	-0.135	0.893	0.894	-0.364	0.716	0.978	-0.860	0.392	0.535
IL-12	44	182.30 (124 to 272)	22	195.04 (145 to 311)	38	156.33 (125 to 234)	-1.818	0.074	0.203	-0.068	0.946	0.979	2.075	0.041	0.082
IL-13	44	27.05	22	27.92	38	25.60	-1.189	0.239	0.449	-0.848	0.398	0.703	0.548	0.585	0.675
IL-15	44	494.18 (358 to 709)	22	730.22 (516 to 807)	38	623.62 (538 to 698)	-3.433	0.001	0.017	-1.789	0.076	0.254	2.816	0.006	0.016
IL-17	44	6.92 (5 to 9)	22	7.35 (6 to 9)	38	4.74 (4 to 6)	-1.296	0.200	0.401	1.343	0.182	0.435	5.316	< 0.0001	< 0.0001
IL-1β	44	88.51	22	96.79	38	65.07	-1.107	0.273	0.480	-0.532	0.596	0.941	0.347	0.729	0.781
IL-1RA	44	3324.90 (2651 to 3896)	22	4551.35 (3866 to 5079)	38	3165.34 (2415 to 3656)	-3.150	0.003	0.019	-0.022	0.982	0.982	5.550	< 0.0001	< 0.0001
IL-2	44	15.06	22	12.54	38	12.21	0.581	0.563	0.704	0.138	0.891	0.979	0.410	0.683	0.759
IL-2R	44	309.27 (263 to 371)	22	368.23 (312 to 487)	38	331.51 (291 to 385)	-3.523	0.0008	0.017	-3.154	0.002	0.016	1.907	0.060	0.106
IL-4	44	20.80 (15 to 31)	22	26.18 (22 to 32)	38	22.71 (18 to 26)	-2.401	0.020	0.084	1.109	0.270	0.578	4.695	< 0.0001	< 0.0001
IL-5	44	3.15	22	6.15	38	4.34	-1.930	0.059	0.195	-1.324	0.188	0.435	0.109	0.914	0.914
IL-6 [2]	39	3282.89	11	2668.43	26	1552.01	-1.711	0.092	0.231	-0.217	0.829	0.979	1.633	0.106	0.177
IL-7	44	70.64 (55 to 98)	22	72.38 (51 to 103)	38	53.57 (45 to 60)	-0.840	0.404	0.574	2.173	0.032	0.121	4.388	< 0.0001	0.0001
IL-8 [2]	42	7578.09	22	5155.16	34	6955.33	-0.133	0.894	0.894	-0.172	0.864	0.979	0.699	0.486	0.608
IP-10 [2]	18	3409.42	1	789.81	9	1743.69	-0.178	0.860	0.894	1.346	0.181	0.435	2.805	0.006	0.016
MCP-1 [2]	42	17090.98	21	17465.90	31	20618.11	-0.157	0.876	0.894	-0.100	0.920	0.979	-0.168	0.867	0.897
MIG	44	109.14 (80 to 175)	22	165.01 (97 to 273)	38	62.11 (47 to 89)	-3.229	0.002	0.019	1.695	0.093	0.279	6.750	< 0.0001	< 0.0001
MIP-1α	44	2174.72	22	2135.07	38	1496.78	-1.314	0.194	0.401	0.264	0.793	0.979	1.283	0.203	0.320
MIP-1β [2]	40	4339.66 (2463 to 6995)	21	7196.90 (4208 to 8883)	35	3194.40 (1816 to 6840)	-2.040	0.046	0.172	0.975	0.332	0.642	2.322	0.023	0.052
RANTES [2]	25	7427.55	12	4547.94	22	5386.97	0.609	0.545	0.704	0.363	0.717	0.978	0.704	0.484	0.608
TNF-α	44	74.64	22	85.97	38	54.32	-1.072	0.288	0.480	0.191	0.849	0.979	0.979	0.330	0.496
VEGF	44	152.68 (101 to 192)	22	130.22 (111 to 208)	38	95.16 (76 to 123)	-1.013	0.315	0.498	2.218	0.029	0.121	4.157	< 0.0001	0.0003

Medians are given in pg/ml.

[2] For some analytes numbers were smaller due to mean fluorescence index (MFI) values exceeding the upper limit of detection, resulting in concentrations being unable to be calculated

The interquartile range (IQR) is only stated where statistical significant difference was evident ($p < 0.05$, $q < 0.1$)

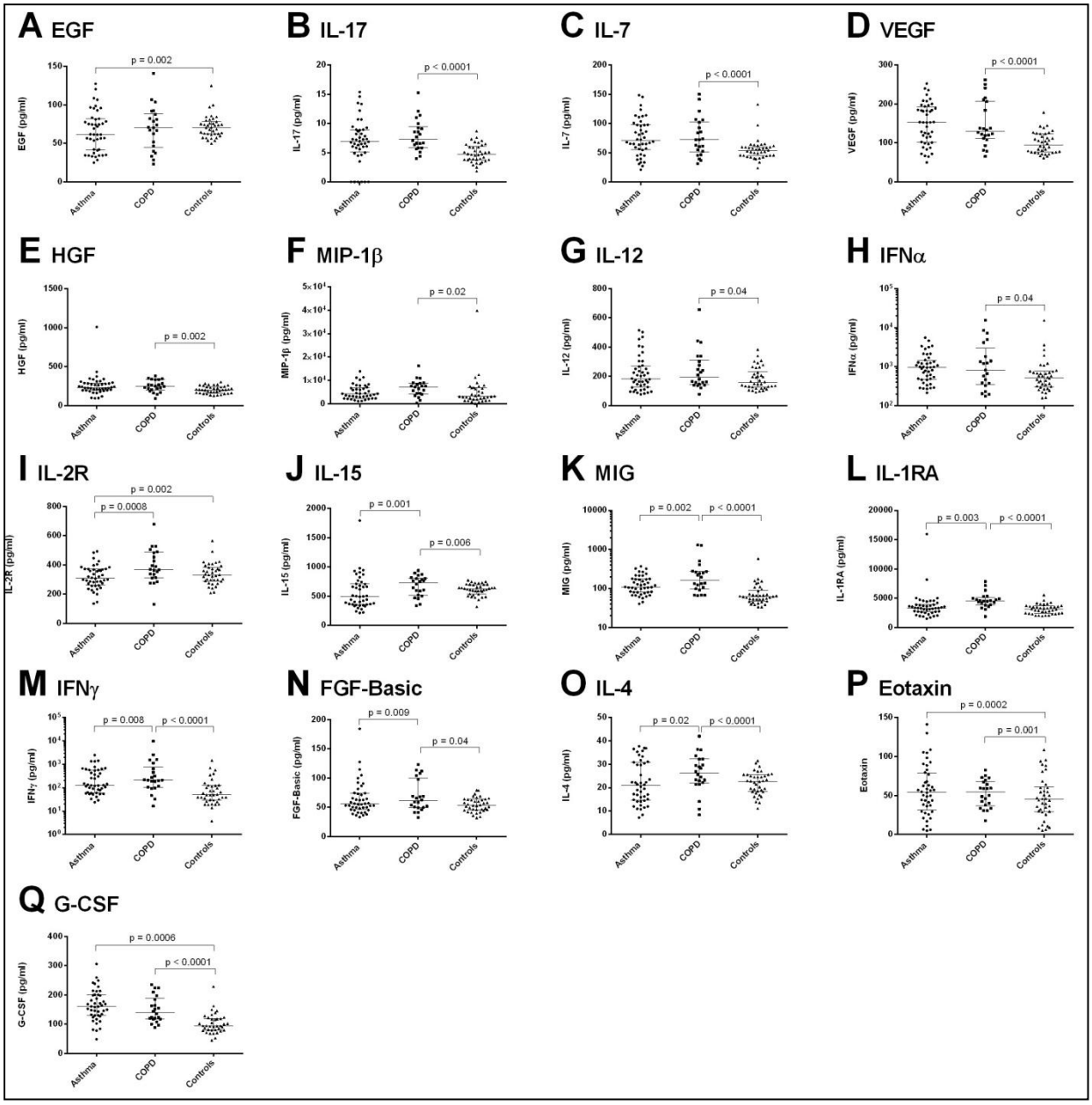


Figure 8.12: Cross-sectional comparison of inflammatory mediators released in response to RV-1B infection in the blood of controls, and those with asthma and COPD. Peripheral whole blood was collected and stimulated with RV-1B for 24 hours, before supernatant aspiration and analysis by multiplex ELISA (n = 44 for asthma, n = 22 for COPD and n = 38 for controls). Data are represented as scatter plots with median + IQR, with p-values calculated using two-group comparisons between each clinical group, with the model including adjustment for age, sex, baseline 25(OH)D status, ethnicity, smoking status and vaccination record, using the programme Qlucore Omics Explorer 2.3. P-values were deemed significant when less than 0.05, with a corresponding q-value of less than 0.1. Any values under the limit of detection (as specified within the product data sheet for each analyte) were given a value of 0.

8.2.10 Analysis of the secretion of inflammatory mediators in the blood following RSV stimulation shows differences between asthma, COPD and controls

Peripheral blood samples were stimulated with RSV, which causes diseases such as bronchiolitis and the common cold. Qlucore Omics Explorer 2.3 was used to carry out comparisons between the three clinical groups, modelling using a two-group comparison (i.e. Student's T-Test) between each group with log-transformed data, and including adjustment for age, sex, baseline 25(OH)D concentration, ethnicity, smoking status and vaccination record. All results obtained from 30-plex ELISA analysis of supernatants from stimulated whole blood are presented in Table 8.11, and significant results are presented in Figure 8.13. When compared to controls, the concentrations of RSV-stimulated G-CSF (asthma $p < 0.0001$; COPD $p = 0.0003$), IL-7 (asthma $p = 0.007$; COPD $p = 0.0002$) and TNF-α (asthma $p = 0.007$; COPD $p = 0.002$) were higher in both asthma and COPD patients, while the concentration of RSV-stimulated eotaxin (asthma $p = 0.005$; COPD $p = 0.007$) was lower in both. The concentrations of RSV-stimulated EGF ($p = 0.007$), IL-1RA ($p = 0.01$) and IL-2R ($p = 0.02$) were lower in asthmatics compared to controls, while concentrations of RSV-stimulated MIG ($p = 0.0006$), IL-17 ($p = 0.0009$), IL-1RA ($p = 0.01$) and MIP-1β ($p = 0.03$) were all higher in COPD patients compared to controls. In a comparison of the immune response to RSV in patients with asthma or COPD, the concentration of IL-1RA ($p = 0.0007$) was lower in asthma compared to COPD. The remaining inflammatory mediators assayed displayed no statistically significant differences between controls, asthmatics, and COPD patients (Table 8.11).

