Immune Responses to Pneumococcal Immunisation in HIV-infected Adults in the UK

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Abstract

Streptococcus pneumoniae is a major cause of morbidity and mortality in HIV infected individuals. Mortality rates remain high despite modern antibiotics, therefore, successful vaccination is key to preventing infection. Vaccination strategies against pneumococcus include a pure polysaccharide vaccine, Pneumovax® (PPV-23), and a polysaccharide-conjugate vaccine, Prevenar-13 ®(PCV-13). PCV-13 is given as part of three vaccine series in infants but is currently recommended only as a single vaccine in adults. The evidence base for either vaccine in adults is limited and guidelines vary. In 2009, national guidelines recommended PPV-23 vaccination but a PCV-13+PPV-23 schedule is now recommended (BHIVA, 2008 & 2015).

The *Assessment of Immune Responses to Routine Immunisations* (AIR) is an observational study that has examined the immune response to UK recommended vaccinations in adults with HIV. Firstly, the AIR study went on to examine the impact of HIV-infection on pneumococcal vaccination with PPV-23, as assessed by pre- and post- vaccine IgG antibodies against 12 pneumococcal (Pn) serotypes (Pn 1, 3, 4, 5, 6B, 7F, 9V, 14,18C, 19A, 19F, and 23F at the WHO (World Health Organization) protective threshold 0.35µg/mL in ≥ 8/12 serotypes threshold using a 19-plex Luminex-based assay. HIV-infected patients responded poorly to a single dose of PPV-23 compared to HIV-negative controls.

AIR then established that PCV-13 could increase the percentage of patients that reach WHO protective thresholds compared to a single dose of PPV-23 and that immune responses to PCV-13 could be maintained for a longer period compared to PPV-23. Response rates could be further improved by booster doses of PCV-13.

Low antigen-specific IgG concentrations are associated with impaired opsonophagocytic killing against pneumococcus, thus a novel opsonophagocytic assay was developed in order to further understand the relationship between the quantity and opsonic functionality of Pn-specific antibody.

Furthermore, assessing responses to pneumococcal vaccination is important in determining immunogenicity. Thus, this thesis also explored the whole vaccine and serotype IgG subclass (IgG1-IgG4) response to PPV-23 and PCV-13 by developing novel Pn-specific IgG subclass assays. Lastly, HIV-infection is characterised by a dysregulated humoral system, therefore, we have examined the impact on different B cell populations at baseline and relationships with total and Pn- specific antibody post-vaccination with PCV-13 are described.

In summary, this study aimed to examine the impact of HIV-infection on pneumococcal vaccination by investigating the quantity and quality of the Pn-specific IgG response, IgG subclass responses, and the effects of a dysfunctional humoral system on total and Pn-specific antibody. Findings would be informative in developing vaccination strategies in HIV-infected adults in the UK.

Publications arising from this thesis

Papers

1) Immunization of HIV-infected adults in the UK with Haemophilus influenzae b/meningococcal C glycoconjugate and pneumococcal polysaccharide vaccines.

MacLennan CA, Richter A, Hodson J, Faustini S, Birtwistle J, Whitelegg A, et al. J Acquir Immune Defic Syndr Nov 2016

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Prevenar-13 Booster Vaccination Overcomes Failure of a Single Dose of Pneumovax-23 or Prevenar-13 in HIV-infected adults.

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Hospital Admission Rates due to Respiratory Diagnoses in HIV-Infected Adults following Pneumococcal Vaccination

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Abbreviations

AIR……..……Assessment of Immune Responses

BAB…….…….....….blood agar base

BCR ……..……..……..……..B-cell receptor

BCTU……...…….……..…Birmingham Clinical Trials Unit

BHI……..……..……..brain heart infusion

BHIVA……..……..……British HIV Association

BSA……..…….....bovine albumin serum

CAP……..……..community acquired pneumonia

cART……...…combination antiretroviral therapy

CFU……..…..……...…colony forming units

CIS……..……Clinical Immunology Service

COSHH…….…….Control of Substances Hazardous to Health

CPG……...……..cytosine guanine dinucleotide

CKD……..……Chronic Kidney Disease

CSF…….cerobrospinal fluid

CWPS…..…….…… cell wall polysaccharide

DD……..……..……double dose

Diph……..……..……..Diphtheria

DMF…….....……...……dimethyl formamide

DMSO…….....……...……dimethyl sulfoxide

FCS……..…… Fetal calf serum

FITC……...……Fluorescein isothiocyanate isomer I

HAART…….....……..Highly Active Antiretroviral Treatment

Hib/HBA…….…..*Haemophilus influenzae* b

HIV…….....Human Immunodeficiency Virus

HLA……...……...Human Leukocyte Antigen

HPA……Health Protection Agency

IgA……..…..Immunoglobulin A

IgG……Immunoglobulin G

IgM…….....…Immunoglobulin M

IL-8 (CXCL8)…… ……..……..Interleukin 8

IPD ……...……..Invasive Pneumococcal Disease

IRAK-4……interleukin-1 receptor-associated kinase 4

JCVI……..…….……..Joint Committee on Vaccination and Immunisation

LytA…….……..autolysin

MAC……..…….....membrane attack complex

Men……..……..……meningococcal

MIA……multiplex immunoassay

mOPA……...…….multiplexed opsonophagocytic assay

NCS…….....……..Newborn Calf Serum

NEMO……..NF-kappa B essential modulator

NKT……..…….natural killer T-cells

OPKA…….……..opsonophagocytic killing assay

Orep……Optochin

OT ……...……opsonophagocytic titre

PALS…….……periarteriolar lymphoid sheath

PavA……..…….pneumococcal adherence and virulence factor

PBMCs……..…….peripheral blood mononuclear cells

PCV-13……..……Prevnar-13

PPV-23……..……..…Pneumovax-23

PsaA……...……pneumococcal surface antigen A

PspA……...……pneumococcal surface protein A

PspC……..……pneumococcal surface protein C

PFA……..……paraformaldehyde

PLL……..……..Poly-L-lysine

Pn……...pneumococcal

PPV-23……...……..…..…Pneumovax-23

QC……..……...……..quality control

RCT……...……....……randomised control trial

REC……...……...Research and Ethics Committee

RT……...……..…….room temperature

SD……..……...……single dose

Spec…….…….Spectinomycin

STGG …….…… skim-milk-trypton-glucose-glycerin

Strep ……...…… Streptomycin

TA……...…….....tetanus

THYA…….…….....Todd-Hewitt Yeast Agar

TLRs……..…….....Toll-like receptors

TLR4……...…… Toll-like receptor 4

TNF-α ……..……Tumor Necrosis Factor –alpha

TOPAS……..TOPAS

Trim ……..……Trimethroprim

TTC…….…….....triphenyltetrazolium chloride

VL……..……..…viral load

UHB……...……University Hospital Birmingham

UoB……..…….....University of Birmingham

WHO……..…..…….....World Health Organization

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Chapter 1. Introduction

1.1 Overview

The overview of this report is the investigation of the immune response to pneumococcal vaccination in the context of HIV-infection. Pneumococcal (Pn)-specific opsonic antibody is essential for effective phagocytosis and bacterial killing. The quantity and quality of specific Pn-antibody required for immunity to pneumococcus in HIV-infected adults is investigated. Thus, this report will primarily focus on B-cell responses to pneumococcal vaccination as dysregulation of the humoral immune system is characterised in HIV-infection. This chapter will discuss *Streptococcus pneumoniae* and the epidemiology of pneumococcal infections, particularly in HIV-infected individuals. The virulence factors of *S. pneumoniae* and the host immune response to these factors will be investigated. Pneumococcal treatment and vaccine options will also be discussed. Furthermore, a section on HIV immunology including CD4 counts, viral loads, and antiretroviral (ARV) treatment will be presented. The AIR (Assessment of Immune Responses to Routine Immunisation) study will also be introduced.

1.2. *Streptococcus pneumoniae*

Streptococcus pneumoniae (S. pneumoniae), a bacterium also known as a pneumococcus, is a major cause of pneumonia, sepsis and meningitis. Mortality rates remain high despite modern antibiotics, so successful vaccination is key to preventing infection.

S. pneumoniae is a gram-positive diplococcus with a polysaccharide capsule that is known to contribute to its virulence.

1.2.1 Taxonomy

The genus *Streptococcus* is a member of the Streptococcaceae family, which is divided into alpha-haemolytic and beta-haemolytic species. *S. pneumoniae* is an alpha-haemolytic species.

1.3 Epidemiology of pneumococcal infection

1.3.1 Pneumococcal infections in the HIV population

HIV-infected patients are at a higher risk of developing pneumococcal infection than the general population. The annual incidence of IPD is 2.5 per 1000 HIV population (aged 15-

44), which is fifty times higher than the general population (1). Furthermore, IPD was higher amongst those individuals who were not on antiretroviral therapy (ART) (2.8 per 1000) and severely immunosuppressed (5.6 per 1000) (1). IPD has increased 50-fold in HIV 15-44 year olds and 25 fold in 45-64 year olds in England and Wales (2000-2009) even in patients who have received ARV treatment and those with higher CD4 counts. (2).

Approximately 1.5% of adults living with HIV in the UK have suffered one or more episodes of invasive pneumococcal disease (IPD) (1), invasive disease includes meningitis and septicaemia. Bacterial pneumonia is the most common infection in HIV positive individuals and 70% of these pneumonias are caused by *S. pneumoniae* (3).

1.3.2 Pneumococcal serotype distribution in carriage, colonisation, infection, and disease

1.3.2.1 PCV-7 and PCV-13 effectiveness in the UK

Since the introduction of PCV-7 (in September 2006) and PCV-13 (in April 2010) to the childhood vaccine schedule in England and Wales in the UK, there has been a general reduction in vaccine serotype-related IPD; however, there has also been an increase in non-vaccine serotype replacement causing IPD including pneumococcal serotypes 7F and 19A (4). Following 4 years post-introduction of PCV-7 in the UK with coverage greater than 90%, vaccine-type IPD had decreased by 86% in all age groups, but there was still a substantial increase in non-vaccine serotype IPD in children $\leq$ 5 years and in adults 65 years and older (5). PCV-13 replaced PCV-7 with high coverage of over 94.4% coverage in England and 95.5% coverage in Wales with the expectation that carriage of pneumococcal serotypes in the nasopharynx would change slightly and that colonisation of IPD-causing serotypes would be substantially reduced (4,6,7). An observational cohort study performed in 2013/14 by Public Health England found that there was an increased incidence of non-vaccine serotype disease since the introduction of PCV-13 and an overall decrease in IPD caused by vaccine serotypes across all age groups (4). Most notably, there was a significant decrease in vaccine serotypes 1, 6A, 7F, and 19A across all age groups, but a significant increase in non-PCV-13 serotypes including 8, 9N, 10A, 12F, 15A, 16F, 24F, 33F, and 35B in individuals over the age of 5 in 2013/14 (4). Although there has been serotype replacement disease, IPD has decreased by 56% in the UK compared to 2006 before the introduction of PCV-7 (4). Furthermore, nasopharyngeal carriage of any of the additional 6 serotypes in PCV-13 that were not included in PCV-7 in children had increased by 38%, but decreased by 95% 2 years post-introduction of PCV-13 replacement of PCV-7 (8). There was a 19% increase of non-

vaccine serotypes in all age groups after the introduction of PCV-7 and a 25% increase in non-PCV-13 vaccine serotypes 4 years post-PCV-7 replacement (4,5).

Interestingly, in individuals older than 45 years old, non-PCV-13 vaccine serotypes causing IPD were the largest and only small reductions were seen within PCV-13 vaccine-type IPD in 2016-17, which suggests that potentially PCV-13 should be given to this age group as individuals may not be protected from the herd effects from the childhood PCV-13 vaccination scheme (9).

As of 2016-17, non-PCV-13 vaccine serotypes caused 78-88% of IPD cases across all age groups in the UK, in particular: <2 years old (y.o.), 2-4 y.o., 15-44 y.o.,45-64 y.o., and ≥65 y.o, but an overall decrease in 5-14 y.o. (9). These serotypes include 8, 9N, 12F, 15A, 22F, and 33F. Two PCV-13 serotypes still causing IPD across all age groups include 3 and 19A as of 2016-17 and they were still detected in nasopharyngeal carriage in children as 2015-16 (9,10). Furthermore, carriage of serotypes 3, 19A, and 19F has also been found 3 to 4 years post-introduction of PCV-13 to the childhood vaccination schedule in Massachusetts in the USA (11).

Common serotypes amongst children in the UK with lower respiratory infections (LRTIs) include serotype 3, 19A, 12F, 22F, and 33F (12). Common serotypes in children that are responsible for meningitis included 10A, 12F, 15B/C, 22F, and 33F (12). Serotypes causing septicaemia cases included 12F, 15B/C, 22F, 23B, and 33F (12).

Worryingly, serotype 3 is still causing 65% of IPD cases in adults over 65 years of age and 24% of IPD cases in 45-64 year olds in England and Wales as of 2016-17 (9). Serotypes 8, 9N, and 12F are classed as emerging serotypes as their prevalence has increased rapidly over the last 3 years and has caused over 40% of total IPD cases. Surprisingly, serotype 8 has caused over 20% of IPD cases in 2016-17. Furthermore, 8 of the 10 most prevalent serotypes that have contributed to IPD cases in 2016-17 are included in the PPV-23 vaccine (9). Furthermore, the increase of these serotypes were prevalent in adults over the age of 65 years old despite the national UK PPV-23 vaccination programme in this patient cohort having been in place since 2003 (9). Even though non-PCV-13 vaccine serotypes have been causing IPD in the UK as of 2016-17, there was no increase in non-PCV-13 vaccine serotypes in the USA until 2015 (13). Interestingly, the emergence of non-PCV-13 vaccine serotypes may have been caused by a wide array of factors including the high incidence of influenza cases during the winter of 2014-15. This was caused by antigenic and genetic mismatch

between the prevalent influenza viruses and the influenza vaccine (14). Furthermore, the UK introduced the live attenuated influenza vaccine for young children in 2013-14, which may have led to the alteration of the nasopharyngeal microbiome thus impacting the carriage of pneumococcus and subsequent disease (15).

Higher valency pneumococcal vaccines may help to reduce IPD infections in some of the emerging non-PCV-13 serotypes including PCV-15, which will include all of the original serotypes included in PCV-13, plus 22F and 33F (16). However, vaccine serotype replacement could still be problematic in the future even with higher valency vaccines, thus, it would be advantageous to find a highly conserved protein present on all 93 pneumococcal serotypes. Some of the best protein candidates include: iron transport lipoproteins, histidine triad proteins, pneumolysin, pneumococcal surface protein A, pneumococcal surface protein C, and pneumococcal surface antigen A, although these pneumococcal protein vaccine candidates are probably at least 5 years away from clinical efficacy trials (16).