Table 8.11: Comparison of inflammatory mediators released following whole blood stimulation by RSV in controls and patients with asthma or COPD

Analytes	Asthma		COPD		Controls		Statistics: Asthma vs. COPD			Statistics: Asthma vs. Controls			Statistics: COPD vs. Controls		
	n	median (IQR)	n	median (IQR)	n	median (IQR)	T-Statistic	P-Value	Q-Value	T-Statistic	P-Value	Q-Value	T-Statistic	P-Value	Q-Value
EGF	44	71.63 (44 to 88)	22	70.18 (53 to 81)	38	68.79 (60 to 83)	-1.034	0.306	0.791	-2.732	0.007	0.044	-1.176	0.243	0.383
Eotaxin	44	53.51 (36 to 76)	22	45.98 (35 to 60)	38	41.60 (26 to 57)	0.241	0.811	0.893	-2.851	0.005	0.044	-2.771	0.007	0.029
FGF-Basic	44	49.58	22	53.30	38	48.95	-1.250	0.216	0.791	-1.314	0.192	0.442	1.372	0.174	0.313
G-CSF	44	154.88 (122 to 180)	22	127.29 (107 to 161)	38	87.39 (77 to 106)	0.822	0.414	0.791	4.728	< 0.0001	0.0002	3.736	0.0003	0.005
GM-CSF	44	5.78	22	5.30	38	6.62	0.809	0.422	0.791	-0.658	0.512	0.668	-1.627	0.107	0.230
HGF	44	235.04	22	227.00	38	195.64	0.239	0.812	0.893	1.702	0.092	0.250	1.944	0.055	0.150
IFN-α [2]	44	635.28	22	469.94	37	343.94	-0.173	0.864	0.893	0.837	0.405	0.606	0.393	0.695	0.835
IFN-γ	44	70.94	22	75.36	38	45.88	0.217	0.829	0.893	1.621	0.108	0.270	1.645	0.104	0.230
IL-10	44	17.37	22	20.33	38	13.13	1.091	0.280	0.791	0.739	0.462	0.629	0.148	0.882	0.930
IL-12	44	92.30	22	113.92	38	109.81	-0.836	0.406	0.791	-0.336	0.737	0.819	1.760	0.082	0.205
IL-13	44	28.88	22	28.95	38	28.77	0.367	0.715	0.893	-0.063	0.950	0.974	0.497	0.620	0.835
IL-15	44	484.10	22	672.87	38	644.96	-2.438	0.018	0.179	-2.059	0.042	0.126	1.459	0.148	0.296
IL-17	44	6.15 (5 to 8)	22	6.63 (5 to 9)	38	4.67 (3 to 5)	0.087	0.931	0.931	2.182	0.031	0.117	3.454	0.001	0.005
IL-1β	44	24.24	22	24.32	38	23.99	0.688	0.494	0.824	0.032	0.974	0.974	0.053	0.958	0.958
IL-1RA	44	2447.36 (1815 to 3193)	22	3203.86 (2615 to 4108)	38	2698.43 (2348 to 3133)	-3.579	0.0007	0.021	-2.579	0.011	0.056	2.535	0.013	0.049
IL-2	44	38.41	22	27.33	38	28.95	0.858	0.394	0.791	0.484	0.629	0.726	-0.178	0.859	0.930
IL-2R	44	282.48 (209 to 312)	22	302.75 (251 to 392)	38	276.89 (251 to 317)	-1.393	0.169	0.791	-2.466	0.015	0.065	0.355	0.724	0.835
IL-4	44	21.78	22	23.97	38	24.31	-0.571	0.570	0.848	1.176	0.242	0.457	2.039	0.044	0.133
IL-5	44	3.56	22	6.51	38	4.52	-1.314	0.194	0.791	-1.173	0.244	0.457	0.128	0.899	0.930
IL-6 [2]	21	15867.82	5	13384.73	1	2704.04	-0.841	0.404	0.791	0.802	0.424	0.606	3.540	0.001	0.005
IL-7	44	95.36 (77 to 132)	22	90.60 (67 to 116)	38	67.81 (64 to 74)	0.547	0.587	0.848	2.801	0.006	0.044	3.851	0.0002	0.005
IL-8 [2]	40	13260.44	22	10354.44	28	11036.73	2.516	0.015	0.179	1.096	0.275	0.459	-1.359	0.178	0.313
IP-10 [2]	11	3131.65	1	1081.24	7	2013.94	-0.383	0.703	0.893	-0.618	0.538	0.673	0.452	0.652	0.835
MCP-1 [2]	42	16466.23	21	15950.52	32	19093.02	0.536	0.594	0.848	0.205	0.838	0.897	-0.364	0.717	0.835
MIG	44	79.82 (63 to 100)	22	97.14 (69 to 119)	38	54.15 (45 to 72)	-0.190	0.850	0.893	2.108	0.037	0.124	3.578	0.001	0.005
MIP-1α	44	265.13	22	220.42	38	250.58	1.320	0.192	0.791	1.097	0.275	0.459	0.765	0.446	0.655
MIP-1β [2]	44	1233.38 (814 to 2325)	22	1510.51 (1098 to 2440)	37	1398.44 (725 to 1925)	-0.387	0.700	0.893	0.907	0.366	0.579	2.258	0.026	0.088
RANTES [2]	21	8117.18	12	5442.68	23	4088.03	1.323	0.191	0.791	0.504	0.615	0.726	-0.744	0.459	0.655
TNF-α	44	15.73 (12 to 21)	22	14.87 (11 to 23)	38	14.16 (11 to 20)	0.925	0.359	0.791	2.766	0.007	0.044	3.173	0.002	0.010
VEGF	44	212.29	22	181.68	38	175.25	0.708	0.482	0.824	1.245	0.216	0.457	1.278	0.205	0.341

Medians are given in pg/ml.

[2] For some analytes numbers were smaller due to mean fluorescence index (MFI) values exceeding the upper limit of detection, resulting in concentrations being unable to be calculated

The interquartile range (IQR) is only stated where statistical significant difference was evident ($p < 0.05$, $q < 0.1$)

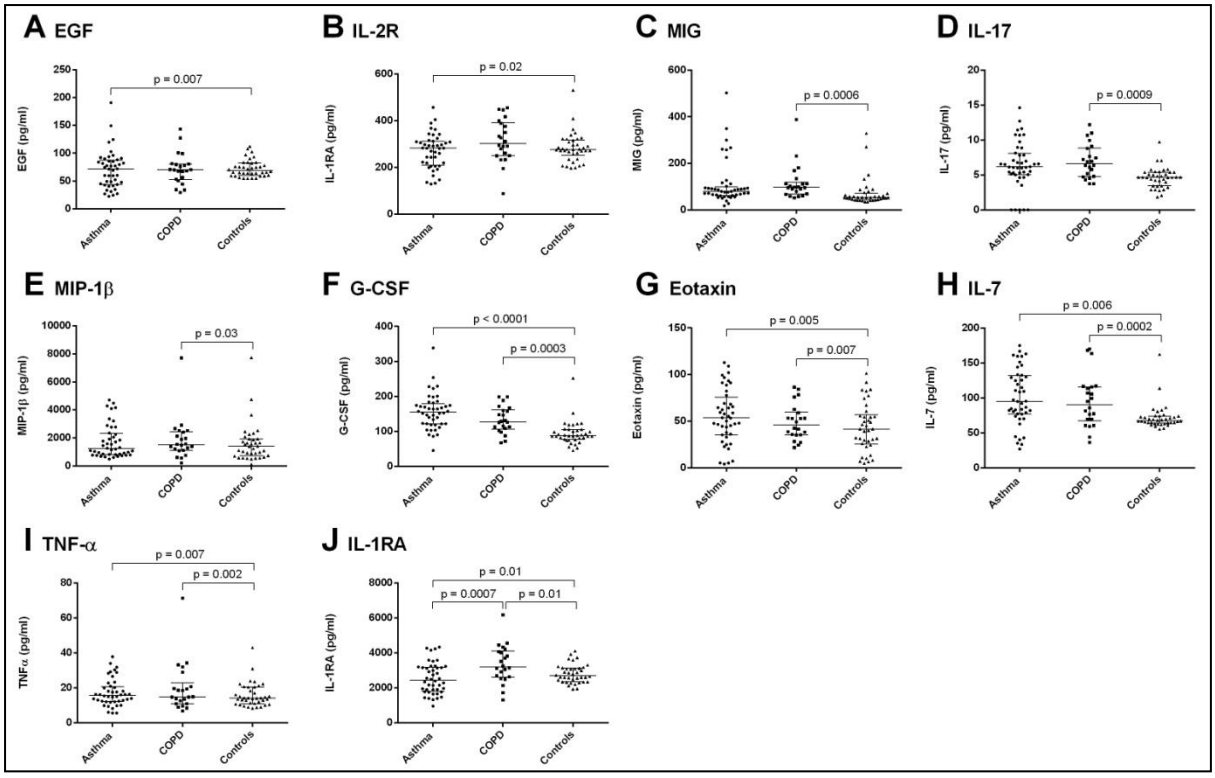


Figure 8.13: Cross-sectional comparison of inflammatory mediators released in response to RSV infection in the blood of controls, and those with asthma and COPD. Peripheral whole blood was collected and stimulated with RSV for 24 hours, before supernatant aspiration and analysis by multiplex ELISA (n = 44 for asthma, n = 22 for COPD and n = 38 for controls). Data are represented as scatter plots with median + IQR, with p-values calculated using two-group comparisons between each clinical group, with the model including adjustment for age, sex, baseline 25(OH)D status, ethnicity, smoking status and vaccination record, using the programme Qlucore Omics Explorer 2.3. P-values were deemed significant when less than 0.05, with a corresponding q-value of less than 0.1. Any values under the limit of detection (as specified within the product data sheet for each analyte) were given a value of 0.

8.2.11 Analysis of the secretion of inflammatory mediators in the blood following *Haemophilus influenzae* stimulation shows differences between asthma, COPD and controls

Peripheral blood samples were stimulated with *H.influenzae*, a Gram-negative bacteria which causes diseases such otitis media and pneumonia. Qlucore Omics Explorer 2.3 was used to carry out comparisons between the three clinical groups, modelling using a two-group comparison (i.e. Student's T-Test) between each group with log-transformed data, and including adjustment for age, sex, baseline 25(OH)D concentration, ethnicity, smoking status and vaccination record. All results obtained from 30-plex ELISA analysis of supernatants from stimulated whole blood are presented in Table 8.12, and significant results are presented in Figure 8.14. When compared to controls, the concentrations of *H.influenzae*-stimulated FGF-Basic ($p = 0.0001$), IL-2R ($p = 0.0003$), eotaxin ($p = 0.09$), IL-15 ($p = 0.01$) and IL-5 ($p = 0.02$) were lower in asthmatics compared to controls, while concentrations of *H.influenzae*-stimulated IL-1RA ($p = 0.0002$), IL-12 ($p = 0.0004$), IL-13 ($p = 0.0006$), VEGF ($p = 0.002$), IL-7 ($p = 0.002$), IL-15 ($p = 0.02$), IL-17 ($p = 0.02$) were all higher in COPD patients compared to controls. In a comparison of the immune response to *H.influenzae* in patients with asthma or COPD, the concentrations of FGF-Basic ($p = 0.0007$), IL-15 ($p = 0.006$), IL-1RA ($p = 0.09$), IP-10 ($p = 0.01$), IL-12 ($p = 0.01$) and IL-2R ($p = 0.02$) were all lower in asthma compared to COPD. The remaining inflammatory mediators assayed displayed no statistically significant differences between controls, asthmatics, and COPD patients (Table 8.12).

Table 8.12: Comparison of inflammatory mediators released following whole blood stimulation by H.influenzae in controls and patients with asthma or COPD

Analytes	Asthma		COPD		Controls		Statistics: Asthma vs. COPD			Statistics: Asthma vs. Controls			Statistics: COPD vs. Controls		
	n	median (IQR)	n	median (IQR)	n	median (IQR)	T-Statistic	P-Value	Q-Value	T-Statistic	P-Value	Q-Value	T-Statistic	P-Value	Q-Value
EGF	44	72.54	22	76.42	24	69.20	-1.579	0.120	0.327	-1.730	0.087	0.236	-0.100	0.921	0.921
Eotaxin	44	60.65 (40 to 92)	22	52.23 (46 to 82)	24	45.30 (27 to 54)	0.601	0.550	0.688	-2.647	0.009	0.073	-1.077	0.285	0.388
FGF-Basic	44	68.51 (50 to 99)	22	105.45 (70 to 155)	24	96.34 (69 to 134)	-3.567	0.0007	0.022	-4.030	0.0001	0.003	1.805	0.075	0.172
G-CSF	44	273.05	22	315.24	24	243.89	-1.295	0.201	0.430	-1.183	0.239	0.422	1.534	0.129	0.257
GM-CSF	44	4.23	22	4.81	24	4.35	-0.105	0.917	0.953	0.065	0.948	0.955	0.659	0.511	0.639
HGF	44	259.08	22	273.82	24	226.90	-0.861	0.393	0.561	-0.461	0.646	0.843	1.480	0.142	0.257
IFN-α	44	185.17	22	192.27	24	178.75	-1.211	0.231	0.431	-1.920	0.057	0.177	1.450	0.151	0.257
IFN-γ	44	23.89	22	67.86	24	22.73	-2.308	0.025	0.106	-1.338	0.184	0.367	2.152	0.034	0.113
IL-10	44	855.18	22	921.94	24	555.17	0.081	0.936	0.953	-0.311	0.756	0.844	1.252	0.214	0.305
IL-12	44	383.89 (252 to 756)	22	1322.71 (410 to 2724)	24	426.36 (340 to 840)	-2.584	0.012	0.074	-2.004	0.048	0.177	3.690	0.0004	0.006
IL-13	44	30.51 (26 to 36)	22	33.81 (26 to 39)	24	27.79 (22 to 29)	-0.800	0.427	0.582	1.202	0.232	0.422	3.547	0.001	0.006
IL-15	44	342.47 (237 to 445)	22	506.97 (387 to 578)	24	422.13 (349 to 580)	-2.872	0.006	0.074	-2.633	0.010	0.073	2.444	0.017	0.073
IL-17	44	8.57 (7 to 12)	22	9.22 (7 to 13)	24	6.78 (6 to 7)	-0.059	0.953	0.953	0.373	0.710	0.844	2.431	0.017	0.073
IL-1β	44	807.13	22	1225.39	24	996.73	-2.135	0.037	0.124	-1.967	0.052	0.177	2.111	0.038	0.113
IL-1RA	44	2942.73 (2351 to 3889)	22	4916.73 (3355 to 7289)	24	3240.52 (2414 to 4274)	-2.699	0.009	0.074	-1.908	0.059	0.177	3.928	0.0002	0.005
IL-2	44	7.86	22	6.85	24	6.31	-0.650	0.518	0.676	-0.895	0.373	0.589	0.452	0.653	0.725
IL-2R	44	376.41 (317 to 435)	22	425.26 (381 to 535)	24	419.98 (386 to 492)	-2.419	0.019	0.094	-3.744	0.0003	0.004	0.321	0.749	0.802
IL-4	44	22.29	22	30.33	24	24.21	-1.177	0.244	0.431	-0.306	0.760	0.844	2.088	0.040	0.113
IL-5	44	4.10 (2 to 7)	22	6.83 (4 to 9)	24	6.45 (6 to 7)	-1.387	0.171	0.427	-2.467	0.015	0.091	-0.807	0.422	0.550
IL-6 [2]	24	13459.55	5	14795.36	8	20621.96	-2.129	0.038	0.124	-1.567	0.120	0.300	1.437	0.154	0.257
IL-7	44	93.17 (67 to 122)	22	88.32 (67 to 107)	24	61.84 (47 to 74)	0.232	0.817	0.908	0.768	0.444	0.635	3.159	0.002	0.013
IL-8 [2]	36	17135.41	20	15225.31	23	19100.40	-0.970	0.336	0.531	-2.118	0.036	0.177	-0.594	0.554	0.665
IP-10 [2]	43	266.10 (137 to 429)	17	483.13 (236 to 916)	22	384.97 (202 to 766)	-2.591	0.012	0.074	-1.368	0.174	0.367	2.024	0.046	0.115
MCP-1 [2]	44	11582.28	21	10723.74	23	10455.53	0.336	0.738	0.885	-0.640	0.524	0.714	-0.525	0.601	0.693
MIG	44	72.94	22	74.11	24	65.71	-1.254	0.215	0.430	-0.057	0.955	0.955	1.591	0.115	0.247
MIP-1α [2]	43	7641.18	20	7315.85	23	6026.64	-0.868	0.389	0.561	-0.940	0.349	0.582	1.299	0.197	0.305
MIP-1β [2]	30	15575.55	13	13967.13	15	12254.51	0.237	0.813	0.908	-0.335	0.738	0.844	-0.105	0.917	0.921
RANTES [2]	24	7616.73	12	4529.40	15	4510.25	1.096	0.278	0.463	-0.244	0.808	0.865	-1.265	0.209	0.305
TNF-α	44	304.56	22	581.76	24	291.46	-1.268	0.210	0.430	-0.808	0.421	0.632	2.070	0.041	0.113
VEGF	44	225.27 (170 to 295)	22	295.27 (196 to 409)	24	202.10 (127 to 298)	-2.088	0.041	0.124	-1.418	0.159	0.367	3.201	0.002	0.013

Medians are given in pg/ml.

[2] For some analytes numbers were smaller due to mean fluorescence index (MFI) values exceeding the upper limit of detection, resulting in concentrations being unable to be calculated

The interquartile range (IQR) is only stated where statistical significant difference was evident ($p < 0.05$, $q < 0.1$)

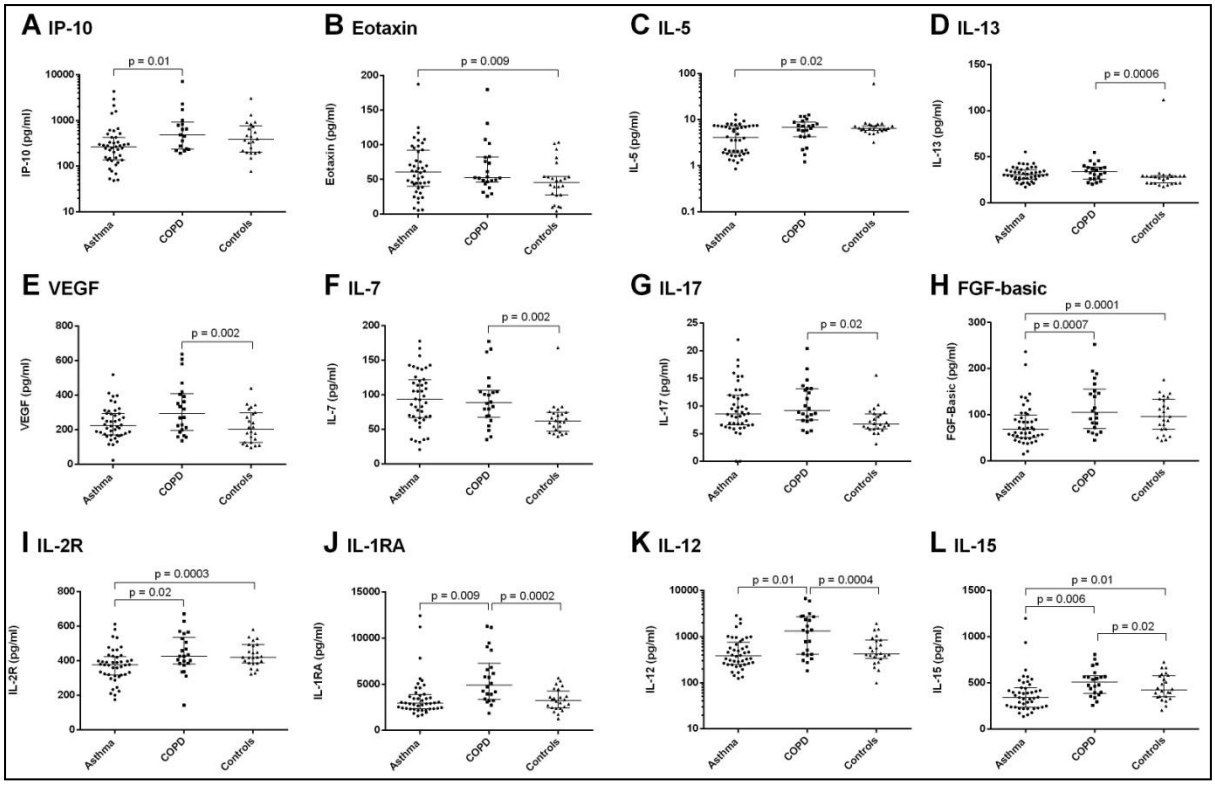


Figure 8.14: Cross-sectional comparison of inflammatory mediators released in response to H.influenzae infection in the blood of controls, and those with asthma and COPD. Peripheral whole blood was collected and stimulated with H.influenzae for 24 hours, before supernatant aspiration and analysis by multiplex ELISA (n = 44 for asthma, n = 22 for COPD and n = 24 for controls). Data are represented as scatter plots with median + IQR, with p-values calculated using two-group comparisons between each clinical group, with the model including adjustment for age, sex, baseline 25(OH)D status, ethnicity, smoking status and vaccination record, using the programme Qlucore Omics Explorer 2.3. P-values were deemed significant when less than 0.05, with a corresponding q-value of less than 0.1. Any values under the limit of detection (as specified within the product data sheet for each analyte) were given a value of 0.

8.2.12 Analysis of the secretion of inflammatory mediators in the blood following *Streptococcus pneumoniae* stimulation shows differences between asthma, COPD and controls

Peripheral blood samples were stimulated with *S.pneumoniae*, a Gram-positive bacteria which causes diseases such as bronchitis and pneumonia. Qlucore Omics Explorer 2.3 was used to carry out comparisons between the three clinical groups, modelling using a two-group comparison (i.e. Student's T-Test) between each group with log-transformed data, and including adjustment for age, sex, baseline 25(OH)D concentration, ethnicity, smoking status and vaccination record. All results obtained from 30-plex ELISA analysis of supernatants from stimulated whole blood are presented in Table 8.13, and significant results are presented in Figure 8.15. When compared to controls, the concentrations of *S.pneumoniae*-stimulated FGF-Basic ($p = 0.0004$) and IL-5 ($p = 0.006$) were lower in asthmatics compared to controls, while concentrations of *S.pneumoniae*-stimulated MCP-1 ($p < 0.0001$), G-CSF ($p = 0.0001$), IL-7 ($p = 0.0006$), IFN-α ($p = 0.0006$), IL-1RA ($p = 0.0007$), IL-4 ($p = 0.0007$), HGF ($p = 0.0009$), IL-13 ($p = 0.001$), MIG ($p = 0.002$), IL-15 ($p = 0.003$), IL-2R ($p = 0.003$), IL-17 ($p = 0.005$), MIP-1α ($p = 0.008$), IL-12 ($p = 0.03$), VEGF ($p = 0.04$), IP-10 ($p = 0.04$) were all higher in COPD patients compared to controls. There was no difference in the immune response to *S.pneumoniae* when comparing patients with asthma and COPD, with all of the remaining inflammatory mediators assayed displaying no statistically significant differences between controls, asthmatics, and COPD patients (Table 8.13).