1.4 Risk factors for pneumococcal disease

1.4.1.Environmental risk factors

Pneumococcus mainly affects the young, elderly, and immunocompromised, notably those with HIV-infection. A bimodal distribution of pneumococcal infection is apparent with children less than 2 years and adults who are 65 years and older carrying the majority of disease (17,18). Other risk factors include alcoholism, cochlear implants, cerebrospinal fluid (CSF) leaks, chronic heart disease, chronic kidney disease (CKD), chronic liver disease, smoking, asplenia, deficiencies in complement and immunoglobulin, diabetes mellitus, influenza, liver and lung disease, sickle cell disease, other respiratory diseases, as well as other immunocompromised states including HIV (12,19–22).

This high susceptibility to pneumococcal infection has a major impact both in terms of quality of life for the patient and significant healthcare service costs , such as lengthy hospital stays, treatment , as well as the possibility of increased mortality due to antibiotic resistance In a Dutch analysis that involved cost-effectiveness of PCV-13, it was found that vaccination with PCV-13 would cost €85.3 million, but would prevent an average of 9850 cases of outpatient community acquired pneumonia (CAP), 1850 cases of inpatient CAP, 2050 episodes of IPD, ,and 630 deaths in the cohort. During the lifetime of the cohort,

patients would gain 5500 life years and 4850 quality adjusted life-years (QALY). Vaccination with PCV-13 would thus save approximately €43.2 million (23).

1.5 Clinical presentation

Pneumococcal pneumonia manifests itself as acute community acquired pneumonia (CAP). Pneumococci colonise the lower airway, but can be expelled via antimicrobial peptides, cough, innate immune defences, and mucociliary clearance (24). If these initial innate immune defences fail, then infection will be established in the host.

Presenting symptoms would include chills, cough, fever, dyspnoea (shortness of breath), and malaise (24). A chest radiograph may indicate pneumococcal pneumonia. Bacterial sepsis is the most common presentation in children less than 5 years of age. However, pneumococcal pneumonia is the most common presentation for adults (18).

If the infection is left untreated, patients may develop blood-tinged and/or brownish purulent sputum, in addition to acute respiratory failure, septicaemia, and multi-organ failure that can lead to death within a short period of days following the onset of clinical symptoms (24).

Patients that present with recurrent pneumococcal pneumonia should be further investigated as this may indicate an undiagnosed immunocompromised condition such as HIV, congenital or acquired B-cell disorders, lymphoreticular disorders, multiple myeloma, and ciliary dyskinesia (defects in the cilia lining of the respiratory tract) (24,25).

1.5.1 Diagnosis

Pneumococci are α-haemolytic and can be cultured on blood agar base plates (BAB) in a 37°C CO_2 incubator. Pneumococci are identified as gram stain positive diplococci (Figure 1.3). Cultures could be sampled from the blood and sputum, as well as other tissue sites before any antibiotics are administered to the patient.

Pneumococcal infection can be detected by urinary antigen secretion of the C-polysaccharide that originates in the pneumococcal cell wall. This assay is an immune-chromatographic test called Binax NOW® (26). This rapid-test also can be used for detecting nasopharyngeal carriage.

A more highly specific- identification of infectious pneumococcal strains would be DNA-sequencing or PCR assays, which are rapid and non-culture based methods.

1.5.2 Treatment

Historically, pneumococcal infection has been treated with penicillin since the late 1940s (24,27). However, penicillin-resistant strains of *S. pneumoniae* have emerged globally since the middle of the 1970s due to structurally modified penicillin binding proteins that allow for peptidoglycan synthesis (24,25). It is suggested that resistance mechanisms can be overcome by administering high doses of penicillin to patients with resistant strains of pneumococci (24,25). Patients that have confirmed cases of mon-meningeal pneumococcal strains can be treated with high dose, β-lactam antibiotics, which may include penicillins, and second or third generation cephalosporins (24).

Pneumococci have developed resistance to antibiotics such as: fluroquinolones, macrolides, trimethroprim, vancomycin, in addition to other various antibiotics (24). This is thought to have arisen partially as a result of aberrant prescription and usage of broad-spectrum antibiotics that do not completely clear pneumococci from the infected patient. Thus, prevention through vaccination is a way of overcoming antibiotic resistance.

1.6 *S. pneumoniae* as a pathogen

There are over 90+ different types of *S. pneumoniae*, which are known to cause various types of non-invasive and invasive illnesses. Pneumococcal serotypes are defined by distinct variabilities in the structures of their polysaccharide capsules. The various serotypes can be identified by the quellung reaction, which is a serum-based test (28). Some serotypes may be closely related, antigenically, and may be placed in a group ie 6A and 6B (29). There are also non-encapsulated pneumococci that are referred to as non-typeable. The different serotypes possess the ability to cause either asymptomatic carriage or various types of illnesses and infections (30).

Such illnesses can include but are not limited to pneumonia, meningitis, sepsis, acute otitis media, etc. It is also important to note that mortality against pneumococcus has not changed since the 1950s. Currently, pneumonia is the sixth leading cause of death in the UK and *S. pneumoniae* is the most common cause of pneumonia (31). It mainly affects the young, elderly, and immunocompromised.

1.6.1 Mode of Infection

Pneumococci infect the host by entering the lower airway. Pneumococcal disease subsequently follows asymptomatic colonisation of the host in the mucosal surfaces of the upper respiratory tract.

S. pneumoniae can be spread via nasopharyngeal carriage via inhalation of aerosols via asymptomatic carriers or a patient with pneumococcal disease. It occurs naturally in approximately 100% of healthy pre-school children at some time and 30% of healthy older children and adults (32). Colonisation declines with age in approximately 10% of adults (33).

Colonisation can progress to invasive (bacteraemia) or non-invasive infections (acute otitis media). Pneumococcal infection can also lead to acute conjunctivitis, arthritis, empyema, meningitis, osteomyelitis, peritonitis, and pneumonia (34).

1.7 *S. pneumoniae* and resistance to killing

1.7.1 Virulence factors

S. pneumoniae possesses a number of physical structures that contribute to its virulence and subsequent evasion of the host species.

1.7.1.1 The capsule

S. pneumoniae is an encapsulated bacteria. The capsule is composed of a polysaccharide layer which is approximately 200-400 nm thick. This structure is considered one of the main virulence factors of the pneumococcus and can be considered as anti-phagocytic (35). The capsule restricts access to cell-bound complement and may also reduce the amount of complement deposited on its surface dependent upon its variable thickness. Therefore, polysaccharide capsule coat thickness is associated with increased colonisation capabilities nd immune evasion.

The C-type lectin, SIGN-R1, expressed by macrophages binds capsular polysaccharide from various serotypes and is essential to pneumococcal clearance (36,37).

Currently, the capsule is a vaccine target. As mentioned before, capsules distinguish various serotypes from one another (29). Thus, vaccinations have been developed to include polysaccharides from multiple serotypes in order to cover invasive strains within the population. In the future, it is more likely that that vaccine targets will include more conserved molecules that cover multiple serotypes. The capsule is essential for colonisation and consequently for its dissemination in the host. Thus, it is an extremely important virulence factor. It is known to escape clearance in the host by its secretion of mucous once invasive disease is established within the host (24,38).

1.7.1.2 The cell wall

Additionally, the cell wall is an important virulence factor as it facilitates attachment of the pneumococcus to activated lung cells. The cell wall consists of phosphorylcholine that binds to the platelet-activating factor (PAF). which is upregulated during inflammation and viral infections, such as HIV (35).

1.7.1.3 Pneumolysin

Pneumolysin, a pore-forming protein, is a toxin that establishes the production of inflammatory products such as TNF-α, IL-1β, NO, and IL-8, prostaglandins, and leukotrienes (35). IL-8 (Interleukin 8 or CXCL8), a chemokine produced by monocytes, macrophages, fibroblasts, endothelial, and epithelial cells, recruits and activates innate immune cells such as neutrophils to sites of infection (39). Pneumolysin, is also known as haemolysin due to its ability to lyse red blood cells. It is toxic to pulmonary endothelial and epithelial cells. It also has mechanisms that can contribute to the evasion of the immune system, including the inhibition of appropriate lymphocyte and phagocyte function, in addition to interfering with the complement pathway (35). Pneumolysin is an important virulence factor with regards to the pathogenesis of meningitis as it can cause damage and induce apoptosis in neural cells (35,40,41). It also can bind Toll-like receptor 4 (TLR4), which protects mice from invasive pneumococcal disease (IPD) (35,42). In humans, TLR defects such as IRAK-4 (interleukin-1 receptor-associated kinase 4) deficiency causes recurrent systemic pneumococcal disease in children (43). IRAK-4 deficiency inhibits appropriate TLR-signaling and function, thus abrogating the innate immune response to pneumococcus , which can lead to fatal pneumococcal disease such as meningitis despite prophylactic vaccination and subcutaneous or intravenous immunoglobulin therapy (44,45). Often IRAK-4 deficiency is undetected in patients as they exhibit normal immunological profiles including T, B-cell , and neutrophil responses, normal total IgG, IgA, and IgM levels, and complement activity (45,46).

Pneumolysin also facilitates protection from complement-mediated clearance. A study found that absence of complement in genetic knock-out mice reduces the need for pneumolysin as an essential virulence factor and that it acts via the classical pathway as opposed to the alternative pathway (36,47). Pneumolysin also acts as a decoy toxin as it is released into the host environment and thus, directs complement activation away from the pneumococcal cell surface, thus allowing pneumococcal survival (36). Increased

complement activation as opposed to inhibition may induce even host-tissue damage, thus contributing even more to pneumococcal pathogenesis.

1.7.1.4 Capsular Surface Proteins

The pneumococcus possesses three families of capsular surface proteins including choline-binding proteins, LPXTG-anchored proteins, and lipoproteins, which may also serve as potential vaccine targets as they are conserved regions.

Choline-binding proteins are attached to the cell wall via non-covalent bonds of the carboxy-terminal end of the protein with the phosphorylcholine of the cell wall. The proteins that are particularly virulent include autolysin (LytA), pneumococcal surface protein A (PspA), and pneumococcal surface protein C (PspC). Interestingly, PspA is expressed by many clinically important pneumococcal serotypes (35), which presents a potential vaccine target. It is known to inhibit complement activation and the deposition of C3b in addition to the alternative pathway component C3 convertase. These contributing factors therefore reduce clearance of various pneumococcal serotypes. PspC is involved in the adhesion of pneumococci to the nasopharynx and some forms of PspC may be involved in the binding of the complement-control protein factor H (35).

LPXTG proteins are composed of hyaluronidase and neuraminidase enzymes. Hyaluronidase leads to the breakdown of mammalian connective tissue and extracellular matrix and is secreted by most clinical isolates. Neuraminidase (of which there are three) cleaves *N*-acetyl-neuraminic acid from glycolipids, lipoproteins, and oligosaccharides that originate on cell surfaces in body fluids (35). For example, neuraminidase A is involved in nasopharyngeal colonisation. These two proteins are involved in colonisation and facilitate the attachment of pneumococci to various host cells.

Lipoproteins are adhesins that are involved in the adhesion of pneumococci to the host cells. An example is pneumococcal surface antigen A (PsaA). PsaA resides in the ABC transporter system and is involved in the transport of manganese. PavA is another adhesion that is involved in the binding of pneumococci to fibronectin, which is a host-cell glycoprotein that is involved in cell adhesion.

1.8 Immunity to *S. pneumoniae*

1.8.1 The Spleen

The spleen is a secondary lymphoid organ found in the upper left quadrant of the abdomen that filters the blood. Its primary function is to remove opsonised bacteria and respond to blood-borne pathogens (48). It also destroys dead erythrocytes (48). It also provides a reservoir of antigen-specific B-cells that respond to bacterial pathogens in the absence of T-cell help (48). The spleen is a highly-structured organ that is composed of the red pulp and white pulp. The red pulp contains macrophages and red blood cells. The white pulp, which is distributed along the central arterioles of the spleen, contains the B and T-cell areas. T-cells are mainly distributed in the periarteriolar lymphoid sheath (PALS) around the central arterioles. B-cells are found primarily in the primary (no germinal centre) and secondary lymphoid tissues (with germinal centres) (48,49). Secondary lymphoid follicles tend to generate activated B-cells. The marginal zone forms the outer region of the PALS, which contains slowly recirculating B-cells in addition to macrophages, which present T-independent antigen to B-cells (49).

1.8.1.1 Germinal Centers

Germinal centers are structures that develop in the B-cell follicles of the secondary lymphoid tissues in response to T-cell dependent antibody reactions within the first three weeks after initial exposure to an antigen (50,51). They will reappear after a repeated encounter with the same antigen and are also involved with the clonal expansion of B-cells, somatic hypermutation in B-cell Ig v-region genes, and positive selection of B-cells that can respond to antigen-specific signals (50). Germinal center B-cells express high affinity antibodies, which subsequently develop and differentiate into antibody-secreting plasma cells and memory B-cells that provide long-term protection against pathogens (52). Furthermore, CD40 is expressed by B-cells and increases B-cell proliferation, Ig class switching, and somatic hypermutation. Subsequent binding of CD40 to CD40 ligand (expressed on helper T-cells) also helps to drive resting B-cells to become activated in response to T-dependent antigens. Furthermore, absence or blockade of CD40 ligation via protein antigens inhibits germinal center formation, thus inhibiting the capacity for high affinity antibody to be generated in an individual (53,54).

1.8.2 Antibody

Antibodies, also known as immunoglobulins, are proteins that are produced in response to specific antigens (48). They are instrumental in the immune response because they

neutralise antigens by binding to their surfaces (55). Antibodies are important in the immune response to pneumococcus because they neutralise the shielding effect of the polysaccharide capsule by opsonisation for ingestion and destruction by phagocytes (56). Some serotypes are more likely to cause IPD, thus these serotypes are covered in the multivalent vaccines. There is cross-protective antibody between vaccine serotypes groups including Pn serotype 6A and 6B, and Pn serotype 19A and 19F (57). However, there is no cross-protective antibody between different serotype groups, which is why it is important to measure each pneumococcal-specific antibody.

1.8.3 Phagocytes

1.8.3.1 Macrophages

Alveolar macrophages are phagocytic cells that kill pneumococci in the lung (24). They also phagocytose neutrophils that have undergone apoptosis as a result of clearing pneumococci. Macrophages also undergo apoptosis themselves in order to kill phagocytosed pneumococci (24,58).

1.8.3.2 Neutrophils

Galectin-3 is a host adhesion molecule expressed and secreted by human monocytes and macrophages that recruits neutrophils in response to pneumococcal infection (59). It is also involved in activating the innate immune response such as degranulation of mast cells and oxidative burst via neutrophils (59) An influx of neutrophils to the site of infection during pneumococcal pneumonia can be due to α- chemokines, as well as many other chemoattractants including CXCL8, most notably induced by pneumolysin (60,61).

1.8.4 Complement

It binds C1q to antibody- polysaccharide complexes or C-reactive protein (CRP) binding to phosphocholine molecules of pneumococci. Pneumococci are opsonised by the complement fragments (C3b, C4b, and C5a) as generated by the complement cascade. In turn, this opsonisation promotes phagocytosis via binding receptors expressed via phagocytic cells. The classical pathway and the induction of natural IgM antibodies are the most important with regards to the clearance of pneumococcus (62).

The complement system is a group of serum molecules that control inflammation and is activated by the innate and adaptive immune system. Complement facilitates the disposal of immune complexes and mediates lysis of pathogens or cells that are opsonised by antibody.

Furthermore, this system is comprised of the classical, alternative, and lectin pathways.

Complement may be able to destroy other microbes through lysis via the membrane attack complex (MAC), but it cannot destroy pneumococcus alone. Opsonisation with pneumococcal-specific antibody is needed (36). Thus, this binding of antibody and complement lead to opsonophagocytosis and killing.

The complement system bridges the two arms of an immune response: the innate and adaptive immune systems. There are three pathways, which include: the classical, the lectin, and the alternative pathway (63).

1.8.5 Cytokines

Cytokines are small proteins that are secreted and released by body cells that induces interactions and communication between cells in an autocrine, paracrine, or endocrine action (64). Upon entrance of pneumococci into the host respiratory tract, anti-inflammatory and pro-inflammatory cytokines are generated as part of the innate immune response (24). TNF-α is an important cytokine that is produced in response to pneumococcal infection as it inhibits and/or eliminates the growth and spread of pneumococci (65). Interleukin-1 also protects against pneumococcus (66). Interleukin-17 (IL-17) is an important cytokine that is produced by $CD4^{+}$T-cells or Th17 cells that has been described as being important for the clearance of pneumococcus in the context of carriage and colonization (67,68).

1.8.6 Toll-like receptors (TLRs)

Toll-like receptors are pattern recognition receptors that are important in pneumococcal infection. Pattern recognition receptors comprise a part of the innate immune system. They are located on the host cell surface. Their primary function is to establish inflammation, which will ultimately lead to the destruction of the bacterial pathogen (36). There are 10 TLR genes expressed in humans which recognise distinct sets of molecular patterns that are derived from Gram-positive and Gram-negative bacteria, fungi, protozoa, and viruses (69).

Human genetic defects in TLR-signalling are associated with an increased susceptibility to pneumococcal infection. TLR-4 is essential in the recognition and innate immune response to gram-negative bacteria; however, it does recognise TLR-4 on macrophages via pneumolysin secretion (42). Furthermore, pneumolysin directly interacts with TLR-4 (70). Malley et al. (2003) found when comparing wild-type (WT) and TLR-4 KO mice in a nasopharyngeal carriage model that TLR4 mice were much more heavily colonised by pneumococcus and much more likely to develop IPD. TLR4 recognises pneumolysin and thus, limits the extent of pneumococcal proliferation within the nasopharynx (42) . TLR9 is required for the

detection of general bacterial DNA and optimal phagocytosis and killing of pneumococci via macrophages that reside in the lung (24).

1.8.7 T-cells

$CD4^+$T-cells are an important component of the adaptive immune response; however, they have also been shown to play a role in the early response to pneumococcal infection (71). $CD4^+$T-cells have been shown to migrate towards pneumococcus *in vitro* and pneumolysin was shown to play a role in this migration . TLR4 is also expressed on T-cells, which may play a role in T-cell migration towards pneumococcus (72). Malley et al (2005) found that $CD4^+$T-cells play an essential role in antibody-independent acquired immunity to pneumococcal colonisation (73).

γδ-T-cells, which are present in respiratory tissues, play an important role in the resolution of pneumococcal infection by the maintenance and regulation of inflammatory cell populations such as alveolar macrophages and pulmonary dendritic cells that may form granulomas, which may contribute to pneumococcal pathogenesis and a delay in the resolution of inflammation (74).

1.9 Vaccines against *S. pneumoniae*

National guidelines published by BHIVA in 2008 (75) recommend HIV patients receive immunisation with polysaccharide pneumococcal vaccine (PPV-23; Pneumovax) (76). However, these guidelines have been established with a limited evidence base. PPV-23 responses in adults with many comorbid illnesses (such as CLL, renal disease, etc.) have a limited response to vaccination and any response is short-lived due no memory B-cells being generated. Polysaccharides stimulate B-cells via cross-linkage of the B-cell receptor (BCR), which drives the production of immunoglobulin (18). No new memory B-cells are generated and the former memory B-cell pool is decreased, which has an effect on subsequent immune responses to booster vaccines. In other words, the memory B-cell pool is terminally differentiated to plasma cells (77).This is a medical challenge as PPV does not stimulate T-cell activation so no long-term memory cells are generated. If patients are re-vaccinated with PPV-23, before 5 years, they get a much lower immune response called hyporesponsiveness. This leaves a number of years where patients are not protected. However, it is important to note that most vaccination studies have been performed at a short duration and further evidence is needed to elucidate whether hyporesponsiveness can last beyond 5 years post-vaccination with PPV-23. A study performed in Fiji in 2016 found that PPV-23 boosting of

children previously vaccinated with PCV-7 did results in hyporesponsiveness; however, there were no clinical consequences of this and after 5 years, immune responses to PCV-13 vaccination were considered normal (78).

It has been suggested that hyporesponsiveness is caused by the large amount of Pn polysaccharide that is contained within the PPV-23 vaccine (approximately 575 μg compared to less than 20 μg in Prevenar-7 (PCV-7)) and repeated antigen exposure and stimulation, which leads to B-cell exhaustion or depletion including memory B-cell and B1b cell subsets that are important for protection against pneumococcal serotypes (79).

In contrast, the conjugate vaccine, Prevnar-13 (PCV-13), which works by stimulating T cells, generates a memory response which can be augmented with repeat vaccination, as undertaken in infants in the UK vaccination schedule. The carrier protein from the conjugate vaccine is presented to a polysaccharide-specific B-cell and is then is internally processed. Carrier protein peptides are then presented to specific T-cells, which then induce T-cell help for the production of plasma and long-lived memory B-cells (18).

PCV-13 covers approximately 61% of the serotype distribution amongst people co-infected with IPD and HIV (1). In fact, three out of every 5 IPD episodes among HIV-positive adults could be prevented by successful vaccination with PCV-13 (1).

In 2015, the British HIV Association (BHIVA) suggested that HIV-infected adults should be vaccinated with a single-dose of PCV-13 irrespective of CD4 count, viral load, and ARV therapy without any booster doses(80). This had changed from the previously recommended single-dose of PPV-23. Most individuals (approximately 80%) who receive PPV-23 develop antibodies within 2-3 weeks, but in immunocompromised individuals such as HIV-patients, these responses decline rapidly or are absent entirely. Furthermore, boosting with PPV-23 is not recommended if previously vaccinated due to issues of hyporesponsiveness (81). HIV-patients have already shown a diminished antibody response to PPV-23, as well as PCV-13, particularly in the AIR (Assessment of Immune Responses to Routine Immunisations) study that has been established at the Queen Elizabeth Hospital HIV Service in collaboration with the University of Birmingham Clinical Immunology Service (CIS). About 50% of patients did not respond to a single-dose of PCV-13, which showed a need for the additional boosters.

Prevention of pneumococcal infection is key with the ever increasing threat of antibiotic resistance. Thus, vaccination with PCV-13 is essential as it covers many of the resistant strains.

In 2012, the JCVI (Joint Committee on Vaccination and Immunisation) reviewed the impact and cost-effectiveness of vaccination with PCV-13 (82). It rejected the implementation of a vaccination programme that would offer PCV-13 to some clinical risk groups and older adults (over 65 years old) in the UK on grounds of the lack of cost-effectiveness and that indirect immunity would offer protection against PCV-13 serotypes across the population. The JCVI considered that the following clinical risk groups would benefit from vaccination with PCV-13 including: patients receiving bone marrow transplants, patients with acute and chronic leukaemia, multiple myeloma, and patients with genetic disorders that severely suppress the immune system (including, IRAK-4, NEMO (NF-kappa B essential modulator), and complement deficiency) (82). NEMO deficiency is caused by a hypomorphic mutation in the NEMO gene, which leads to impaired NFκB activation, thus leading to a decreased activation of mature naïve B cells (83). Consequently, this decreased activation of mature naïve B cells leads to impaired antibody class switch recombination and antibody synthesis (83).

Currently, the Green Book advises that clinical risk groups include individuals with: asplenia, chronic respiratory disease, chronic heart disease, chronic kidney disease, chronic liver disease, diabetes, immunosuppression, cochlear implants, cerebrospinal fluid leaks, who undergo bone marrow transplantation, and those with genetic deficiencies (84). As of November 2015, the JCVI have not changed their position with regards to the advice of adult pneumococcal vaccination and still support PPV-23 vaccination in adults (85)

There is no cross-protection between serotypes (however, this is not the case within serotype groups such as 6A/6B and 19A/19F) when individuals are vaccinated with pneumococcal vaccines. This is why vaccines need to include multiple serotypes (Figure 1.1). Also, different serotypes cause different types of disease (29,86,87). For instance, certain serotypes may be more likely to cause IPD and other types of pneumococcal disease.

Table 1.1. Serotypes included in available pneumococcal vaccines

Pneumococcal Vaccine	Serotypes included in vaccine
PPV-23	1,2,3,4,5,6B,7F,8,9N,10A,11A,12F,14,15B,17F,18C,19A,19F,20, 22F, 23F, and 33F.
PCV-7	4, 6B, 9V, 14, 18C, 19F, and 23F
PCV-10	1, 4, 5, 6B, 7F 9V, 14, 18C, 19F, and 23F
PCV-13	1, 3,4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F, and 23F

1.9.1 Measuring antibody responses to pneumococcal vaccination

Quantification of the IgG response to pneumococcal vaccination assesses protective antibody levels and can be used to monitor high-risk or immunocompromised patients when their protective levels drop below WHO threshold levels. Two ways that one can measure vaccine-induced antibodies to pneumococcus is through the quantity of capsular polysaccharide-specific antibody produced and the quality or ability of the antibody to opsonise and promote phagocytosis of the pneumococcal serotypes.

1.9.1.1 Quantification of pneumococcal-specific IgG- pneumococcal immunoassays

The earliest method for measuring the antibody response to pneumococcus was a radioimmunoassay, which was then replaced by enzyme-linked immunosorbent assays (ELISAs) (88).

The World Health Organization (WHO) established a pneumococcal-serotype specific IgG ELISA method, which was standardised for use in clinical and research laboratories around the world in 2002 (89). Although this method could still generate serotype-specific IgG results, the assay would need to be run multiple times in tandem. Thus, an alternative method using a multiplex microsphere assay using pneumococcal polysaccharide coated Luminex beads allows for the simultaneous measurement of IgG against a wide array of pneumococcal serotypes in one assay (90). This method requires a substantially smaller

volume of patient serum and is much less time-consuming. The dynamic range of the multiplex immunoassay is much greater than the standard ELISA, thus allowing for greater sensitivity and the subsequent removal of additional sample dilutions for patient samples where there is a high concentration of pneumococcal serotype-specific IgG.

The development of the pneumococcal multiplex immunoassays has increased significantly over the last 15 years with the establishment of a nonaplex assay developed at Public Health England in Manchester, 14-plex assay established at the University of Utah School of Medicine in Salt Lake City, USA, and a 19-plex assay established at the University of Birmingham, Clinical Immunology Service, in Edgbaston, UK (91–95). The most important requirement of the multiplex assay is that the results compare to the ELISA technique. Some studies have shown that there is variability between the correlation of the ELISA and multiplex method for some pneumococcal serotypes; however, there are other studies that have demonstrated a robust correlation between the two methods, thus making multiplex immunoassays a reliable alternative to the WHO ELISA method (92,94,96).

1.10 Immunity against HIV

HIV infection induces immune suppression and chronic inflammation, which ultimately leads to the exhaustion of the immune system (97). Due to this significant dysregulation of the immune system, HIV-infected patients are particularly vulnerable to acute respiratory infections including pneumococcal disease (76).

HIV is characterised by the progressive loss of $CD4^+$T-cells. Normally, these lymphocytes upon stimulation with antigen release B-cell growth factors, IL-2, and interferons that regulate the growth, maturation, and activation of cytotoxic T-cells, macrophages, and natural killer T-cells (NKT) (98). During the initial weeks of HIV-infection, there may be a small increase in the rise of $CD8^+$T-cells that may be in the peripheral circulation; however, during the asymptomatic stages of the disease, many individuals will have normal levels of circulating $CD8^+$T-cell subsets (98). Another hallmark of infection in progressive HIV-infection would be polyclonal B-cell activation that would result in a dramatic increase in serum immunoglobulin levels (98).

Normally, during the adaptive immune response, an acute infection will be cleared within 4-7 days (22).

However, during acute HIV infection, $CD4^+$T-cells are depleted dramatically within the first 2 to 6 weeks of infection. This acute phase of infection is usually accompanied by a flu-like illness also known as seroconversion , in about 80% of HIV-infected individuals (100). High titres of HIV virus are present in the blood during this period. Furthermore, an increased number of $CD8^+$T-cells are found in the peripheral blood (98). Following this period, the adaptive immune response is established and $CD4^+$T-cells are restored. However, viral load is not reduced (asymptomatic phase). During the symptomatic phase, as $CD4^+$T-cell counts fall to around 500 cells per µl, opportunistic infections begin to overwhelm the immune system (1200 cells per µl is normal). This period can last anywhere between 6 months and 20+ years post-primary infection. A person is said to have AIDS when $CD4^+$T-cell counts fall below the 200 cells per µl threshold. Most individuals will develop AIDS unless the HIV viral load is suppressed for a lifetime with anti-retroviral treatment (ARV).

If HIV-infection is left untreated, AIDS can develop within the patient. An untreated patient can survive for up to 6-12 months following opportunistic infections, 9-12 months following Pneumocystis jirovecii pneumonia, and 20-30 months following Kaposi's sarcoma (34).

1.10.1 CD4 counts and Viral load (VL)

HIV-infected $CD4^+$T-cells located within the plasma have a short half-life of approximately 2 days (101). The majority of detectable virus (approximately 95%) within the plasma is thought to be derived from these $CD4^+$T-cells. $CD4^+$T-cells found within the T-cell zones of lymphoid tissue may become infected as a result of activation during an immune response (102). HIV may lie dormant within memory $CD4^+$T-cells and macrophages, which are reservoirs of infection (103).

Survival for HIV-infected patients will vary depending on whether or not they have initiated ARV treatment, previous treatment and viral resistance, viral load, HLA type, and viral mutation rate (98).