Table 8.13: Comparison of inflammatory mediators released following whole blood stimulation by S.pneumoniae in controls and patients with asthma or COPD

Analytes	Asthma		COPD		Controls		Statistics: Asthma vs. COPD			Statistics: Asthma vs. Controls			Statistics: COPD vs. Controls		
	n	median (IQR)	n	median (IQR)	n	median (IQR)	T-Statistic	P-Value	Q-Value	T-Statistic	P-Value	Q-Value	T-Statistic	P-Value	Q-Value
EGF	44	92.17	22	104.22	24	102.83	-2.490	0.016	0.118	-2.104	0.038	0.226	1.522	0.132	0.188
Eotaxin	44	76.57	22	70.83	24	55.30	0.901	0.371	0.606	-0.143	0.886	0.917	1.693	0.094	0.141
FGF-Basic	44	102.93 (72 to 210)	22	192.93 (125 to 260)	24	199.89 (153 to 255)	-2.687	0.009	0.118	-3.672	0.0004	0.011	0.377	0.707	0.816
G-CSF	44	489.98 (375 to 637)	22	550.35 (435 to 874)	24	365.10 (290 to 461)	-1.241	0.220	0.439	0.297	0.767	0.885	4.017	0.0001	0.002
GM-CSF	44	14.62	22	19.16	24	19.89	-0.854	0.397	0.606	-2.311	0.023	0.226	-0.626	0.533	0.667
HGF	44	318.92 (265 to 384)	22	369.08 (259 to 420)	24	311.78 (222 to 364)	-0.955	0.344	0.606	0.650	0.517	0.741	3.435	0.0009	0.004
IFN-α	44	201.75 (180 to 241)	22	224.14 (199 to 273)	24	207.06 (176 to 239)	-2.080	0.042	0.158	-1.246	0.215	0.592	3.561	0.0006	0.004
IFN-γ	44	416.43	22	598.99	24	473.55	-1.680	0.098	0.293	-0.525	0.601	0.806	1.978	0.051	0.090
IL-10	44	195.17	22	235.04	24	179.69	-0.207	0.837	0.914	-0.343	0.733	0.879	1.767	0.081	0.134
IL-12	44	1994.91 (1358 to 3491)	22	2966.51 (1985 to 3990)	24	1660.49 (1264 to 3056)	-1.280	0.206	0.439	-0.732	0.465	0.735	2.160	0.034	0.072
IL-13	44	34.87 (29 to 42)	22	39.52 (32 to 47)	24	31.51 (26 to 37)	-0.579	0.565	0.737	1.140	0.257	0.592	3.364	0.001	0.004
IL-15	44	441.60 (340 to 595)	22	646.79 (501 to 760)	24	587.23 (415 to 746)	-2.497	0.015	0.118	-1.516	0.132	0.567	3.079	0.003	0.008
IL-17	44	11.06 (9 to 14)	22	12.61 (9 to 17)	24	8.96 (7 to 10)	0.590	0.558	0.737	0.993	0.323	0.626	2.857	0.005	0.013
IL-1β [2]	44	5590.92	18	6954.36	24	7912.10	-0.186	0.853	0.914	-0.832	0.407	0.679	0.398	0.691	0.816
IL-1RA	44	4884.65 (4079 to 7007)	22	7406.78 (5572 to 9135)	24	4867.91 (3773 to 6799)	-2.341	0.023	0.136	-1.363	0.176	0.585	3.520	0.0007	0.004
IL-2	44	12.49	22	11.85	24	10.42	-0.069	0.945	0.945	-1.061	0.291	0.624	-0.031	0.976	0.999
IL-2R	44	448.83 (362 to 510)	22	536.99 (424 to 645)	24	481.56 (413 to 541)	-2.617	0.011	0.118	-1.220	0.225	0.592	3.050	0.003	0.008
IL-4	44	27.11 (20 to 36)	22	34.92 (29 to 39)	24	29.31 (25 to 32)	-1.483	0.144	0.356	0.647	0.519	0.741	3.498	0.0007	0.004
IL-5	44	5.28 (2 to 9)	22	7.97 (6 to 10)	24	7.60 (6 to 9)	-1.444	0.154	0.356	-2.776	0.006	0.097	0.001	0.999	0.999
IL-6 [2]	21	16647.71	5	22730.67	7	22015.85	-1.636	0.107	0.293	-0.834	0.406	0.679	1.130	0.262	0.341
IL-7	44	105.68 (83 to 141)	22	106.66 (80 to 132)	24	72.78 (56 to 90)	-0.073	0.942	0.945	1.176	0.242	0.592	3.567	0.0006	0.004
IL-8 [2]	35	20826.98	19	20535.87	22	24331.05	-1.191	0.239	0.447	-1.409	0.162	0.585	0.160	0.873	0.935
IP-10 [2]	41	632.65 (266 to 1313)	10	586.25 (376 to 2118)	17	511.70 (308 to 1633)	-1.863	0.068	0.225	-0.153	0.878	0.917	2.018	0.047	0.088
MCP-1 [2]	43	15346.36 (11050 to 20254)	21	18227.68 (13323 to 21173)	23	11176.44 (9317 to 18290)	-0.373	0.710	0.888	-0.374	0.709	0.879	4.638	< 0.0001	0.0004
MIG	44	120.11 (87 to 176)	22	157.88 (111 to 331)	24	83.94 (66 to 162)	-2.214	0.031	0.154	0.026	0.980	0.980	3.193	0.002	0.007
MIP-1α [2]	42	15098.68 (10270 to 26392)	21	16834.47 (13986 to 35005)	23	9216.26 (6934 to 13850)	-0.642	0.523	0.737	0.971	0.334	0.626	2.696	0.008	0.019
MIP-1β [2]	29	17223.22	11	17821.09	14	11198.04	0.841	0.404	0.606	2.114	0.037	0.226	1.320	0.190	0.259
RANTES [2]	32	6963.67	12	5786.00	19	4131.37	0.278	0.782	0.914	-0.221	0.826	0.917	0.273	0.786	0.873
TNF-α [2]	41	5061.24	13	4954.13	18	4229.18	0.251	0.803	0.914	0.500	0.618	0.806	1.711	0.091	0.141
VEGF	44	493.48 (350 to 583)	22	566.44 (446 to 813)	24	501.44 (357 to 796)	-2.125	0.038	0.158	-1.921	0.057	0.287	2.035	0.045	0.088

Medians are given in pg/ml.

[2] For some analytes numbers were smaller due to mean fluorescence index (MFI) values exceeding the upper limit of detection, resulting in concentrations being unable to be calculated

The interquartile range (IQR) is only stated where statistical significant difference was evident ($p < 0.05$, $q < 0.1$)

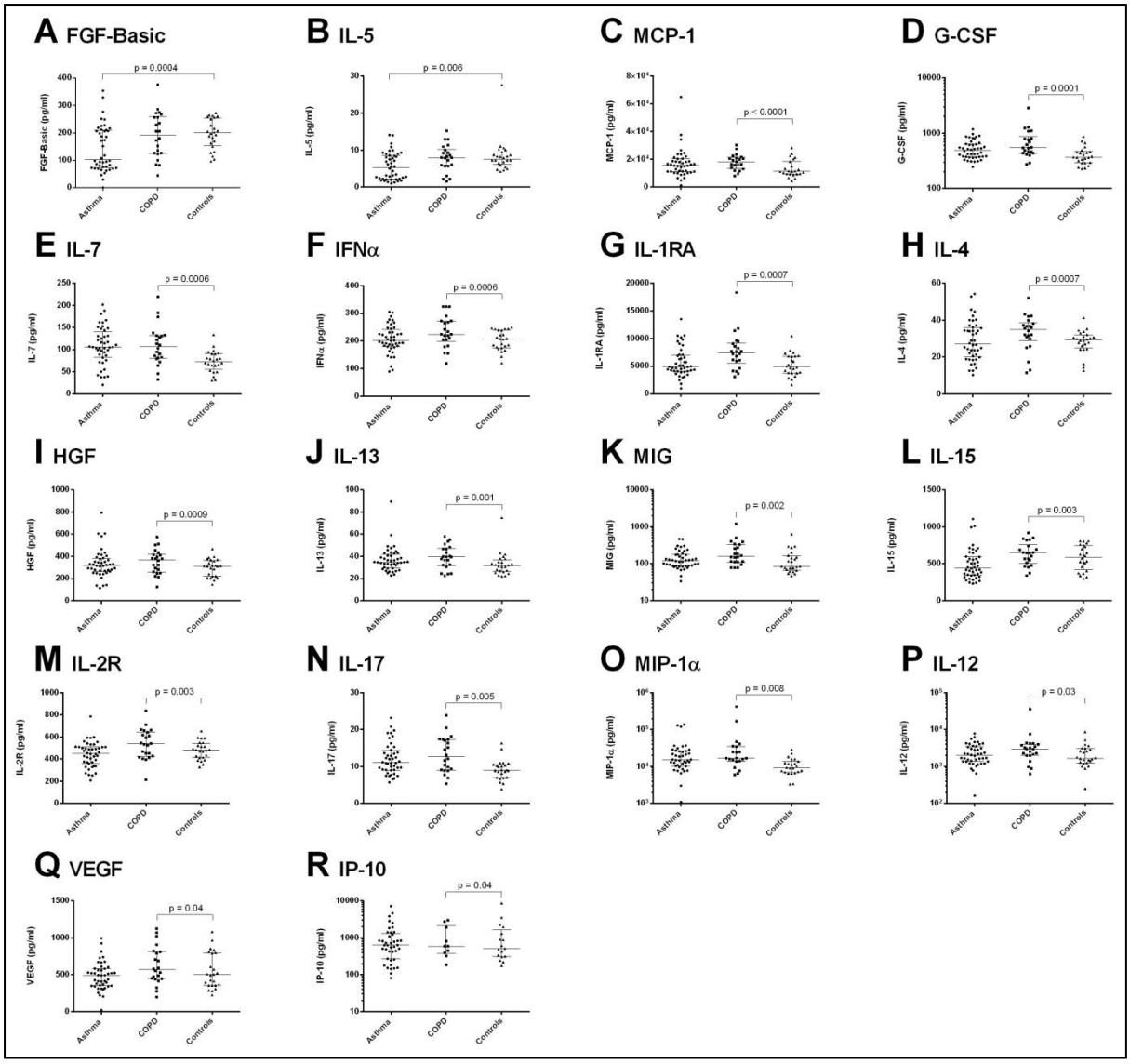


Figure 8.15: Cross-sectional comparison of inflammatory mediators released in response to S.pneumoniae infection in the blood of controls, and those with asthma and COPD. Peripheral whole blood was collected and stimulated with S.pneumoniae for 24 hours, before supernatant aspiration and analysis by multiplex ELISA (n = 44 for asthma, n = 22 for COPD and n = 24 for controls). Data are represented as scatter plots with median + IQR, with p-values calculated using two-group comparisons between each clinical group, with the model including adjustment for age, sex, baseline 25(OH)D status, ethnicity, smoking status and vaccination record, using the programme Qlucore Omics Explorer 2.3. P-values were deemed significant when less than 0.05, with a corresponding q-value of less than 0.1. Any values under the limit of detection (as specified within the product data sheet for each analyte) were given a value of 0.