1.10.2 B-cells and HIV

HIV infection is characterised by dysregulated humoral immune responses, which include hypergammaglobulinemia or hyper IgM syndrome that may induce inhibitory, blocking antibody. Hypergammaglobulinemia is a B-cell class switching defect where patients have a normal B and T-cell repertoire with normal to high levels of IgM in their serum. Typically, patients mount poor antibody responses to antigens that require T-cell help and only produce other immunoglobulin isotypes in minute amounts (104). It has been established

that memory B-cell populations are dramatically reduced and hyperactivated naïve B-cells are very common in the context of HIV-1 infection (105). Furthermore, the loss of memory B-cells may be due to overexpression of CD70 on activated T-cells, which can lead to terminal differentiation into plasma cells and/or spontaneous apoptosis (105–108). Activated naive B –cells have been suggested as the causative agent of hypergammaglobulinemia in HIV-1 infected patients (106). This cell population presents viral antigens via a BCR-independent mechanism (109). Furthermore, activated naïve B-cells from HIV-1 infected patients have the ability to secrete abnormal IgG due to reduced numbers of memory B-cells in circulation (106). Inhibitory, blocking antibody inhibits complement and antigen-specific antibody deposition, thus affecting effective opsonisation and subsequent phagocytic killing, particularly with regards to *S. pneumoniae.*

HIV-infection is also characterised by hyperactivation, which causes cytokines and growth factors to trigger the activation of B-cells, which is known to inhibit effective B and T-cell interactions. This could potentially lead to impaired responses to both pneumococcal vaccines.

Hypergammaglobulinemia and high spontaneous antibody production occur in the early stages of HIV-infection before the depletion of fully functional CD4+T-cells (110–114). This results in the production of excessive, but non-functional antibodies.

In addition to impaired B-cell responses, CD4+T-cell responses are reduced during HIV-infection since HIV infects this cell type. Subsequently, infected CD4+T-cells are killed via viral cytopathic effects or indirectly by activation-induced apoptosis (115). Thus, B-cell responses are essential with regards to regulating opportunistic infections that accompany HIV infection. However, due to the dependency on B- cells in the context of controlling infection, B-cells become exhausted. Also, in cases of chronic viraemia, B-cells lose their function even when patients are on antiretroviral treatment. As a result of HIV-infection, B-cell polyclonal activation occurs and antibodies are generated in abundance, although, they may not be fully functional (116).

Past research suggests that there is a correlation between invasive pneumococcal disease (IPD) and dysfunctional B-cell responses. It has been suggested that effective ARV treatment cannot reconstitute B-cell function (110). Low antigen-specific IgG concentrations are found in serum and there is evidence of poor opsonophagocytic killing against various

serotypes of *S. pneumoniae*, which suggests that the quality and number of antigen-specific antibodies is impaired (110).

After antigen stimulation by specific pneumococcal capsular polysaccharides, naïve B-cells can differentiate into IgM+ memory B-cells, which can produce pneumococcal-specific IgM without T-cell help. Later, during hypermutation and class-switching, pneumococcal specific IgM B-cells will differentiate into pneumococcal specific IgG or IgA memory B cells or plasma cells (110).

HIV+ patients may experience a decline in CD27+ memory B-cells (IgD+IgM+CD27+). Patients deficient in this cell phenotype experience are more vulnerable to lethal pneumococcal infections, particularly in the elderly population. IgM defects may impact B-cell maturation and the production of antigen-specific IgG or IgA and function. Thus, this leads to dysfunctional late humoral responses which cause severe bacterial infections. Therefore, IgM is essential in order to mount a successful immune response against pneumococcal infection. Also, patients with low levels of IgM memory B-cells have impaired immunity to pneumococcal infections and often develop recurrent respiratory infections.

IgA is also important in the humoral defence against pneumococcal infection, but more specifically at the mucosa, including the nasal and salivary mucosal sites (110). Patients with IgA deficiency mount impaired vaccine responses to pneumococcal vaccination, thus, they have high rates of recurrent infection and bronchiectasis (110).

Patients should mount an IgM+ memory B-cell response post-vaccination after 6-7 days; however, IgM+ memory B-cells are depleted and pneumococcal-specific antibody responses to vaccination are impaired in HIV-patients (even those that have received long-term ARV therapy).

A reduced number of CD4+T-cells, especially follicular helper CD4+T-cells, may also account for the impaired immune response to pneumococcal vaccination (110).

Additionally, there is a marked increase in spontaneous B-cell apoptosis and an impaired proliferation response to T-cell independent B-cell antigens in HIV-infection.

HIV leads to chronic immune activation via ongoing viral replication (117). Before effective ARV regimes came into effect, B-cell hyperactivation and impaired antibody responses were

widely reported in HIV-infected individuals. B-cells in the peripheral blood of healthy controls are phenotypically resting naïve or memory B-cells that are characterised by either a switched (IgA, IgG, or IgE) or unswitched (IgM or IgD) antibody isotype (117). However, in HIV-infected individuals, there are several additional B-cell subpopulations that reside in the peripheral blood including immature transitional B-cells, exhausted B-cells, activated mature B-cells, and plasmablasts due to the dysregulation caused by the disease (117–120).

Additionally, HIV-induced lymphopenia leads to the production of immature transitional B-cells in HIV infected individuals (117).

High viral loads associated with HIV-infection may lead to the expansion of several aberrant B-cell sub-populations, including immature transitional, hyperactivated, and exhausted B-cells, which collectively contribute to impaired humoral responses to vaccination (117) .

Loss of CD21 expression of peripheral blood B cells is a reliable marker of ongoing HIV replication and disease progression. Furthermore, CD21 lo B-cells consist of two heterogeneous populations including: CD27+ B-cells (as a result of HIV-induced activation and differentiation into plasmablasts) and CD10+ B-cells (immature transitional B-cells that are generated in response to the low numbers of T-cells that accompany HIV-infection . Thus, an exhausted B-cell subpopulation results.

Many patients in the AIR cohort will have initiated ARV treatment; however, ARV treatment may not actually assist the memory B-cell population. It has been evidenced that B-cell deficiencies persist even after several years of effective ARV treatment (117,121,122).

However, it has been documented that there are some B-cell abnormalities that are decreased by ARV treatment including hypergammaglobulinaemia and HIV-induced chronic immune cell activation, which is a key feature of HIV pathogenesis. There is an increased rate of B-cell death during ongoing viral replication, which leads to an overall net loss of B-cells. An increase in CD4+T-cell counts following the reduction in HIV viraemia by ART is associated with a decrease in the frequency of immature transitional B-cells. However, some B-cell deficiencies are not reversed by ARV treatment including the loss of memory B-cells and the decrease in memory B-cell function in chronically-HIV infected individuals.

Conversely, ARV treatment has been shown to increase the numbers of CD27+ resting memory B-cells following treatment; however, this reconstitution is slow and incomplete (117).

Studies have evidenced that the loss of responsiveness to pneumococcal vaccination was not reversed by ARV therapy (123,124). Furthermore, loss of memory function is associated with a decreased frequency of IgM+ memory B-cells, which makes individuals more susceptible to pneumococcal infection. However, early initiation of ART does seem to reverse the low frequency of IgM+ memory B-cells, which is a major defect of chronic HIV infection (125).

1.11 HIV treatment

Despite antibiotic treatment and preventative pneumococcal vaccination, the burden of pneumococcal disease is approximately 35-fold higher in HIV-infected patients when compared to age-matched HIV-uninfected individuals (2). Thus, it is essential that ARV treatment is given to patients as it has been shown to decrease rates of IPD in the HIV-infected population (126).

Current and potential therapies have been established as a result of investigating the way that HIV gains entry to the host cell and replicates inside the cell (98). Vaccines to induce the production of neutralising antibodies have not been as successful due to viral syncytium formation and its facilitation of cell-to-cell spread of the virus (98). However, fusion inhibitors such as enfuvirtide, inhibits entry of the virus via cell-to-cell transmission by blocking viral gp41 fusion with the host cell membrane (98). Maraviroc, an entry inhibitor, is an example of an allosteric co-receptor entry inhibitor that binds to the CCR5 co-receptor. Viral replication can also be inhibited by blocking the activity of the reverse transcriptase e.g. zidovudine. Zidovudine, the first drug approved to treat HIV, is a thymidine analogue that leads to the production of inactive proviral DNA (98). Protease inhibitors can also be used to block the assembly of new HIV virions (98). However, many infected patients can develop resistance to these drugs within a few days of initiating treatment.

Combination antiretroviral therapy (cART) including efavirenz (non-nucleoside reverse transcriptase inhibitor) with tenofovir or emtricitabine (nucleotide analogue reverse transcriptase inhibitor (NRTIs)) is the gold standard for HIV treatment and was established in 2006 (98).

Further combination with integrase and protease inhibitors can also be used in order to reduce viral loads within the patient.

Upon initiation of ARV treatment, the viral load will drop below the limit of detection (<39 copies per mL) and the $CD4^+$T-cell count will rise in most patients. This is due to the inhibition of re-infection of $CD4^+$T-cells and trafficking of these cells from the lymphoid tissues within the peripheral blood (98).

Once a patient has initiated ARV therapy, it is important to routinely monitor total $CD4^+$T-cells, VL, and serum β_2 microglobulin levels (important for the loading and transportation of class I to the host cell surface) (49,98). Furthermore, it is also important to monitor antiretroviral resistance as the high rate of HIV replication generates multiple variants. This contributes considerably to HIV-related mortality.

As of 2013, 107,800 HIV-infected individuals were living in the UK (127). Of this population, 6,000 new individuals were newly diagnosed and 320 individuals were diagnosed with AIDS (127). As of 2013, 530 individuals in the UK died as a result of HIV-associated infections (127). As of 2014, 36.9 million individuals were living with HIV with 2 million newly diagnosed individuals worldwide (128). In 2014, 1.2 million individuals died as a result of HIV-associated infections worldwide (128).

1.12 Pneumococcal Treatment in HIV

In the UHB HIV Service, first line treatment for pneumococcal pneumonia in HIV is Co-trimoxazole. When the use of Co-trimoxazole is contraindicated, Clindamycin can be used in conjunction with Primaquine. Pentamidine may also be used. Maintenance of treatment may include treatment with Co-trimoxazole with Dapsone and Pentamidine used as alternative therapies. Treatment may vary depending on hospital protocols.

Antibiotic prophylaxis is not recommended for preventative treatment of bacterial pneumonia (129).

1.13 Existing Knowledge of Pneumococcal Vaccination in HIV-patients

Previously, it has been suggested that two doses of a conjugate pneumococcal vaccine are more immunogenic than one dose (130). Furthermore, a three dose primary course plus booster dose of PCV-13 is recommended in HIV-infected children (131). Also, a dose-ranging study has been performed that investigated the use of a pneumococcal conjugate

vaccine. A dose-dependent response was observed. Local reactions were seen with the 4x dose as opposed to the 2x dose (132).

The lack of evidence is not confined to HIV and a control population will provide important information on the longevity and quality of the response to booster vaccines, in healthy individuals, as well as investigating the effect of HIV on the immune response. Ultimately, a randomised control trial (RCT) such as AIR II will help to inform researchers, clinicians, and policy makers whether or not a second or even third dose of PCV-13 is needed to sustain a long-term Pn-specific antibody response.

Historically, HIV-infected individuals have suboptimal responses to pneumococcal vaccination. There is a lack of evidence in HIV- infected individuals with regards to protective and functional antibody responses, as well as the longevity of the immune response to pneumococcal vaccination.

Pneumococcal vaccination studies using PPV-23 have been trialled in HIV-infected individuals; however, protective antibody levels were significantly lower in HIV-positive individuals for all of the serotypes covered in the vaccine (133). In another study, PPV-23 was found to be ineffective in HIV-infected Ugandan adults. Increased rates of pneumococcal disease were reported in vaccine recipients (134) . Anti-polysaccharide IgG levels, poor opsonophagocytosis, and IPD was associated with PPV-23 vaccination in HIV-infected Africans (135).

Trials investigating pneumococcal conjugate vaccine were to be assessed in the HIV-infected population. In one study, HIV-infected adults were randomised to receive different combinations of vaccines including placebo-placebo, placebo-polysaccharide, conjugate-conjugate and conjugate-polysaccharide. The second dose was given 8 weeks post the initial vaccination. The conjugate-conjugate and conjugate-polysaccharide vaccine combinations had higher antibody concentrations and opsonophagocytic killing (OPKA) titres after the second dose (136). Another combined vaccination schedule was trialled in France in HIV-infected adults. PCV-7 was administered as an initial vaccination at week 0 followed by a booster dose of PPV-23 at week 4. The frequency and magnitude of anti-PS IgG responses were greater when compared with the PPV-23 alone (137).

Another study suggested that past receipt of PPV-23 did not affect the response to conjugate vaccination (138). The studies carried out in the current AIR study suggest that it will and

has affected the response to conjugate vaccination, particularly if the individual has been vaccinated within 2-5 years of receiving PPV-23 ie hyporesponsiveness.

A study performed by the Health Protection Agency (HPA) in 2011 found that 61% of HIV-positive adults suffered from IPD episodes and therefore, called for the usage of PCV-13 in HIV-infected adults, as the benefits from herd protection from the immunisation of children did not reach this population (139).

Two separate studies have been conducted by Pfizer in 2012. In Study 6115A1-3017, HIV-infected adults previously immunised with PPV-23 were given 3 doses of PCV-13 at 6-month intervals. Patient responses remained above initial baseline levels (140). In Study 6115A1-3002, HIV-infected children, adolescents, and adults who had not received any pneumococcal previously were vaccinated with 3 doses of PCV-13 and a single dose of PPV-23 given at one month intervals (140). Robust immune responses were seen after the initial dose with PCV-13, although there were only modest increases after doses 1,2, and 3. Although these studies have investigated the immune response to single vaccine doses at various time points, they have not investigated the immune response to double doses nor the longevity of the immune response. There has been much debate with regards to achieving the optimal vaccination schedule in HIV-infected adults in the UK.

PPV-23 has limited efficacy in HIV-infected adults and Pn-specific antibody levels may decline over time. This may also occur with regards to HIV-infected patients that receive a single dose (SD) of PCV-13. However, boosting with PCV-13 appears to elicit a better immunological response than when boosting with PPV-23 (80,141).

In a placebo-controlled RCT using PCV-7 in Malawi in HIV-infected adults, patients were given two doses with a one month gap. Although it reduced vaccine serotype IPD by 74%, it did not show any advantage over a single dose schedule (142). Limitations to this study include the fact that the investigators did not test Pn-specific concentrations and investigate the longevity of the immune response, nor did they look at timing of the booster vaccination.

It has been suggested that patients receive an initial dose of PCV-13 and then a booster dose of PPV-23 to include vaccine coverage against additional serotypes. However, the caveat to this vaccination schedule is that patients will not be able to be re-vaccinated for 2 to 5 years even if they are unprotected. One study evidenced that Pn-specific antibody concentrations were enhanced once given a conjugate Pn-polysaccharide vaccine and then a booster of

PPV-23 nine months later. However, limitations to this study include the fact that they only tested 4 serotypes and post-vaccination concentrations were only higher for 3 of the serotypes that were tested (143).

Another study investigating this prime-boost approach suggested that HIV-infected adults who received an initial dose of PCV-7 and then a booster dose of PPV-23 after 4-weeks. The study found that after 8-weeks, patients had a 2-fold increase of Pn-specific IgG concentrations and sustained these levels at week 24 (144). A limitation to this study was that the investigators neglected to look at the longevity of the response at one and two years post-vaccination with regards to hyporesponsiveness and a decline in Pn-specific IgG concentrations.

One RCT found that when boosting with PCV-7 after receiving an initial dose of PPV-23 (3-8 years prior), HIV-infected patients had a greater Pn-specific IgG response at 2 months post-vaccination than those that were boosted with PPV-23. Furthermore, at 6 months, patients who had received a booster dose of PCV-7 had similar Pn-specific IgG concentrations to those that received a booster dose of PPV-23 (145). However, this decline in Pn-specific antibody concentrations may be due to hyporesponsiveness.

In contrast, an RCT carried out in Brazil with HIV-infected adults suggested that the administration of a booster dose of PPV-23 after PCV-7 was not advantageous over a booster dose of PCV-7. Patients that received a booster dose of PCV-7 sustained Pn-specific IgG concentrations greater than a four-fold increase compared to patients that received a booster dose of PPV-23 (146).

Current BHIVA guidelines suggest that HIV-infected adults receive a SD of PCV-13 regardless of their ARV/HAART usage, VL, and/or CD4 count consistent with our group's findings, in addition to those of Iyer et al. (147). BHIVA suggests that HIV-infected adults (below the age of 65) should be given PCV-13 at least 3 months post-vaccination with PPV-23 and those that are 65 and older should be given a SD of PPV-23. They also recommend against repeat vaccination with PPV-23 and/or PCV-13, but fail to give evidence which suggests why it is not advantageous (80).

By comparing a single pneumococcal booster dose with a double pneumococcal booster dose (DD) in an RCT such as AIR II, we will be able to see whether or not boosting is safe in HIV-infected and HIV-uninfected adults. Pneumococcal boosting studies with PCV-13 have

not been trialled extensively to date in adults (aged 18-65) or HIV-infected adults, thus, the study findings would influence and impact local, national, and global healthcare policies.

1.14 Aims and Objectives:

The aim of this project is to investigate the immune response to pneumococcal vaccination in the context of HIV infection. This will be achieved by employing a variety of immunological and microbiological techniques to assess the quantity and quality of pneumococcal-specific antibody produced in response to vaccination. Also, we will assess how HIV and its associated co-morbidities impact the cellular response to immunisation. To do this, the following questions will be addressed:

- What is the quantity of pneumococcal-specific antibody produced in response to pneumococcal vaccination?
    - What is the duration of the immune response at 12, 24, 36, 48, and 60 months post-vaccination?
- There may be pneumococcal-specific opsonic antibody present, but is it functional? Is it able to enhance or mediate phagocytosis and cell-mediated killing?
- How does HIV impact the cellular response to pneumococcal vaccination? What is the phenotype of B and T-cells in the context of HIV infection when compared to normal controls?

Chapter 2: Impact of HIV-infection on the vaccine response to pneumococcal polysaccharide vaccination

Publication: *MacLennan CA, Richter A, Hodson J, Faustini S, Birtwistle J, Whitelegg A, et al. Immunization of HIV-infected adults in the UK with Haemophilus influenzae b/meningococcal C glycoconjugate and pneumococcal polysaccharide vaccines. J Acquir Immune Defic Syndr Nov 2016*

Oral presentation: *12th European Meeting on the Molecular Biology of the Pneumococcus EuroPneumo 2015, St. Catherine's College, University of Oxford, July 2015.*

2.1 Aims

The aim of this chapter was to investigate how HIV-infection affects the response to pneumococcal vaccination. The study was set up by Professor(s) Calman MacLennan and Mark Drayson and I was brought into this study as part of my PhD project in order to test the archived serum samples (pre- and post-vaccination), as well as the annual serum samples collected in order to investigate the longevity of the immune response to pneumococcal vaccination. Furthermore, I established a multiplexed opsonophagocytic-killing assay (OPKA) (discussed later in Chapter 5) and multiplexed Pn-specific IgG1 and IgG2 Luminex multiplex immunoassays (MIAs) (discussed later in Chapter 6).

2.2 Objectives

The objective of this study was to:

1) Investigate Pn-specific IgG pre-and post- vaccination (4-weeks) serum antibody concentrations following a single dose of Pneumovax-23® (PPV-23) in HIV-infected and HIV-negative individuals.

2.3 Ethical Approval

Ethical approval for the collection of blood from HIV-infected and non-HIV- infected individuals in the UK was granted by the University Hospital Birmingham (UHB) Research and Development Trust, NHS Research and Ethics Committee (REC) under the AIR (Assessment of Immune Responses to Routine Immunisations in HIV-infected patients) study. Ethical approval for the use of blood and serum samples was also granted by the University of Birmingham (UoB) Clinical Research Office who also sponsored the trial.

UHB R&D Trust: RRK3743

Main REC Ref: REC-09/H12041/53

Sponsor's Protocol Number: RG_09_034, ERN_14-1166

2.4 AIR Study (2009-2010) Background

The current AIR (**A**ssessment of **I**mmune **R**esponses to Routine Immunisations in HIV-infected adults) I study, was undertaken at the University Hospital Birmingham (UHB) and the Whittall Street Clinic (a genitourinary medicine clinic in the Birmingham city centre) in conjunction with investigations carried out via the Clinical Immunology Service (CIS) at the University of Birmingham (UoB), set out to investigate the immune responses to routine vaccinations recommended nationally for HIV-infected patients by the British HIV Association (BHIVA).

There was a clinical need for the study due to the increased risk of vaccine-preventable infections and mortality associated with HIV-infected individuals. HIV-infected individuals are also more susceptible to encapsulated bacteria, most notably *Haemophilus influenza* and *Streptococcus pneumoniae* even though they may be on a highly activated antiretroviral therapy (HAART) regime. There are available vaccines against these extracellular pathogens but the impact of HIV infection on the response to vaccination is poorly understood. The AIR study was established in order to understand the implications of HIV-infection on the immune response to vaccinations within the patient cohort.

Patients attending clinic were routinely offered Hepatitis B and annual influenza vaccinations. However, patients enrolled on the study additionally received vaccines against pneumococcus (Pneumovax-23®, PPV-23), a combined vaccine called Menitorix which protects against bacterial infections caused by *Haemophilus influenza* b and meningococcal C, and the low dose Diphtheria, Tetanus, and Polio vaccine. For the purposes of this chapter and thesis, only the Pn-specific IgG antibody concentrations will be reported.

2.5 Materials and Methods

This study was conducted between September 2009 and December 2010 at the UHB HIV Department as part of the AIR study. HIV-infected and HIV-negative individuals were enrolled from the UHB HIV service, as well as the Whittle Street genitourinary medicine clinic (Birmingham, UK). Participants received a single dose of PPV-23 (0.5 mL). Inclusion

criteria were proven HIV-positive test or HIV-negative test (control group) and older than 18 years of age. Individuals who were previously administered a pneumococcal vaccine were excluded from this study.

2.5.1 HIV testing

HIV antibody testing (Abbott Laboratories; Abbott Park, IL, USA) and HIV-1 viral load (VL) measurements were carried out on study patients. Study patients were bled pre-vaccination and 4 to 6 weeks post-vaccination.

2.5.2 19-plex multiplex Luminex assay IgG Antibody Quantification

Pn-specific IgG antibody levels to 12 Pn serotypes (1, 3, 4, 5, 6B, 7F, 9V, 14, 18C, 19A, 19F, and 23F) were measured in serum using the Luminex multiplex immunoassay (MIA).

The 19-plex assay is a multiplexed antibody assay that measures vaccine specific IgG antibodies using a Luminex 100/200 analyser (Merck Millipore, Texas, USA) in conjunction with Bioplex Manager 6.1 software (Bio-Rad Laboratories, Inc.). This assay is instrumental in measuring a patient's antibody response to vaccination and it gives researchers and clinicians alike an insight into the status of his/her respective immune systems. It can and has been used as a tool to measure protective antibody levels and indicate when booster vaccinations should be administered to particular patients. This assay is routinely used in the Clinical Immunology Service (CIS) at the University of Birmingham (UoB) and measures twelve *S. pneumoniae* serotypes simultaneously including(Pn1,3,4,5,6B,7F,9V,14,18C,19A,19F ,23F) all found in PCV-13 (148). It also measures tetanus, diphtheria, meningococcal and *Haemophilus influenzae* b (Hib) –specific IgG.

S. pneumoniae antigens (Pn1,3,4,5,6B,7F,9V,14,18C,19A,19F, 22F, and 23F) were acquired from the American Type Culture Collection (ATCC; Virginia, USA). 22F was not analysed in the assay. Instead, it was used to adsorb cross-reactive antibodies. Tetanus toxoid was obtained from Quadratech (Epsom, UK).

There is much controversy with regards to correlates of protection against pneumococcus. Two levels have been proposed for the Pn serotypes: 1.3 µg/mL (AAAAI) (149) and 0.35 µg/mL (WHO)(150) . However, a recent study of PCV-13 in infants raises concerns about whether or not these levels are appropriate for assessing pneumococcal vaccine responses in HIV-infected patients (151).

2.5.2.1 PLL (Poly-L-Lysine) conjugations

Hib and pneumococcal (5 mg/mL) polysaccharides, meningococcal (2 mg/mL), and tetanus and diphtheria toxoid (50 mg/mL) were added to 0.01% NaOH. Cyanuric chloride (Sigma, UK) was used to conjugate the polysaccharide to 0.1% PLL (Sigma, UK). The conjugated polysaccharide was then purified using G25 PD-10 Sephadex desalting columns (GE Healthcare, Chalfont St. Giles, UK).

2.5.2.2 Bead Conjugations

Hib and pneumococcal (5 mg/mL) polysaccharides, meningococcal (2 mg/mL), and tetanus and diphtheria toxoid (50 mg/mL) were added to 0.01% NaOH. Cyanuric chloride (Sigma, UK) was used to conjugate the polysaccharide to 0.1% PLL (Sigma, UK). The conjugated polysaccharide was then purified using G25 PD-10 Sephadex desalting columns (GE Healthcare, Chalfont St. Giles, UK).

2.5.2.3 19-plex assay method

Samples were prepared, diluted, and loaded onto a 96 well dilution plate (Millipore, UK) using the Dynex machine (Dynex Technologies Ltd., Worthing, UK) one day prior to running the assay. Serum samples were diluted 1 1:100 of 1% BSA, 0.05% Tween 20, cell wall polysaccharide (CWPS) (Statens Serum Institute, Copenhagen, Denmark) (5 µg/mL), and Pn 22F (5 µg/mL). Pn22F was added to absorb non-specific antibodies (152) . Seven four-fold serial dilutions at a 1:20 dilution were carried out manually using 007 serum, a human pneumococcal standard reference serum, as the control standard (153). A 96- well filter plate (Millipore) was pre-wet with PBS-Tween and 25 µl of the master bead mix was added to each well. The loaded plate was then washed with PBS 0.05 % Tween using a vacuum pump (Millipore). 25 µl standards, controls, and samples were then transferred from the dilution plate to the luminex plate and then incubated in the dark on the orbital shaker (500 rpm) for 1 hour at RT. The luminex plate was then washed with PBS 0.05 % Tween and then incubated with 100 µl of the secondary antibody (mouse anti-human IgG or IgM phycoerythrin (PE)-conjugate (Southern Biotech) at a 1:200 dilution for 30 minutes in the dark on an orbital shaker (500 rpm). Following washes with PBS 0.05 % Tween, beads were then resuspended in 125 µl of PBS 0.05 % Tween in each well in order to analyse on the Luminex 100/200 instrument. A standard curve of median fluorescence intensities (MFIs) was generated for 007sp antibody concentrations in order to assign specific antibody concentrations for test samples.

2.5.3 Statistical Methods

Non-parametrical analysis was used to compare Pn-specific IgG levels between HIV-negative and HIV-infected patients due to skew in levels and truncation of results by the clinical limits of the Luminex MIA assays. Furthermore, Pn-specific IgG levels were treated as continuous variables and compared against HIV status, CD4 count, VL, and usage of ART. Mann-Whitney and Kruskal-Wallis tests were used. Pn-specific IgG was used as a binary variable according to whether patients were above WHO and/or AAAAI protective thresholds and comparisons were carried out across the groups using a Fisher exact test.

Pre- and post-vaccine Pn-specific IgG levels, fold change post-vaccination, and the percentage of previously un-protected patients reaching protective threshold Pn-specific IgG levels were determined using IBM SPSS Statistics (IBM Corporation, Armonk, NY). P-values were Bonferroni corrected for 36 comparisons (12 antigens by 3 outcomes), with $P<0.0009$ considered as statistically significant. HIV-infected patients were stratified according to CD4 count (TruCount assay; Becton Dickinson, San Jose, CA) (<350, 350-490, 500-690, and $\geq$700 cells per mL).

2.6 Patient Population

211 HIV-infected adults and 73 HIV-uninfected adults (controls) were recruited for vaccination with a single dose of PPV-23. The study patients and controls were recruited under the AIR study. HIV-uninfected patients were enrolled from the UHB genitourinary medicine clinic attendees that tested negative for HIV-infection. This was carried out in order to reduce study bias. Most of the AIR HIV-patients were on HAART in accordance with the BHIVA-recommended national guidelines (83%-176 total). Most patients received a non-nucleoside reverse transcriptase inhibitor (NNRTI) 73% (128 total) had viral loads (VL) of less than 50 copies per mL. 62 HIV-infected patients had detectable viral loads greater than 50 copies/mL. The median VL was 5060 copies/mL (interquartile range (IQR) 482-51,933 copies/mL). Patients had a median CD4 count of 500 cells/µl (IQR 350-630 cells/µl). Patients were then stratified according to CD4 counts, which resulted in four subgroups of approximately 50 patients per group (<350 (47 patients), 350-490 (56 patients), 500-690 (68 patients), and $\geq$700 (40 patients)). In the HIV-infected cohort, 42% of patients were Caucasian, 43% African, 10% Caribbean, 6% Asian, and 6% other/unknown. In the HIV-uninfected cohort, 52% of patients were Caucasian, 18% African, 8% Asian, and 8% other/unknown.