8.3 Discussion

It has been demonstrated here that there are significant differences in inflammatory responses mounted to a range of stimuli in the blood of asthmatics, COPD patients and controls with neither condition. Overall, the biggest differences were seen between COPD patients versus patients with asthma and versus controls with neither condition, with increased release of inflammatory mediators observed. The discussion of these results is separated into sections, looking at comparisons between the three study populations in circulating inflammatory mediators and stimulated results in turn, before a summary at the end (Table 8.14).

Circulating inflammatory markers, COPD vs. controls

An increased concentration of IL-1RA was observed when comparing the serum inflammatory mediators present in COPD patients versus controls. IL-1RA inhibits signalling by IL-1β, with the balance between the two playing an important role inflammation, and the susceptibility to and severity of many diseases [686]. While the anti-inflammatory effects of IL-1RA are potentially beneficial in dampening chronic inflammation, as shown in the treatment of the systemic inflammation of rheumatoid arthritis [687, 688], and demonstrated with an associated reduction in airway hypersensitivity in asthmatic guinea pigs [689], higher concentrations of IL-1RA in mice have been associated with increased susceptibility to *Listeria monocytogenes* infection, with IL-1RA KO demonstrating enhanced resistance to listeriosis [690]. Additionally, gene expression analysis of peripheral blood neutrophils has shown increased levels of IL-1RA mRNA in patients with severe COPD compared to moderate COPD, with an associated reduction in lung function, suggesting an important role in the pathophysiology of COPD [691]. A study in serum has also shown increased concentrations of IL-1RA, with associated increased rate of exacerbations and reduced FEV1 [692].

In addition to IL-1RA, serum concentrations of IL-17 and IL-2R were higher in COPD patients compared to controls. IL-17 is a pro-inflammatory cytokine, implicated in neutrophil activation and recruitment, induction of pro-inflammatory cytokine release, and up-regulation of airway remodelling [693]. Th17 cells have been demonstrated to be increased in the bronchial mucosa of COPD patients [694], with a positive correlation to airflow limitation [695], as well as IL-17-secreting CD8+ T-cells [696], thus resulting in increased expression of IL-17 in sputum and serum of COPD patients [692, 697]. IL-2R, on the other hand, has not been specifically linked to COPD or respiratory disease before, but the CD25 component of this heterotrimeric receptor has been implicated as a potential inflammatory marker [698-701].

Interestingly, EGF and MIP-1β were both shown to be present in reduced concentrations in the serum of COPD patients compared to controls. MIP-1β is a chemoattractant for lymphocytes, monocytes and eosinophils. It has been shown to be elevated in the serum and sputum of COPD patients, contributing to airway inflammation [623, 702-704]. One study looking at gene expression in peripheral blood neutrophils only showed increased expression of MIP-1β in very severe COPD, and not in moderate COPD [691]. EGF is important in epithelial repair, and thus the decreased expression seen here may indicate a reduction in epithelial repair and thus be a contributing factor to the tissue damage that occurs in the airways of COPD patients. However, COPD has typically been associated with elevated levels of EGF, contributing to airway remodelling, goblet cell metaplasia, and increased chemokine expression [692, 704, 705]. Surprisingly, IL-6 and IL-8 were also both shown to be reduced in the serum of COPD patients compared to controls. IL-6 is a pro-inflammatory cytokine which has previously been shown to be increased in the sputum and serum of COPD patients, with an inverse correlation with lung function and high expression associated with increased mortality and poor clinical outcomes [706-711]. While some studies have demonstrated no difference in serum IL-6 between COPD patients and healthy people [623, 692], no other studies have demonstrated lower concentrations. Likewise, IL-8, a neutrophil chemoattractant, is strongly associated with COPD, having been shown to be upregulated in sputum, BAL and serum [623, 640, 692, 712], and also demonstrated to be negatively correlated with FEV1/FVC [640, 692], and positively correlated with exacerbation rate [692]. One study, however, did show no difference in the gene expression of IL-8 in peripheral blood neutrophils between healthy people and COPD patients [691].

Stimulated inflammatory responses, COPD vs. controls

The immune response to a range of stimuli was markedly different in the blood of COPD patients compared to controls, most notably in response to whole pathogens and TLR ligands associated with viral infection. The response to TLR ligands associated with bacterial infection showed fewer differences between the two groups. Secretion of IFN-α was increased in COPD patients in response to whole blood stimulation with LPS and Pam3CSK4, while LPS stimulation also resulted in increased secretion of the pro-inflammatory cytokines IL-4, IL-15 and IL-12. Pam2CSK4 induced lower MCP-1 and FGF-Basic secretion compared to controls.

A wide range of inflammatory mediators were present in higher concentrations in the supernatants of whole blood of COPD patients in response to the virus-associated TLR ligands polyI:C and R848 compared to controls. Stimulation with polyI:C or R848 induced increased secretion of the

proinflammatory cytokines IL-15, IL-17 and IL-6, the chemokine MIG, the growth factor VEGF, and IL-1RA compared to controls. MIG is a chemoattractant for a range of leukocytes, thus increasing inflammation, while VEGF increases endothelial permeability and plays a role in the production of pro-inflammatory cytokines, and has been implicated in systemic inflammation and COPD pathogenesis [713-715]. IL-1RA acts as an anti-inflammatory cytokine, blocking the effects of IL-1, with over-expression associated with inhibition of primary immune responses to pathogens [716]. Stimulation with polyI:C also resulted in increased secretion of the chemokines IP-10, MIP-1β, MIP-1α, and the pro-inflammatory cytokines TNF-α and IFN-γ in COPD patients compared to controls, while R848 caused elevated secretion of the pro-inflammatory cytokines IL-2 and IL-12 (as well as the IL-2 receptor), the growth factors HGF and FGF-Basic, cytokines involved in B-cell activation and proliferation IL-4, and IL-7, the anti-inflammatory cytokine IL-10 and the anti-viral agent IFN-α. Surprisingly, R848-induced secretion of IL-8 was reduced in COPD patients compared to controls.

Similar to the effects caused by TLR ligation by polyI:C and R848, RV-16, RV-1B and RSV all induced increased secretion of MIG, IL-17 and IL-1RA in COPD patients compared to controls. As seen with polyI:C stimulation, all three viral pathogens induced increased secretion of MIP-1β, with RV-16 and RV-1B also increasing IFN-γ secretion, and RSV infection causing increased concentrations of TNF-α in COPD patients compared to controls. More similarities were seen with R848 stimulation, with all three viral pathogens inducing increased IL-7 secretion in COPD patients. Additionally, both RV-16 and RV-1B stimulation caused increased secretion of IL-4, IFN-α, FGF-Basic and HGF, as seen following R848 stimulation, while RV-16 also induced increased concentrations of IL-2R and IL-2, and RV-1B increased concentrations of IL-12. The only inflammatory mediator seen to have increased secretion in COPD patients compared to controls in response to viral pathogens but not to virus-related TLR ligands, and thus suggesting a role for other immune receptors, was G-CSF, a growth factor which plays a role in neutrophil trafficking and has been shown to exacerbate inflammatory conditions such as rheumatoid arthritis [717, 718], as well as being associated with increased risk of mortality following influenza infection [719].

The bacterial pathogens also induced higher secretion of a range of inflammatory mediators in COPD patients compared to controls. Similar to LPS stimulation, both *S.pneumoniae* and *H.influenzae* caused increased secretion of the pro-inflammatory cytokines IL-12 and IL-15, with *S.pneumoniae* also stimulating increased secretion of IFN-α and IL-4. Unrelated to any of the bacteria-related TLR ligands, stimulation with both *S.pneumoniae* and *H.influenzae* resulted in increased secretion of the pro-inflammatory cytokines IL-17, IL-7 and IL-13, which are involved in B-cell growth and

proliferation, as well as VEGF, and IL-1RA. Stimulation with *S.pneumoniae* also resulted in increased secretion of the chemokines MCP-1, MIG, MIP-1α and IP-10, IL-2R, and the growth factors HGF and G-CSF in COPD patients compared to controls.

Overall, these results indicate a more marked inflammatory response to a range of stimuli in COPD patients compared to controls. Data comparing the immune response in COPD patients with healthy controls are lacking, but it has previously been suggested that the cytokine response in COPD is dysregulated, with a disproportionate response to immune stimuli. Studies looking at cells from tracheobronchial tissue, induced sputum supernatants, BAL and peripheral blood neutrophils of COPD patients have demonstrated an increase in RV-induced expression of inflammatory mediators such as IL-6 and IL-8 [163, 720-722], with one study in mice demonstrating a skewed Th2 response to RV infection, with increased IL-13 and IL-5 and associated impairment of viral clearance [723]. This is the first study to look at a broad range of stimuli and inflammatory mediators, and it also demonstrates a more marked inflammatory response, consisting of pro-inflammatory cytokines, chemokines and growth factors. Cytokine and chemokine release plays a critical role in the clearance of both bacterial and viral infections, but an exaggerated response may have a detrimental effect, with increased tissue damage, airway inflammation and obstruction, and elevated mucus production, thus resulting in a more severe disease phenotype [724, 725].

Circulating inflammatory markers, asthma vs. controls

A range of differences were also observed between asthmatics and controls in this study. In the serum of asthmatic patients, as seen in COPD, EGF and MIP-1β were shown to be present at reduced concentrations compared to controls. Both EGF and its receptor (EGFR) have previously been demonstrated to have elevated expression in the respiratory epithelium of asthma patients, correlating with disease severity [726-728], with the EGFR antagonist AG1478 inhibiting bronchial hyper-responsiveness and inflammation [729], thus making these results surprising. Additionally, the serum concentration of TNF-α was also lower in asthmatics compared to controls.

Stimulated inflammatory responses, asthma vs. controls

Ex vivo whole blood inflammatory responses to a range of stimuli were altered in the blood of asthma patients compared to controls, although the differences were not as large in number as seen in COPD. The only difference seen in the inflammatory response to the bacteria-related TLR ligands in asthma and controls was decreased eotaxin secretion in asthmatics in response to both LPS and Pam2CSK4 stimulation. Eotaxin secretion was consistently decreased in response to the whole

range of stimuli in both asthma and COPD patients compared to controls. It is a chemoattractant, which also activates and promotes degranulation of eosinophils, immune cells which have been shown to have increased expression in asthma and COPD, as also shown in this study in Figure 7.6. Thus, it was surprising to see such a consistent abrogated eotaxin response to a range of stimuli compared to controls, also opposing the observation of persistent eosinophilia following rhinovirus infection in asthma patients [171]. However, this decreased eotaxin expression may play a role in the increased susceptibility to viral infections seen in asthma and COPD patients, with eosinophils having an important protective role especially against RNA viruses such as RSV [730]. Additionally, the difference in eotaxin concentration between population groups in this study, while statistically significant, was only small in magnitude, thus questioning the biological significance of these findings.