2.7 Vaccine Safety

No serious adverse events occurred following vaccination with PPV-23 or PCV-13.

2.8 Results: HIV-negative patients respond better to one dose of PPV-23 than HIV-positive patients

2.8.1 Vaccine responses to AAAAI protective thresholds

Pre-vaccination Pn-specific IgG concentrations were significantly lower in the HIV-infected group for Pn serotypes 7F ($p<0.001$) according to the AAAAI protective threshold of 1.3 µg/mL (Table 2.1; Figure 2.2). HIV-negative patients responded significantly better to Pn1 ($p<0.0001$), 7F ($p<0.001$), 18C ($p<0.001$), and 23F ($p<0.001$) (Table 2.1, Figure 2.3). It should be noted that although not significant post-Bonferroni correction, HIV-negative patients responded better to Pn serotypes 4 ($p=0.044$), 5 ($p=0.003$), 6B ($p=0.031$), 9V ($p=0.002$), 19A ($p=0.035$), and 19F ($p=0.008$) (Absolute values; Table 2.1, Figure 2.3). Furthermore, there was a greater fold change for HIV-negative patients post-vaccination for Pn1 (8.01; $p<0.001$). However, it is worth noting that there was a $\geq$4-fold change in Pn-specific IgG concentrations for HIV-infected patients for Pn5 (4.15; ns), 7F (5.56, ns), and 18C (5.47, ns) (Table 2.1).

2.8.2 Vaccine responses to WHO protective thresholds

Pre-vaccination Pn-specific IgG concentration were significantly lower in the HIV-infected group for Pn serotypes 5 ($p=0.020$), 7F ($p<0.001$), 9V ($p=0.003$), and 23F ($p=0.010$) according to the WHO protective threshold (Table 2.3, Figure 2.4). HIV-negative patients responded significantly better to Pn1 ($p=0.019$), 7F ($p=0.032$), 9V ($p=0.007$), 18C ($p=0.002$), and 19A ($p=0.028$) (Table 2.4/Table 2.5, Figure 2.5).

2.8.3 Lack of association between CD4 Count and VL on Pneumococcal Vaccine Responses

There was a distinct lack of association between pre-vaccination, post-vaccination, and fold-change in Pn-specific IgG levels, CD4 count stratification, full VL suppression, or ARV therapy (Table 2.4/Table 2.5). In order to assess the effect of ARV therapy on Pn-specific IgG levels, study participants with a CD4 count of $\geq$ 500 cells/mL and fully suppressed VL were compared with all of the other HIV-infected subgroups. There was very little difference between the two groups. A normal CD4 count is approximately 1200 cells/µl (normal range 500-1500 cells/ µl) (148).

Table 2.1. Pn-specific IgG Levels to Pneumococcal Vaccination in Study Participants According to HIV Status

Pre-Vaccine		Pn1	Pn3	Pn4	Pn5	Pn6B	Pn7F	Pn9V	Pn14	Pn18C	Pn19A	Pn19F	Pn23F
HIV	*HIV-*	0.24 (0.10-0.76)	0.29 (0.10-1.78)	0.14 (0.07-0.33)	0.38 (0.19-1.06)	0.36 (0.14-1.49)	0.77 (0.39-1.49)	0.52 (0.18-1.33)	1.03 (0.32-3.94)	0.44 (0.18-1.95)	0.78 (0.25-2.71)	0.63 (0.19-2.26)	0.55 (0.22-1.60)
Status	*HIV+*	0.24 (0.08-0.67)	0.24 (0.08-0.67)	0.12 (0.04-0.35)	0.24 (0.09-0.72)	0.32 (0.10-1.12)	0.35 (0.15-1.07)	0.25 (0.10-0.70)	1.51 (0.23-5.65)	0.43(0.08-1.52)	0.66 (0.15-2.23)	0.40 (0.14-1.38)	0.31 (0.10-0.96)
	p-Value	*0.782*	*0.446*	*0.398*	*0.015*	*0.600*	***<0.001 (**)***	*0.002*	*0.516*	*0.168*	*0.243*	*0.146*	*0.005*
Post-Vaccine		**Pn1**	**Pn3**	**Pn4**	**Pn5**	**Pn6B**	**Pn7F**	**Pn9V**	**Pn14**	**Pn18C**	**Pn19A**	**Pn19F**	**Pn23F**
HIV	*HIV-*	**2.74** (0.93-10.00)	0.49 (0.16-3.93)	0.36 (0.21-1.06)	**4.63** (0.58-10.00)	**1.67** (0.41-5.72)	**5.24** (2.43-10.00)	**1.89** (0.82-10.00)	**7.76** (1.22-10.00)	**5.96** (1.13-10.00)	**3.72** (1.00-8.50)	**2.19** (0.57-7.08)	**2.35** (0.79-6.69)
Status	*HIV+*	1.11 (0.46-3.55)	0.53 (0.20-2.10)	0.30 (0.10-0.79)	**1.34** (0.41-5.55)	0.97 (0.25-3.20)	**1.51** (0.65-4.75)	1.15 (0.35-3.82)	**5.12** (1.38-10.00)	**2.01** (0.29-9.48)	**2.19** (0.57-6.97)	1.07 (0.30-4.32)	1.08 (0.31-2.72)
	p-Value	***<0.0001 (**)***	*0.791*	*0.044*	*0.003*	*0.031*	***<0.001 (**)***	*0.002*	*0.255*	***<0.001 (**)***	*0.035*	*0.008*	***<0.001 (**)***
Fold Change		**Pn1**	**Pn3**	**Pn4**	**Pn5**	**Pn6B**	**Pn7F**	**Pn9V**	**Pn14**	**Pn18C**	**Pn19A**	**Pn19F**	**Pn23F**
HIV	*HIV-*	**8.01** (3.92-16.67)	1.63 (1.00-3.95)	2.65 (1.60-5.93)	**4.15** (2.07-12.88)	2.20 (1.30-10.02)	**5.56** (2.15-11.88)	2.81 (1.58-11.25)	3.43 (1.27-8.19)	**5.47** (2.17-16.66)	3.31 (1.29-6.22)	2.94 (1.52-6.19)	2.55 (1.80-8.91)
Status	*HIV+*	3.42 (1.54-10.32)	1.79 (1.00-3.90)	2.00 (1.24-4.08)	**4.00** (1.62-11.07)	1.87 (1.18-4.69)	3.50 (2.300-7.65)	3.82 (1.57-8.29)	1.99 (1.00-4.63)	3.35 (1.56-7.30)	2.00 (1.20-5.58)	2.06 (1.29-4.82)	2.40 (1.40-5.56)
	p-Value	***<0.0001 (**)***	*0.387*	*0.030*	*0.299*	*0.156*	*0.060*	*0.863*	*0.017*	*0.013*	*0.181*	*0.060*	*0.126*

Data are reported as median (quartiles). Bold text are stated as above the Pn protective threshold (Pn=1.3 ug/mL) and/or a >4-fold change.

P-values from Mann-Whitney tests

***Significant after Bonferroni correction for 36 comparisons (12 antibodies by 3 measures) at p<0.00088*

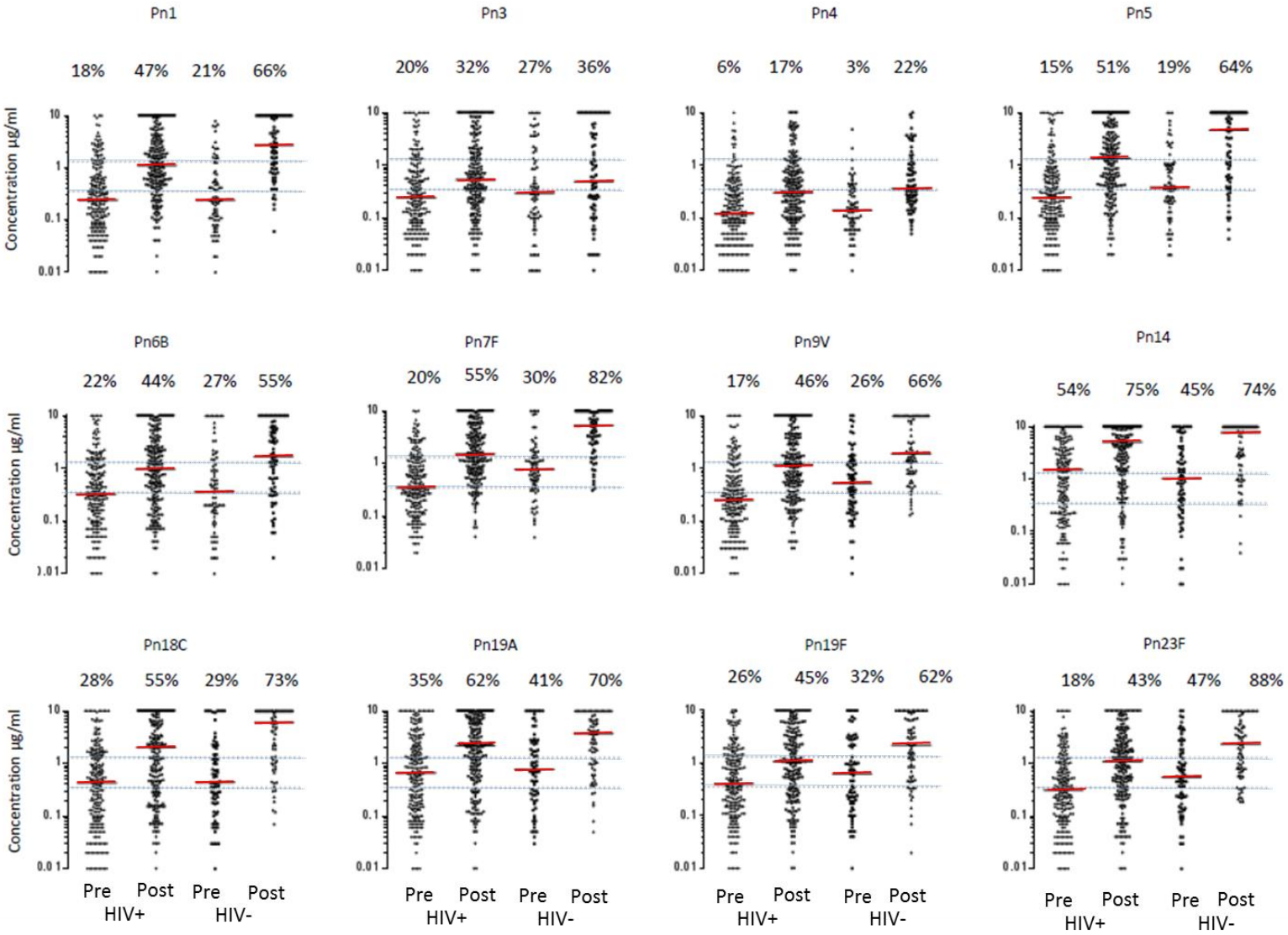


Figure 2.1 Pn-specific IgG antibody levels pre-and post-pneumococcal vaccination with PPV-23 (µg/mL). The WHO protective threshold is denoted at 0.35 µg/mL. The AAAI protective threshold is denoted at 1.3 µg/mL. Each point represents one study participant. Solid horizontal lines (in red) indicate medians. Dotted horizontal lines (blue) indicate protective thresholds for post-vaccination Pn-specific IgG titres.

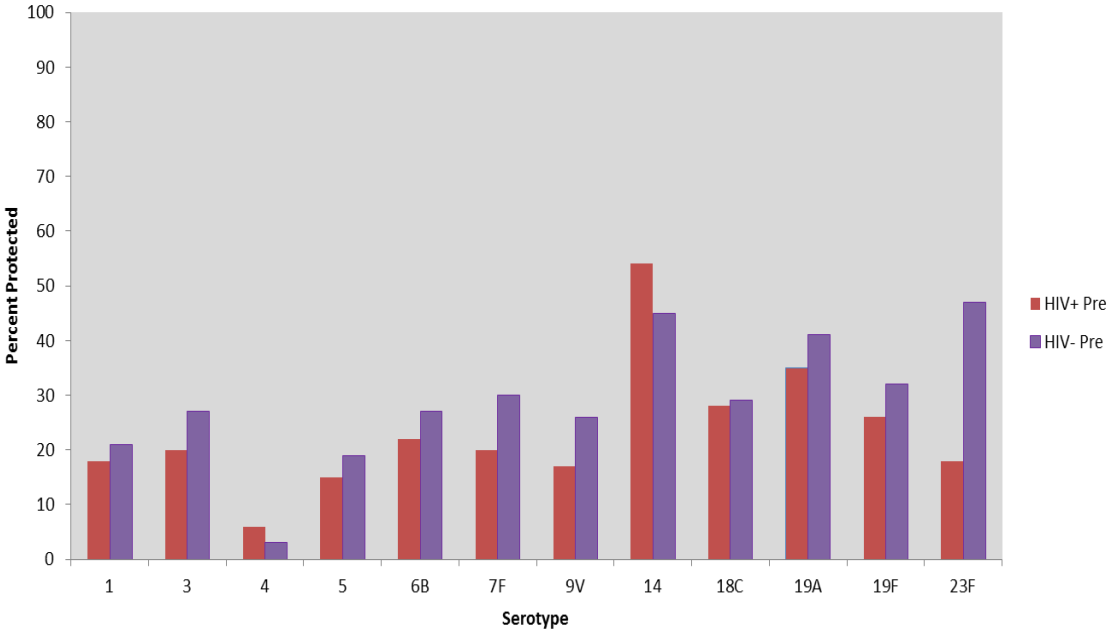


Figure 2.2. Pn-specific IgG concentrations pre-vaccination with one dose of PPV-23. HIV+(HIV-infected) n=211, HIV-(HIV-negative) n=73. Percent protected according to the AAAAI 1.3 µg/mL protective threshold.