Stimulation with the virus-related TLR ligand polyI:C resulted in a markedly different immune response in asthma compared to controls. Secretion of the pro-inflammatory cytokines TNF-α, IL-6, IL-2, IL-1β and IL-15, IL-7, the growth factors G-CSF, VEGF, HGF and FGF-Basic, and the chemokines MIP-1β and MIP-1α were all elevated compared to controls following polyI:C stimulation. The only differences observed following R848 stimulation were an increase in IFN-α, but decreased secretion of eotaxin and IL-8 in asthma compared to controls, as also seen in COPD patients. Similar to the effects caused by TLR ligation by polyI:C, the viral pathogens all induced increased secretion of G-CSF in asthma patients compared to controls, with RV-16-induced IFN-α and VEGF, and RSV-induced IL-7 and TNF-α also elevated. All three viral pathogens had decreased IL-2R and EGF secretion compared to controls, with RSV-induced IL-1RA also reduced.

The immune response induced by bacterial pathogens was also different in asthmatic subjects compared to controls, with secretion of FGF-Basic and IL-5 following both *H.influenzae* and *S.pneumoniae* decreased in asthmatics. *H.influenzae*-induced secretion of IL-2R, eotaxin and IL-15 was also reduced in asthma patients compared to controls.

Therefore, in comparison to COPD, where the inflammatory responses were mostly exaggerated compared to that induced in samples from controls, asthmatic patients demonstrated a more complex pattern. Little difference was observed in the response to TLR ligands, except polyI:C, where an exaggerated inflammatory response was detected. Inflammatory mediator release following infection with viruses was diminished in the case of mediators such as IL-2R and EGF, but increased in the case of G-CSF, while the inflammatory response was diminished for bacterial

pathogens. As such, the results presented here do not entirely corroborate the view that the innate immune response is deficient in asthma patients. A previous study has demonstrated decreased release of IFN-β following infection of bronchial epithelial cells with RV-16 in asthmatic cells compared to healthy controls [172]. The type I IFNs are innate cytokines which induce the anti-viral state, and are thus very vital in anti-viral immunity. However, in our study, RV-16 induced higher secretion of IFN-α, another type I IFN, in asthmatic subjects compared to controls. Other studies have shown discrepancies in the release of pro-inflammatory cytokines, with no differences observed in the release of IL-6 and IL-8 in nasal lavage and induced sputum inoculated with RV-16 [168], but increased release of IL-1β and IL-1RA in RV-16-infected nasal lavage samples in asthma patients compared to healthy controls [167]. In bacteria, one study has shown a decreased pro-inflammatory response in whole blood non-specifically stimulated with PMA in asthmatic patients, but no difference in the response to *S.pneumoniae* infection [731]. However, many of these studies looked at the airway epithelium rather than peripheral blood, which may explain the disconnect between their results and those presented here.

Therefore, the study presented here has helped to elucidate the differences in the inflammatory response between asthmatics and controls to a range of stimuli. Unlike COPD, there does not appear to be a clear picture of simply having a decreased or exaggerated immune response to contribute to the more severe and increased duration of symptoms experienced by asthma patients during infection by respiratory pathogens.

Circulating inflammatory markers and stimulated inflammatory responses, asthma vs. COPD

Fewer differences were observed between asthma and COPD, than by comparing to controls. In serum, the concentration of EGF was higher in COPD patients compared to asthmatics. Following TLR stimulation, differences between COPD and asthma were only shown in the inflammatory response to LPS and R848 stimulation. Following stimulation by both TLR ligands, asthma patients secreted lower levels of FGF-Basic, IL-12, IL-2R, IL-1RA, EGF and IL-15 compared to COPD, with LPS-induced IL-6 also decreased. Secretion of IL-1RA was reduced in asthma compared to COPD following stimulation by all three viral pathogens, with RV-16 and RV-1B-induced IL-2R, MIG, IL-1RA, IL-15, IFN-γ and FGF-Basic also decreased. Finally, *H.influenzae*-induced secretion of FGF-Basic, IL-15, IL-1RA, IP-10, IL-12 and IL-2R were decreased in asthma compared to COPD patients, with no differences seen following *S.pneumoniae* infection. Therefore, as seen in the comparison against controls, the inflammatory responses in COPD patients are also heightened compared to asthma patients.

Conclusions

Here it has been demonstrated that there is a vast difference in the inflammatory mediator profile of serum and stimulated blood between asthma, COPD and controls. The main limitation of this study is that it was carried out in peripheral blood. Asthma and COPD are both respiratory diseases, and the pathogens used were all respiratory pathogens, thus investigation of the inflammatory response mounted and the differences between the diseases and controls would have been more valuable at the site of infection. However, blood provides a good model for the systemic inflammation occurring following infection, and is a useful marker in assessing the inflammatory response occurring. Blood is also much better tolerated than the use of techniques such as experimental rhinovirus challenge, sputum induction, and bronchoscopy which are utilised in assessing the airway epithelium directly, and thus this enables greater numbers to be recruited.

Analysis may also have been underpowered for some analytes, such as IL-6, RANTES and IP-10, due to concentrations frequently exceeding the upper limit of detection, with KNN-imputation used during analysis to attempt to compensate for this. If samples had been diluted to allow such analytes to fall within the detectable range, many more analytes would have been at concentrations below the sensitivity threshold for the assay. Additionally, it would not have been feasible to run singleplex ELISAs with samples diluted accordingly for each individual assay for the number of analytes that we wished to quantify and the number of samples to be tested. While samples could be diluted and re-analysed in the future for the analytes which were consistently highly expressed, currently this is not possible due to budgetary constraints. Of note, as well as the use of KNN-imputation to compensate for missing data, adjustment for potential confounders such as age, sex and ethnicity was also carried out to ensure any inter-group differences observed were due to respiratory physiology rather than effects of demographics. Additionally, to allow for the large number of comparisons carried out, the Benjamini-Hochberg procedure for multiple testing correction was applied to analysis to control the false discovery rate at 10% [467].

The other limitation in this study is in the use of ViDiFlu participants as controls. These participants were used as a sample of convenience, representing a population without diagnosed asthma or COPD, as these were exclusion criteria for the trial. However, the majority of participants in the ViDiFlu trial were older adults, and thus their immune response may not be comparable with that of younger healthy adults. For example, immunosenescence has been demonstrated [204], with numerous changes in the immune response and immune regulation, such as decreased antigen presenting ability by dendritic cells [208], less cytolytic activity by NK cells [210], and reduced T-cell

memory [211]. Additionally, a low-grade chronic inflammatory state has been described, with increased expression of bacterial receptors [87]. In mice, an age-related delay in cytokine release and cellular infiltration has been observed following infection with RSV and influenza [732, 733] , while older humans have been shown to have a diminished IFN-γ and IL-13 response in whole blood inoculated with RV-16 [734]. Systemic levels of circulating IL-6, TNF-α and IL-1 have also been demonstrated in older adults [706], which may, for example, obscure any increase in IL-6 compared to healthy controls as has been reported in COPD patients in other studies [708, 710]. However, the median age of ViDiFlu participants was not significantly different compared to the COPD participants in this study, so any differences seen between these two groups were likely down to the COPD alone, and not a result of an aged control population. Of note, ViDiFlu patients did not undergo spirometry at any point during their study visits, and as such undiagnosed asthma or COPD could not be ruled out in this study population. Additionally, other co-morbidities are likely to be present in this population due to the older age group sampled, with conditions such as diabetes, atherosclerosis, stroke and heart disease potentially influencing inflammatory indices.

Therefore, it has been demonstrated here that the inflammatory response to a range of pathogens is altered in patients with COPD or asthma compared to controls (Table 7.13) , which may contribute to the more severe pathophysiology experienced by these patient groups during acute respiratory tract infections and exacerbations.

Table 8.14: Summary of the differences in inflammatory mediators between study populations grouped by stimulant

	Asthma vs. COPD (reference)	Asthma vs. Control (reference)	COPD vs. Control (reference)
Circulating	↓ EGF ↓ Neutrophils ↓ Lymphocytes	↓ EGF ↓ MIP-1β ↓ TNF-α ↑ Eosinophils	↓ EGF ↓ MIP-1β ↓ IL-6 ↓ IL-8 ↑ IL-1RA ↑ IL-17 ↑ IL-2R ↑ Lymphocytes ↑ Neutrophils ↑ Monocytes ↑ Eosinophils
Viral TLR			↑ IL-15 ↑ IL-17 ↑ IL-6 ↑ IL-1RA ↑ VEGF ↑ MIG
Viruses	↓ IL-1RA	↑ G-CSF ↓ IL-2R ↓ EGF	↑ IL-7 ↑ G-CSF ↑ MIG ↑ IL-17 ↑ IL-1RA ↑ MIP-1β
Bacterial TLR			↑ IFN-α
Bacteria		↓ FGF-Basic ↓ IL-5	↑ IL-1RA ↑ IL-12 ↑ IL-13 ↑ VEGF ↑ IL-7 ↑ IL-15 ↑ IL-17

Circulating: serum and coulter counts; Viral TLR: PolyI:C- and R848-stimulated blood; Viruses: RV-16-, RV-1B- and RSV-stimulated blood; Bacterial TLR: LPS-, Pam2CSK4- and Pam3CSK4-stimulated blood; Bacteria: H.influenzae- and S.pneumoniae-stimulated blood.

Results only shown if differences statistically significant ($p < 0.05$, $q < 0.1$) for all stimuli in that group

9. Discussion and Concluding Remarks

This thesis tested the hypothesis that vitamin D metabolites augment antimicrobial responses to respiratory pathogens, and suppress immunopathological inflammation in diseases such as asthma and COPD, thus preventing acute respiratory tract infections and exacerbations, and ameliorating the symptoms associated with these events. An *in vitro* cell-line model was utilised to quantify gene expression, a whole blood assay was developed to enable quantification of the release of inflammatory mediators following *ex vivo* stimulation with TLR ligands or pathogens, and clinical trial blood and sputum samples were used to assess cellular profiles. The main result presented in this thesis demonstrates that *in vitro* incubation with 25(OH)D or $1,25(OH)_2D$ in A549 cells reduces rhinovirus-16 infection. Immunological analysis of clinical trial samples did not demonstrate any consistent effect of *in vivo* vitamin D_3 supplementation on circulating, TLR-stimulated or pathogen-stimulated inflammatory profiles, or on inflammatory indices in induced sputum. The only effect demonstrated was a reduction in the number of B-cells in older adults with neither asthma nor COPD given high-dose vitamin D supplementation, although type I error cannot be ruled out with this finding.