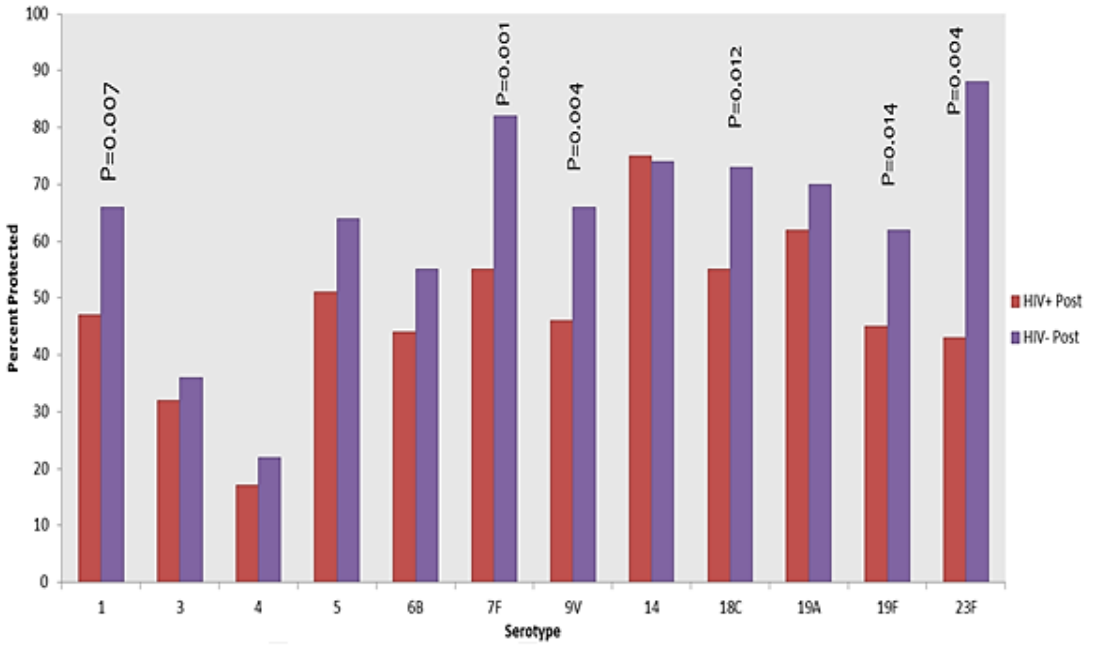


Figure 2.3. HIV-negative patients respond better to one dose of PPV-23 than HIV-positive patients- AAAAI threshold. Percentage of patients protected 4-weeks post-vaccination with PPV-23. There was a greater percentage of HIV-negative patients that showed statistical significance for Pn 1, 7F, 9V, 18C, 19F, and 23F. HIV+(HIV-infected) n=211, HIV- (HIV-negative) n=73. Percent protected according to the AAAAI 1.3 µg/mL protective threshold.

Table 2.2 Percent Protected Pn-specific IgG concentrations (AAAAI threshold) according to HIV status

Pre-Vaccine		Pn1	Pn3	Pn4	Pn5	Pn6B	Pn7F	Pn9V	Pn14	Pn18C	Pn19A	Pn19F	Pn23F	8 Pn's[#]
HIV	*HIV-*	21%	27%	3%	19%	27%	30%	26%	45%	29%	41%	32%	29%	12%
Status	*HIV+*	18%	20%	6%	15%	22%	20%	17%	54%	28%	35%	26%	18%	7%
	p-Value	***0.600***	***0.191***	***0.530***	***0.462***	***0.424***	***0.076***	***0.121***	***0.225***	***1.000***	***0.398***	***0.360***	***0.069***	***0.220***
Post-Vaccine		**Pn1**	**Pn3**	**Pn4**	**Pn5**	**Pn6B**	**Pn7F**	**Pn9V**	**Pn14**	**Pn18C**	**Pn19A**	**Pn19F**	**Pn23F**	**8 Pn's[#]**
HIV	*HIV-*	66%	36%	22%	64%	55%	82%	66%	74%	73%	70%	62%	63%	53%
Status	*HIV+*	47%	32%	17%	51%	44%	55%	46%	75%	55%	62%	45%	43%	33%
	p-Value	***0.007****	***0.665***	***0.376***	***0.057***	***0.104***	***<0.001***	***0.004****	***0.876***	***0.012****	***0.259***	***0.014****	***0.004****	***0.002****
Absolute Increase		**Pn1**	**Pn3**	**Pn4**	**Pn5**	**Pn6B**	**Pn7F**	**Pn9V**	**Pn14**	**Pn18C**	**Pn19A**	**Pn19F**	**Pn23F**	**8 Pn's[#]**
HIV	*HIV-*	45%	8%	19%	45%	27%	52%	40%	29%	44%	29%	30%	34%	41%
Status	*HIV+*	30%	12%	11%	36%	21%	35%	29%	22%	27%	27%	19%	25%	26%
	p-Value	-	-	-	-	-	-	-	-	-	-	-	-	-
Previously		**Pn1**	**Pn3**	**Pn4**	**Pn5**	**Pn6B**	**Pn7F**	**Pn9V**	**Pn14**	**Pn18C**	**Pn19A**	**Pn19F**	**Pn23F**	**8 Pn's[#]**
HIV	*HIV-*	57%	13%	20%	56%	40%	75%	56%	53%	62%	51%	44%	50%	47%
Status	*HIV+*	37%	17%	12%	43%	28%	44%	35%	47%	38%	42%	27%	32%	28%
	p-Value	***0.009****	***0.669***	***0.116***	***0.097***	***0.126***	***<0.001***	***0.010****	***0.578***	***0.004****	***0.294***	***0.034****	***0.021****	***0.005****

Data reported as the proportions of patients with antibody levels above the protection thresholds (Pn=1.3)

p-values from Fisher's Exact tests

[‡] The proportion of patients with antibody levels below the threshold pre-vaccine, who had levels above the thresholds post-vaccine

[#] Patients with 8 Pn antibodies above the thresholds

**Significant at p<0.05*

***Significant after Bonferroni correction for 36 comparisons (12 antibodies by 3 measures) at p<0.00088*

Table 2.3 Percent Protected Pn-specific IgG concentrations (WHO threshold) according to HIV status

Pre-Vaccine		Pn1	Pn3	Pn4	Pn5	Pn6B	Pn7F	Pn9V	Pn14	Pn18C	Pn19A	Pn19F	Pn23F	8 Pn's[#]
HIV Status	*HIV-*	42%	45%	23%	56%	51%	75%	60%	74%	60%	67%	56%	64%	47%
	HIV+	40%	43%	25%	40%	49%	50%	40%	71%	53%	62%	55%	46%	30%
	p-Value	***0.783***	***0.784***	***0.875***	***0.020****	***0.892***	***<0.001*** **	***0.003****	***0.763***	***0.340***	***0.482***	***0.892***	***0.010****	***0.015****
Post-Vaccine		**Pn1**	**Pn3**	**Pn4**	**Pn5**	**Pn6B**	**Pn7F**	**Pn9V**	**Pn14**	**Pn18C**	**Pn19A**	**Pn19F**	**Pn23F**	**8 Pn's[#]**
HIV Status	*HIV-*	92%	59%	52%	84%	78%	97%	90%	93%	90%	92%	84%	85%	84%
	HIV+	80%	62%	46%	78%	72%	89%	76%	89%	73%	80%	72%	74%	72%
	p-Value	***0.019****	***0.679***	***0.415***	***0.401***	***0.357***	***0.032****	***0.007****	***0.371***	***0.002****	***0.028****	***0.060***	***0.076***	***0.060***
Absolute Increase		**Pn1**	**Pn3**	**Pn4**	**Pn5**	**Pn6B**	**Pn7F**	**Pn9V**	**Pn14**	**Pn18C**	**Pn19A**	**Pn19F**	**Pn23F**	**8 Pn's[#]**
HIV Status	*HIV-*	49%	14%	29%	27%	27%	22%	30%	19%	30%	25%	27%	21%	37%
	HIV+	40%	19%	21%	38%	22%	38%	36%	18%	20%	18%	18%	27%	42%
	p-Value	-	-	-	-	-	-	-	-	-	-	-	-	-
Protection for Previously Unprotected Patients[‡]		**Pn1**	**Pn3**	**Pn4**	**Pn5**	**Pn6B**	**Pn7F**	**Pn9V**	**Pn14**	**Pn18C**	**Pn19A**	**Pn19F**	**Pn23F**	**8 Pn's[#]**
HIV Status	*HIV-*	86%	35%	45%	69%	58%	89%	79%	84%	76%	75%	63%	65%	72%
	HIV+	69%	37%	30%	66%	46%	78%	61%	64%	46%	51%	42%	54%	60%
	p-Value	***0.043****	***0.852***	***0.049****	***0.837***	***0.248***	***0.362***	***0.085***	***0.155***	***0.006****	***0.038****	***0.065***	***0.659***	***0.198***

Data reported as the proportions of patients with antibody levels above the protection thresholds (Pn=0.35)
p-values from Fisher's Exact tests
[‡]The proportion of patients with antibody levels below the threshold pre-vaccine, who had levels above the thresholds post-vaccine
[#]Patients with 8 PN antibodies above the thresholds
**Significant at p<0.05*
***Significant after Bonferroni correction for 36 comparisons (12 antibodies by 3 measures) at p<0.00088*

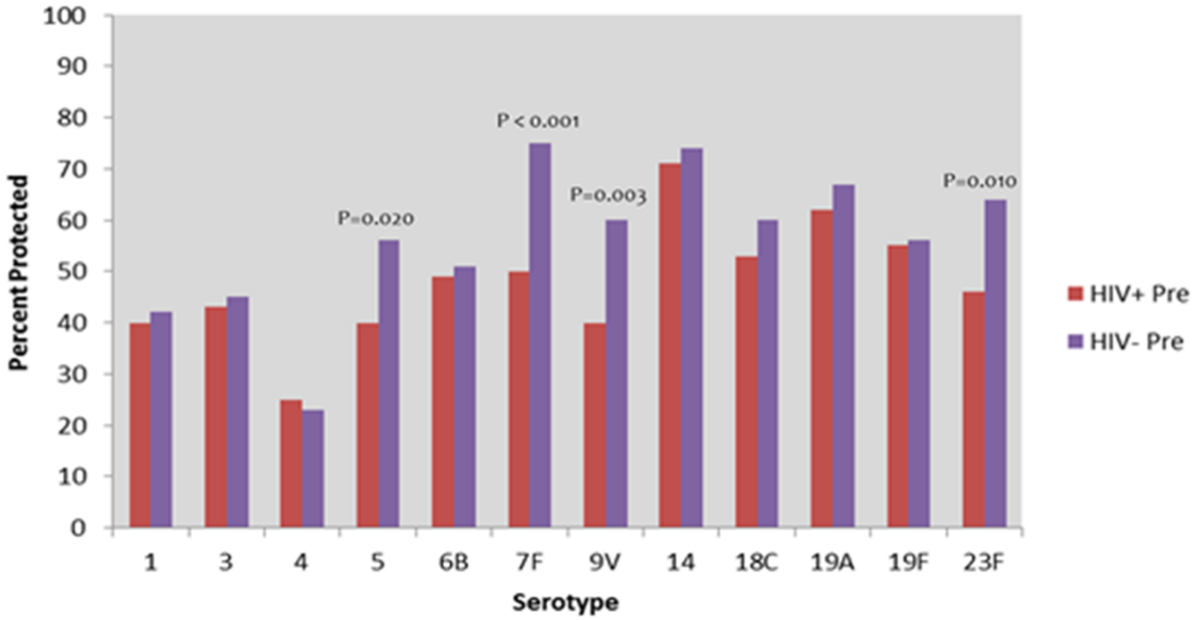


Figure 2.4. Pn-specific IgG concentrations pre-vaccination with one dose of PPV-23 (WHO threshold) HIV+(HIV-infected) n=211, HIV- (HIV-negative) n=73. Percent protected according to the WHO 0.35 µg/mL protective threshold.

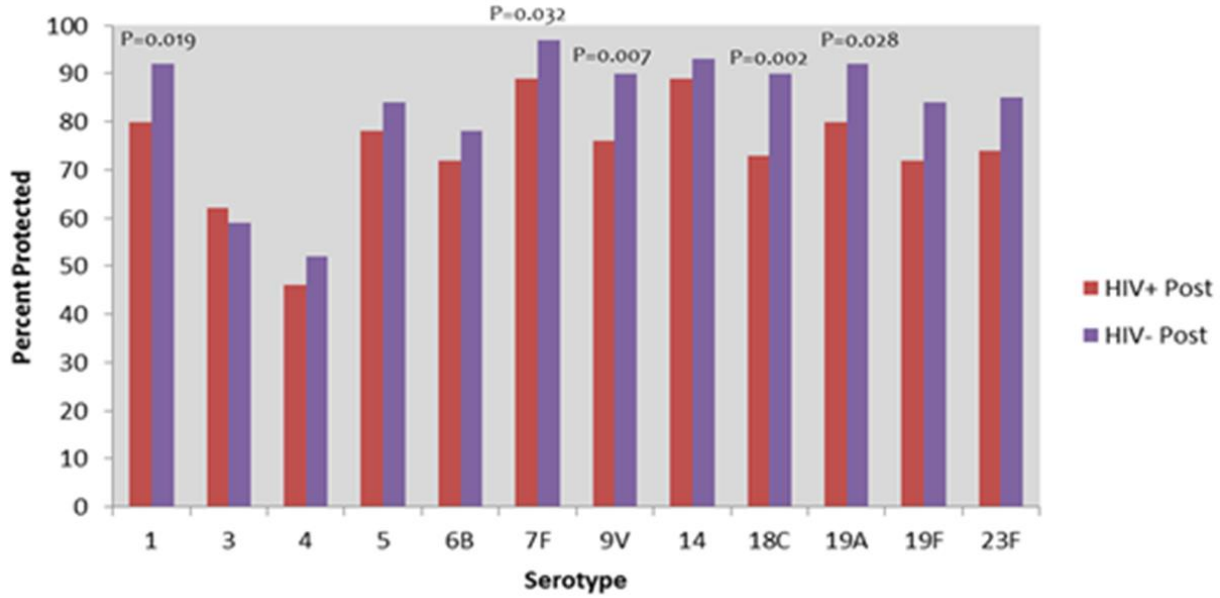


Figure 2.5. HIV-negative patients respond better to one dose of Pneumovax-23 (PPV-23) than HIV-positive patients- WHO threshold. The percentage of patients protected 4-weeks post-vaccination with PPV-23. There was a greater percentage of HIV-negative patients that showed statistical significance to Pn 1, 7F, 9V, 18C, 19A, and 23F. HIV+(HIV-infected) n=211, HIV- (HIV-negative) n=73. Percent protected according to the WHO 0.35 µg/mL protective threshold.