Main Findings

The *in vitro* study carried out offers a mechanistic explanation as to how vitamin D supplementation may prevent acute respiratory tract infections, as has been observed in some clinical trials [300-305]. We have shown for the first time that co-culture with a physiological concentration of 25(OH)D increases resistance of A549 cells to infection with rhinovirus-16, with this effect also seen when incubating with $1,25(OH)_2D$ as a positive control. This was associated with attenuation of RV-induced ICAM-1 expression, the main receptor for major serotypes of rhinovirus [524, 525]. Other studies have also demonstrated upregulation of ICAM-1 following infection by RSV [735-737] and rhinovirus [457, 543, 545, 738], with augmented ICAM-1 expression resulting in cellular transmigration and increased epithelial adherence [735, 739, 740]. This upregulated expression of ICAM-1 may facilitate further infection by rhinovirus, and the subsequent spreading of rhinovirus to adjacent cells, with the increased influx of leukocytes contributing to localised inflammation and immunopathology. Therefore, 25(OH)D inhibition of RV-induced ICAM-1 upregulation may prevent viral spreading and reduce any immunopathology experienced, potentially reducing the severity of the disease. Additionally, co-culture with 25(OH)D was demonstrated to lessen RV-induced A549 cytotoxicity, thus further inhibiting the facilitation of viral spreading.

RV-induced upregulation of ICAM-1 has been demonstrated to be mediated by IL-1α [736], IL-1β [736, 738] and NFκB [111, 457, 526, 737]. However, we did not observe any effect of co-incubation with vitamin D metabolites on IL-1β concentrations released, suggesting that vitamin D is not inhibiting upregulation of ICAM-1 through effects on this cytokine. It has previously been demonstrated that $1,25(OH)_2D$ is able to increase the expression of the NF-κB inhibitory protein IκBα [327, 549], thus inhibiting NF-κB signalling, but still maintaining the anti-viral state [327]. However, while a trend towards increased expression of IκBα was observed in this study following co-culture of A549 cells with 25(OH)D or $1,25(OH)_2D$, statistical significance was not reached.

Significantly, *in vitro* experiments also demonstrated an effect of vitamin D metabolites in inhibiting RV-induced upregulation of the platelet activating factor receptor (PafR). This has been demonstrated to be a major receptor for *Streptococcus pneumoniae* and *Haemophilus influenzae*, facilitating adherence and colonization of the respiratory tract, with expression upregulated by RSV, RV-14, influenza and coronavirus infections [88, 101, 102]. Additionally, ICAM-1 may play a role in bacterial adherence and infection, with *H.influenzae* demonstrated to use ICAM-1 as a receptor [741]. As such, vitamin D may be beneficial in preventing secondary bacterial infections, via inhibition of viral-induced upregulation of receptors such as PafR and ICAM-1. Since viral-induced PafR expression has been associated with NF-κB activation [102, 109], the effect of vitamin D metabolites observed here may again be mediated through increased IκBα expression.

Therefore, *in vitro* work has demonstrated that vitamin D metabolites are able to reduce infection of A549 cells by rhinovirus-16, while also inhibiting virus-induced upregulation of the receptors ICAM-1 and PafR, although the mechanisms behind this are not entirely clear. However, clinical trials carried out in conjunction with these experiments did not show any reduction in the incidence of upper respiratory tract infections or exacerbations in asthmatics, COPD patients, or people living in sheltered accommodation (results not yet published). Immunological assays using blood samples from trial participants stimulated *ex vivo* with a panel of TLR ligands and pathogens also did not demonstrate any effects of vitamin D supplementation on the release of inflammatory mediators. Finally, the cellular profiles and underlying inflammation in the sputum and blood of patients with asthma or COPD were not affected by vitamin D supplementation, with only the number of B-cells reduced in the intervention arm in older adults living in sheltered accommodation. However, while an effect of vitamin D on B-cell proliferation, antibody production and memory cell differentiation has been shown elsewhere [255, 608], with the vast number of analyses carried out in this study and

the fact that only one parameter showed any difference following vitamin D supplementation, type I error cannot be ruled out, despite having a q-value of less than 0.1.

The discord between the positive effect of co-culture with 25(OH)D in decreasing RV-16 infection in A549 cells, and the lack of any effect of vitamin D supplementation in the immunological assays and the majority of the clinical trials results could be due to a number of reasons. In the *in vitro* work carried out, only one pathogen was studied. It is possible that vitamin D has differentially protective effects for different respiratory pathogens, with one study demonstrating reduced incidence of influenza A following vitamin D supplementation with 1200 IU/day, but no effect on incidence of influenza B [303]. Only RV-16 was used to infect A549 cells in cell culture work, whereas the clinical trials patients would have been exposed to a wide range of different respiratory pathogens. As such, it is possible that vitamin D supplementation did reduce incidence of rhinovirus infection, but had no effect on other acute respiratory tract infections experienced. Rhinovirus is the most common ARI, as shown elsewhere [7, 517] and in the ViDiFlu study, with 6 out of the 11 PCR positive episodes which achieved Jackson symptom criteria of being a URI associated with rhinovirus infection. However, the lack of any effect of vitamin D on other respiratory pathogens, such as parainfluenza, enterovirus, RSV and influenza, could have masked any effect of vitamin D on rhinovirus infection. Additionally, effects of vitamin D could be group, species or even serotype specific, with only RV-16 investigated, which is a major group, A species serotype of rhinovirus. Finally, the use of symptom scores to define URIs may not have been accurate in clinical trial patients, with the potential to miss true URIs or record false URIs. The gold standard of PCR was performed in nose and throat swabs from a subset of ViDiFlu participants (n = 166), with 95% of those presenting with a negative symptom score also being PCR negative for the presence of a panel of pathogens (RSV, influenza A and B, parainfluenza 1-3, adenovirus, enterovirus, rhinovirus, metapneumovirus). However, only 52% of those presenting with a positive symptom score were PCR positive for any of those pathogens, suggesting that false positives may have been reported in URI incidence in all three trials. Likewise, 39% of PCR positive swabs were associated with negative symptom scores, indicating that true URIs may have been missed.

Host factors may also offer an explanation as to the discord in results from cell culture work and analysis of clinical trials samples. Firstly, *in vivo* supplementation represents a much more complex system than co-culture in a cell line. In the host, all cell types are present, with interplay and cross-talk between epithelial cells and leukocytes to regulate immune homeostasis, inflammation and tissue repair [742], whereas the use of a A549 cells involves only one cell type, in this case

adenocarcinomic human alveolar basal epithelial cells. The vitamin D supplementation regimen may also have had an effect. A549 cells were co-cultured with a 100 nmol/L concentration of 25(OH)D, thus experiencing a sustained steady high concentration of 25(OH)D for the duration of their culture. *In vivo* concentrations of 25(OH)D on the other hand fluctuated, from a peak of likely greater than 100 nmol/L at one week post-dose, where concentrations following bolus supplementation have been shown to be highest [316], to a much lower trough concentration 2 months post-dose, which is the point at which blood and sputum samples were taken for use in immunological assays and serum 25(OH)D concentrations were quantified. Therefore, the difference between the intervention and placebo arms was probably much greater immediately post dosing, and it is possible that if the immunological tests had been carried out at this point a greater difference between the vitamin D status of the two arms could have resulted in an effect of vitamin D on the release of inflammatory mediators being observable. While bolus dosing is beneficial due to improved compliance, a smaller daily dose may provide a more stable serum 25(OH)D concentration. It has also been hypothesized that high-dose bolus supplementation with vitamin D may result in induction of CYP24 [743], the 24-hydroxylase enzyme which is able to catabolise $1,25(OH)_2D$ into $1,24,25(OH)_3D$, as well as metabolising 25(OH)D to the relatively inactive metabolite $24,25(OH)_2D$ [318, 744, 745]. Therefore, the amount of active $1,25(OH)_2D$ is reduced, both by accelerating its catabolism and by decreasing the availability of the 25(OH)D substrate for 1-hydroxylation. This could explain the results observed in the ViDiFlu trial, with acute upper respiratory tract infection incidence increased in the intervention arm.

While supplementation did result in a statistically significant increase in vitamin D status from baseline levels to 12 month follow-up in all studies, the difference in serum 25(OH)D concentration between the two arms at 12 months was modest in size in some cases, and did not reach statistical significance in the COPD sputum sub-study (Table 9.1). The average difference in mean vitamin D status at the 12 month time-point between the 2 arms of each study was 26.7nmol/L. Using a cut-off of 50nmol/L to define deficiency and sufficiency, at the 12 month time-point the means for the intervention arm for all studies were above this threshold. However, in half of the studies, the means for the 12 month time-point in the placebo arm were also above this threshold, thus potentially masking any effects of vitamin D supplementation. Additionally, there were a number of participants above the sufficiency threshold of 50nmol/L in the placebo arms of each sub-study at 12 month follow-up, and a number of participants who failed to reach sufficiency in the intervention arm.

Table 9.1: Mean serum 25(OH)D concentration at 12 month follow-up for both arms of all trials

Study	12 month placebo / low-dose vitamin D [1]			12 month intervention / high-dose vitamin D [1]			Statistics	
	n with 25(OH)D > 50nmol/L	n with 25(OH)D < 50nmol/L	mean (nmol/L)	n with 25(OH)D > 50nmol/L	n with 25(OH)D < 50nmol/L	mean (nmol/L)	difference between means, nmol/L (95% CI)	p-value
ViDiAs sputum sub-study	9	14	42.39	18	3	71.14	28.75 (15.5 to 42.0)	< 0.0001
ViDiCO sputum sub-study	8	5	54.62	17	7	63.13	8.51 (-7.1 to 24.2)	0.28
ViDiAs WBA sub-study	5	18	37.57	20	2	70.59	33.03 (23.3 to 42.8)	< 0.0001
ViDiCO WBA sub-study	2	11	32.46	5	3	61.25	28.79 (8.2 to 49.4)	0.009
ViDiFlu residents WBA sub-study	16	8	62.63 [1]	32	1	86.76	24.13 (12.3 to 36.0)	0.0001
ViDiFlu staff WBA sub-study	5	4	52.56	6	0	89.83	37.28 (4.6 to 69.9)	0.03

In all sub-studies, 25(OH)D was measured in serum samples.

[1] ViDiFlu residents on different dosing regimen, with 12 month placebo participants having also received vitamin D supplementation (low-dose) throughout the study

Abbreviations used:25(OH)D: 25-hydroxyvitamin D; CI: confidence interval; WBA: whole blood assay

The serum 25(OH)D concentration also does not necessarily correlate with the concentration experienced in the airways, the site of infection for ARIs, with one study demonstrating significantly lower 25(OH)D and 1,25$(OH)_2$D concentrations in BAL compared to serum [746]. As such, the effect of vitamin D supplementation on the airway concentration of vitamin D cannot be determined in this study, and thus it is possible that the immune cells of the respiratory tract did not reach a high enough 1,25$(OH)_2$D concentration to exert any immunomodulatory effects and alter ARI incidence. For the cell culture work on the other hand, we can be sure that the epithelial cells being challenged with RV-16 had experienced a sustained high concentration of 1,25$(OH)_2$D or 25(OH)D. Finally, the metabolites were used individually in separate cell culture experiments for the *in vitro* work, whereas *in vivo* there is likely a complex interplay between all of the different vitamin D metabolites, with effects on metabolism and catabolism.

A number of other clinical trials have also not demonstrated any effect of vitamin D supplementation, with no effects on hospital readmission rates due to respiratory tract infections [309], self-reported URTI incidence, duration or severity [310], pneumonia incidence [311, 312], influenza-like illness incidence or URTI duration [313, 314], or the rate of asthma exacerbations, lung function and airway hyperreactivity [315]. The reasons behind why effects of vitamin D have been observed in some clinical trials but not in others, including the ones reported in this study, remains to be elucidated. The inconsistencies in dosing regimens in intervention studies may play a role, with the optimum dose of vitamin D yet to be determined, and issues surrounding bolus dosing versus daily dosing, while the follow-up period for each trial may also be a determinant, with sufficient duration required to allow prolonged vitamin D repletion.

Stratification of analyses by genotype or baseline vitamin D deficiency may also provide explanations as to why some trials report positive results and some do not. In the three clinical trials reported in this study, COPD patients did show that effects of vitamin D supplementation were modified by genotype. However, these effects were not observed in the other 2 trials, and no other published intervention studies into vitamin D supplementation in acute respiratory tract infections have determined the genotype of participants. Likewise, baseline vitamin D deficiency may be an important determinant which was not addressed in a number of other intervention studies. In the ViDiCO study, vitamin D supplementation was shown to reduce the risk of exacerbation in patients with baseline deficiency (aHR 0.57, 95% CI 0.35 to 0.92, p = 0.021), and in the study by Lehouck *et al.* exacerbations were decreased in COPD patients with profound baseline deficiency [308]. However, one study has demonstrated that in individuals with 25(OH)D concentrations greater than 140nmol/L the risk of active tuberculosis was increased [317], suggesting that supplementation may be detrimental in vitamin D replete individuals by raising their vitamin D status by too much. In order to address these potential determinants, trials would have to recruit participants based on their genotypes and baseline vitamin D status to provide enough power, which would add to the complications of trial design and recruitment procedures.

Finally, and separate from the main aim of this study in determining effects of vitamin D, clinical trial samples were further utilised to perform cross-sectional analyses to compare the underlying inflammatory profiles in patients with asthma or COPD, and those with neither condition. Interesting differences were observed, with the sputum of asthmatics having increased proportions of macrophages and higher concentrations of IL-2, IL-4, IL-13 and GM-CSF compared to COPD patients, while the sputum of COPD patients had an increased number of neutrophils present and

elevated concentrations of IFN-α, MIP-1α, IL-8, IL-6, G-CSF, IL-1RA, eotaxin, MCP-1, HGF and IL-1β. Differences were also observed in the concentrations of inflammatory mediators released following *ex vivo* stimulation of the blood, with COPD patients demonstrating a more marked inflammatory response compared to controls, potentially increasing immunopathology in this disease. In asthmatics, a more complex pattern was demonstrated, with increased release of inflammatory mediators following viral stimulation, but a decrease following bacterial stimulation when compared to controls. In a comparison of the inflammatory response in asthma versus COPD, the release of inflammatory mediators such as IL-2R, IL-12, IL-15 and IL-1RA was higher in COPD following stimulation with pathogens, LPS and R848, again demonstrating a heightened inflammatory response with the potential to contribute to immunopathology.

Strengths and limitations of the studies carried out

The main limitation of the studies presented in this thesis was insufficient power. During trial design, power calculations were based on being able to detect an effect of vitamin D supplementation on the primary outcomes of time to first exacerbation/URI. Due to patients being lost to follow-up, delays in the implementation of immunological assays, and budgetary constraints on the quantity of the analyses carried out, sufficient numbers may not have been gained to reach significance thresholds. In an attempt to increase power, data from asthmatics, COPD patients and ViDiFlu staff members who all underwent similar vitamin D dosing regimens were pooled, raising another possible limitation. The inflammatory response for these three distinct populations was likely to differ markedly, as shown in the cross-sectional comparisons carried out, with effects of vitamin D potentially depending on disease phenotype. This effect was partly observed by the finding that vitamin D reduced exacerbations in COPD patients with baseline deficiency, but had no effect in deficient asthma patients. However, this was compensated for by using a statistical analysis platform which allowed for adjustment of confounders, with study eliminated during all analysis to remove any influence of having asthma or COPD. Additionally, although not presented here, analyses of sputum inflammatory mediators and cellular profiles, and *ex vivo* responses to stimulation of peripheral blood were carried out stratified by disease type, with no effect of vitamin D supplementation observed.

Power was also reduced in the analysis of inflammatory mediator release, with analytes such as IL-6, IP-10 and RANTES frequently presenting with concentrations exceeding the upper limit of detection,. While KNN-imputation was used during analysis to attempt to compensate for this, it would have been preferable to dilute samples and re-analyse them. However, if samples had been diluted to

allow such analytes to fall within the detectable range, many more analytes would have been at concentrations below the sensitivity threshold for the assay. Additionally, it would not have been feasible to run singleplex ELISAs with samples diluted accordingly for each individual assay for the number of analytes that we wished to quantify and the number of samples to be tested. While samples could be diluted and re-analysed in the future for the analytes which were consistently highly expressed, currently this is not possible due to budgetary constraints.

As already touched upon, the use of a bolus-dosing regimen in clinical trials patients is also a potential limitation to study. This regimen likely resulted in fluctuations in serum 25(OH)D concentration, with potential induction of CYP24, and increased catabolism of the active metabolite $1,25(OH)_2D$. Another hypothesis relating to worth of bolos-dosing is that immune cells require parent vitamin D_3 which they then 25-hydroxylate themselves [747]. Since the half-life of vitamin D_3 is only approximately 24 hours, bolus-dosing would not be sufficient to influence circulating vitamin D_3 levels, and thus immunomodulatory actions on immune cells would not be possible.

However, there were also a number of strengths in the studies carried out. The clinical trials were all well-designed, in that they were double-blind, with directly observed dosing and detailed assessments carried out. A vast range of assays and techniques were used to try and capture any immunomodulatory effects of vitamin D, with quantification of circulating and sputum supernatant inflammatory mediators, cellular profiles of both the airways and circulation, and stimulated responses to both TLR ligands and whole pathogens. No other clinical trials into vitamin D supplementation in the prevention against acute respiratory infections have carried out concomitant analysis of both sputum and blood samples from clinical trials patients, to try to offer a mechanistic explanation behind any primary effects observed. The *ex vivo* stimulation of whole blood was also novel in this context, analysing the effects of *in vivo* vitamin D supplementation on the inflammatory response to a wide range of stimuli. This investigation is especially important, since pathogenic stimulation has been demonstrated to upregulate CYP27B1, thus increasing the potential to detect an immunomodulatory effect of vitamin D supplementation even in participants without on-going infection or inflammation. The *in vitro* investigations were also the first of their kind to be carried out, with an effect of physiological concentrations of 25(OH)D observed in reducing rhinovirus infection, as well as associated and potentially important inhibition of RV-induced ICAM-1 and PafR expression, and improved cell viability. While only rhinovirus was looked at, this is the most common respiratory pathogen, thus justifying this choice.

Future Work

As such, while the majority of the results presented here were negative, the questions and hypothesis addressed in the studies carried out were worth asking, and lots of new and interesting data has been produced. In spite of the number of observational and intervention studies demonstrating a protective effect of vitamin D in the prevention of acute respiratory infections, it cannot be ruled out that vitamin D is not able to have such a profound effect on incidence and severity of ARI, with a number of trials, including the ones presented here, indicating that this may be the case.

The main thing to be elucidated before any more intervention studies into the effects of vitamin D on ARIs is the optimum dosing regimen to use. From what I have already discussed, it seems likely that daily dosing would provide a better system when assessing the effects of supplementation. However, this brings about issues surrounding compliance, with it being impossible to directly observe the consumption of a daily dose, and an increased chance of participants forgetting or losing doses. Future clinical trials may also benefit from only recruiting participants with baseline vitamin D deficiency, since the effects of vitamin D supplementation may be more marked or more beneficial in this population. Similarly, genotyping participants may provide insight into whether effects are influenced by the genetics of an individual. Large-scale trials would be needed to address these questions, with high enough numbers recruited to reach sufficient power.

Finally, the *in vitro* work carried out should be expanded on in the future to further elucidate the effects of vitamin D in the prevention against rhinovirus infection, as well as other pathogens. The first step would be to repeat the experiments in primary human bronchial epithelial cells, to see if these results can be replicated. A549 cells are a transformed cancerous cell-line, and as such are not as physiologically accurate as using primary cells. It has also been shown both here and elsewhere that A549 cells have relatively high expression of CYP24A1 and low expression of CYP27B1, suggesting that the presence of the active metabolite $1,25(OH)_2D$ may be diminished in this cell type, thus preventing some of the immunomodulatory effects of vitamin D to be observed [430]. The abundance of CYP24A1 in this cell-line may also effect the time-frame of any effects, potentially being the cause of the transient effects of co-culture with vitamin D metabolites on viral mRNA expression, RV-induced PafR expression and A549 viability observed in this study. A number of additional experiments should also be carried out to explain the mechanisms by which vitamin D

metabolites are able to reduce rhinovirus infection. Firstly, the involvement of the NF-κB activation pathway in the effects of vitamin D could be further elucidated in a variety of ways. Phosphorylation of IκBα could be determined by western blot, to assess whether vitamin D is increasing IκBα mRNA by inhibiting its phosphorylation and degradation, thus resulting in reduced activation of NF-κB. Silencing and antagonism of IκBα, ICAM-1, type I IFNs and LL-37, as well as NF-κB itself, would also help to determine how important each of these components are in the observed vitamin D mediated resistance to RV infection. An adhesion assay looking into the effects of vitamin D metabolites on virus-induced bacterial adhesion would help to establish the importance of vitamin D metabolites in preventing secondary bacterial infections, following on from our findings that 25(OH)D was able to inhibit RV-induced upregulation of the bacterial receptor PafR. Finally, a wider variety of respiratory pathogens could also be utilised. As already discussed, vitamin D may be differentially protective against different pathogens, thus the use of other common respiratory viruses such as RSV, influenza and coronavirus could also be investigated.

Conclusion

This study has demonstrated that *in vitro* co-incubation of A549 cells with 25(OH)D increases transient resistance to infection with rhinovirus-16, while also inhibiting RV-induced upregulation of ICAM-1 and PafR. These findings did not translate to any effects in the immunological analysis of clinical trial samples from participants with asthma, COPD or neither condition, with no changes in the cellular profile of the airways or circulation, the inflammatory profile of induced sputum supernatants, or the inflammatory mediator release following *ex vivo* stimulation of peripheral whole blood. While one effect was observed between low-dose and high-dose vitamin D supplementation, with a decrease in the number of B-cells in the blood of older adults living in sheltered accommodation following a combined daily and bolus high-dosing supplementation regimen, type I error cannot be ruled out with this finding.

Therefore, further studies should be carried out to determine whether vitamin D supplementation would be beneficial in preventing acute respiratory tract infections, especially in vulnerable populations such as the elderly and those with chronic inflammatory conditions such as asthma and COPD.

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