Table 2.4. Median Pn-specific IgG levels (µg/mL) in HIV-infected patients according to CD4 count, VL, and ART

Pre-Vaccine		Pn1	Pn3	Pn4	Pn5	Pn6B	Pn7F	Pn9V	Pn14	Pn18C	Pn19A	Pn19F	Pn23F
CD4 (cells/ µL)	<350	0.20 (0.07-0.62)	0.14 (0.08-0.56)	0.11 (0.04-0.22)	0.22 (0.08-0.61)	0.30 (0.09-1.08)	0.26 (0.12-0.72)	0.19 (0.07-0.58)	0.90 (0.12-3.76)	0.44 (0.07-1.24)	0.69 (0.15-2.08)	0.31 (0.13-1.00)	0.41 (0.20-1.04)
	350-499	0.28 (0.10-0.84)	0.39 (0.12-1.32)	0.12 (0.04-0.30)	0.28 (0.11-0.68)	0.46 (0.19-1.40)	0.45 (0.13-1.17)	0.32 (0.13-0.93)	1.22 (0.20-6.07)	0.52 (0.13-1.68)	0.81 (0.28-2.15)	0.76 (0.14-1.77)	0.35 (0.12-1.34)
	500-699	0.25 (0.07-0.66)	0.30 (0.13-0.80)	0.17 (0.06-0.38)	0.18 (0.06-0.59)	0.30 (0.12-1.08)	0.35 (0.18-1.08)	0.30 (0.10-0.59)	2.15 (0.41-6.09)	0.47 (0.09-1.45)	0.46 (0.13-1.87)	0.36 (0.16-1.16)	0.24 (0.06-0.76)
	700+	0.31 (0.14-0.73)	0.13 (0.05-0.74)	0.14 (0.04-0.47)	0.29 (0.12-1.06)	0.21 (0.08-0.87)	0.44 (0.24-0.76)	0.22 (0.09-0.65)	1.87 (0.37-5.71)	0.20 (0.07-1.62)	0.85 (0.14-3.55)	0.38 (0.12-1.33)	0.22 (0.10-0.60)
	p-Value	**0.456**	**0.053**	**0.473**	**0.228**	**0.322**	**0.458**	**0.501**	**0.267**	**0.652**	**0.491**	**0.469**	**0.278**
Viral Load (VL)(copies /mL)	<50	0.29 (0.09-0.78)	0.24 (0.08-1.01)	0.13 (0.04-0.41)	0.29 (0.09-0.87)	0.30 (0.09-1.11)	0.35 (0.17-1.14)	0.27 (0.10-0.72)	1.58 (0.22-6.36)	0.45 (0.09-1.62)	0.68 (0.16-2.04)	0.37 (0.13-1.17)	0.28 (0.10-0.66)
	50+	0.23 (0.08-0.63)	0.26 (0.09-0.76)	0.11 (0.05-0.25)	0.20 (0.09-0.47)	0.38 (0.14-1.14)	0.36 (0.12-1.03)	0.24 (0.08-0.57)	1.38 (0.36-3.85)	0.37 (0.08-1.40)	0.55 (0.15-2.57)	0.45 (0.15-1.43)	0.34 (0.09-1.27)
	p-Value	**0.629**	**0.883**	**0.531**	**0.197**	**0.327**	**0.354**	**0.767**	**0.787**	**0.704**	**0.747**	**0.541**	**0.284**
ART	No	0.21 (0.07-1.03)	0.48 (0.17-0.78)	0.13 (0.05-0.36)	0.19 (0.07-0.72)	0.43 (0.18-1.50)	0.36 (0.15-0.80)	0.28 (0.08-0.66)	1.86 (0.40-3.89)	0.49 (0.08-1.40)	0.50 (0.15-3.08)	0.48 (0.19-1.38)	0.66 (0.12-2.60)
	Yes	0.26 (0.09-0.66)	0.21 (0.07-0.80	0.12 (0.04-0.35)	0.27 (0.10-0.69)	0.32 (0.10-1.09)	0.35 (0.15-1.15)	0.25 (0.10-0.70)	1.51 (0.23-5.95)	0.42 (0.09-1.56)	0.70 (0.16-2.15)	0.39 (0.12-1.29)	0.28 (0.09-0.75)
	p-Value	**0.825**	**0.075**	**0.964**	**0.396**	**0.189**	**0.449**	**0.795**	**0.702**	**0.971**	**0.871**	**0.352**	**0.057**
Post-Vaccine		**Pn1**	**Pn3**	**Pn4**	**Pn5**	**Pn6B**	**Pn7F**	**Pn9V**	**Pn14**	**Pn18C**	**Pn19A**	**Pn19F**	**Pn23F**
CD4 (cells/ µL)	<350	1.60 (0.44-2.89)	0.52 (0.21-1.70)	0.26 (0.10-0.83)	1.72 (0.28-6.64)	0.95 (0.26-2.66)	1.12 (0.49-3.75)	0.85 (0.26-4.41)	5.12 (0.80-10.00)	1.66 (0.34-5.21)	2.36 (0.43-6.26)	0.88 (0.24-3.14)	1.32 (0.52-3.07)
	350-499	1.51 (0.45-3.73)	0.63 (0.32-2.05)	0.31 (0.12-0.73)	1.70 (0.57-6.09)	1.60 (0.46-3.79)	1.68 (0.80-6.47)	1.72 (0.56-6.76)	4.52 (1.08-10.00)	2.31 (0.56-7.74)	2.21 (0.74-7.86)	1.98 (0.47-6.28)	1.15 (0.48-2.58)
	500-699	0.85 (0.44-3.16)	0.69 (0.20-4.65)	0.38 (0.16-1.04)	1.12 (0.31-4.14)	0.94 (0.21-3.70)	1.88 (0.62-3.52)	1.31 (0.52-3.43)	5.13 (1.93-10.00)	2.06 (0.28-10.00)	1.99 (0.55-6.82)	1.05 (0.37-4.26)	1.09 (0.24-2.92)
	700+	0.81 (0.52-4.79)	0.26 (0.09-1.14)	0.22 (0.10-0.75)	1.00 (0.42-4.54)	0.57 (0.12-2.45)	1.41 (0.63-5.38)	0.57 (0.23-1.41)	5.22 (1.13-10.00)	0.77 (0.16-10.00)	1.99 (0.42-8.16)	0.80 (0.25-3.50)	0.64 (0.28-1.99)
	p-Value	**0.826**	**0.030***	**0.753**	**0.488**	**0.165**	**0.422**	**0.009***	**0.794**	**0.503**	**0.935**	**0.433**	**0.302**
Viral Load (VL)(copies /mL)	<50	1.14 (0.47-3.47)	0.59 (0.21-2.10)	0.32 (0.11-0.83)	1.54 (0.42-4.71)	0.98 (0.26-3.71)	1.55 (0.69-5.71)	1.24 (0.44-4.42)	4.75 (1.19-10.00)	1.98 (0.34-10.00)	2.20 (0.66-6.68)	1.00 (0.27-4.52)	1.00 (0.30-2.66)
	50+	1.07 (0.47-3.27)	0.44 (0.19-1.73)	0.26 (0.10-0.77)	1.32 (0.31-6.11)	0.97 (0.25-2.65)	1.47 (0.53-4.21)	0.99 (0.25-2.30)	5.12 (1.49-10.00)	2.06 (0.27-6.35)	2.09 (0.53-7.50)	1.17 (0.34-4.04)	1.14 (0.34-3.17)
	p-Value	**0.773**	**0.612**	**0.548**	**0.832**	**0.931**	**0.391**	**0.166**	**0.85**	**0.62**	**0.882**	**0.695**	**0.469**
ART	No	1.31 (0.29-5.45)	0.67 (0.35-5.03)	0.30 (0.09-0.79)	0.87 (0.31-10.00)	1.65 (0.43-3.33)	1.47 (0.41-3.53)	1.10 (0.25-1.87)	5.30 (1.71-10.00)	2.41 (0.29-10.00)	3.24 (0.76-7.98)	1.65 (0.61-5.87)	1.78 (0.69-4.74)
	Yes	1.07 (0.47-3.27)	0.47 (0.18-2.05)	0.31 (0.12-0.80)	1.35 (0.41-4.74)	0.90 (0.25-2.90)	1.55 (0.69-4.80)	1.16 (0.41-4.08)	4.98 (1.07-10.00)	1.94 (0.31-8.97)	2.03 (0.53-6.68)	1.03 (0.26-3.87)	0.89 (0.28-2.49)
	p-Value	**0.934**	**0.144**	**0.865**	**0.784**	**0.192**	**0.37**	**0.421**	**0.923**	**0.68**	**0.356**	**0.12**	**0.019***

Data are reported as median (quartiles).

HIV Status: p-values from Mann-Whitney U tests

CD4 cell counts: p-values from Kruskal-Wallis tests

**Significant at p<0.05*

***Significant after Bonferroni correction for 36 comparisons (12 antibodies by 3 measures) at p<0.00088*

Pn: Pneumococcal

ART: Antiretroviral therapy

VL: Viral Load

Table 2.5 Median Fold Changes- Pn-specific IgG levels in HIV-infected patients according to CD4 count, VL, ART

Fold Change		Pn1	Pn3	Pn4	Pn5	Pn6B	Pn7F	Pn9V	Pn14	Pn18C	Pn19A	Pn19F	Pn23F
CD4 (cells/ µL)	<350	5.50 (2.15-16.12)	2.21 (1.00-4.31)	2.44 (1.25-4.00)	4.00 (1.72-21.17)	2.33 (1.56-6.42)	3.50 (2.04-7.61)	4.24 (2.14-8.75)	2.83 (1.02-4.90)	2.75 (1.55-10.38)	2.57 (1.20-5.45)	2.40 (1.18-4.69)	2.05 (1.39-5.45)
	350-499	3.42 (1.36-9.21)	1.44 (0.99-3.67)	2.32 (1.62-4.14)	4.36 (1.99-11.13)	2.56 (1.20-6.36)	4.05 (1.77-9.38)	4.81 (1.86-9.95)	1.95 (1.02-6.29)	4.26 (2.33-6.75)	1.85 (1.18-4.34)	2.04 (1.35-4.97)	2.96 (1.35-7.00)
	500-699	3.00 (1.53-10.12)	2.09 (1.00-4.77)	1.93 (1.04-4.92)	4.25 (1.73-12.20)	1.67 (1.09-4.56)	4.06 (2.07-7.00)	4.05 (1.50-9.01)	1.76 (1.00-4.19)	3.41 (1.48-7.25)	3.00 (1.22-6.02)	2.40 (1.49-5.10)	2.63 (1.43-5.57)
	700+	3.03 (1.83-6.01)	1.72 (1.07-2.71)	1.82 (1.20-3.33)	2.69 (1.35-6.89)	1.53 (1.15-3.97)	2.49 (1.73-5.50)	1.98 (1.39-4.06)	1.73 (1.09-3.28)	2.60 (1.27-7.13)	1.75 (1.18-3.32)	1.65 (1.14-3.57)	2.35 (1.55-4.62)
	p-Value	*0.224*	*0.442*	*0.414*	*0.167*	*0.188*	*0.591*	***0.019****	*0.248*	*0.476*	*0.342*	*0.431*	*0.941*
Viral Load (VL)(copies /mL)	<50	3.45 (1.54-8.62)	2.04 (1.07-4.03)	2.00 (1.28-3.88)	3.28 (1.51-8.92)	2.18 (1.27-4.84)	3.47 (2.00-6.72)	4.07 (1.62-9.42)	1.73 (1.00-4.65)	3.39 (1.60-7.00)	1.99 (1.21-5.51)	2.01 (1.26-4.71)	2.60 (1.50-6.86)
	50+	3.35 (1.67-13.63)	1.50 (1.00-3.67)	2.15 (1.11-4.33)	5.21 (2.00-13.20)	1.61 (1.15-4.32)	3.59 (1.61-9.01)	3.56 (1.50-6.83)	2.57 (1.02-4.56)	3.20 (1.35-8.00)	2.22 (1.16-5.63)	2.27 (1.30-5.00)	2.09 (1.32-4.80)
	p-Value	*0.602*	*0.344*	*0.885*	*0.062*	*0.157*	*0.936*	*0.116*	*0.243*	*0.702*	*0.985*	*0.492*	*0.223*
ART	No	3.00 (1.49-15.11)	1.71 (1.00-3.63)	2.14 (1.24-4.00)	5.38 (1.64-13.33)	1.62 (1.13-5.54)	3.57 (1.93-6.27)	2.63 (1.49-4.67)	2.50 (1.08-4.56)	3.40 (1.88-8.13)	2.48 (1.16-8.06)	2.75 (1.62-5.00)	2.12 (1.21-4.47)
	Yes	3.47 (1.55-10.12)	1.79 (1.00-3.95)	2.00 (1.23-4.09)	3.63 (1.61-10.75)	2.00 (1.21-4.68)	3.49 (2.00-7.00)	4.03 (1.63-8.63)	1.89 (1.00-4.65)	3.33 (1.56-7.02)	1.99 (1.20-5.36)	1.98 (1.23-4.71)	2.45 (1.41-5.77)
	p-Value	*0.866*	*0.819*	*0.998*	*0.276*	*0.691*	*0.676*	*0.102*	*0.544*	*0.555*	*0.384*	*0.132*	*0.478*

Data are reported as median (quartiles).
HIV Status: p-values from Mann-Whitney U tests
CD4 cell counts: p-values from Kruskal-Wallis tests
**Significant at p<0.05*
***Significant after Bonferroni correction for 36 comparisons (12 antibodies by 3 measures) at p<0.00088*
Pn: Pneumococcal
ART: Antiretroviral therapy
VL: Viral Load

2.9 Discussion

2.9.1 HIV-infected patients mount a poor immune response to PPV-23

HIV-uninfected subjects produced a protective response to the plain polysaccharide vaccine PPV-23, but HIV-infected subjects had only a poor response to PPV-23 with a lack of association between vaccine responses and CD4 count, ARV therapy, and/or VL.

The AIR study cohort is a unique HIV-infected cohort in the UK as patients were able to receive booster vaccines if they failed to mount a response to an initial dose of PPV-23 or PCV-13. Previous studies tend to focus on single vaccinations. One study found similar responses to PPV-23 in HIV-infected (recently seroconverted) and HIV-negative patients (154). However, it's important to note that other studies investigating PPV-23 vaccination in HIV-infected patients have established opposing responses. Two studies have indicated a strong immunogenic response to PPV-23 (155,156). However, another two studies have indicated a poor immunogenic response similar to our study results (157,158).

Consistent with our study findings, there have been studies examining pneumococcal vaccination in HIV infection that found no correlation between the CD4 count and the pneumococcal antibody response (156,158). However, there were two studies that found poor responses to PPV-23, which was associated with low CD4 counts (159,160).

ARV therapy did not have an effect on the response to PPV-23 vaccination in the AIR study cohort and this has been established in other studies, one of which found that there is no benefit from delaying pneumococcal vaccination until the patient has received 6 to 12 months of ARV therapy (160,161).

The lack of association between vaccine responses to PPV-23 and CD4 counts, VL, and ARV therapy supports the policy of early vaccination following a positive HIV diagnosis. The results from this AIR study suggest that there is a need to investigate the functionality of $CD4^{+}$T-cells and B-cells that are also characterised as being dysfunctional in HIV-infection (117,162).

Inability to achieve cohort protection to PPV-23 led to a change in the study protocol to give patients the pneumococcal conjugate vaccine, Prevenar-13 – PCV-13, used in infants in the UK. Infants' immune systems cannot respond to PPV-23, but do respond to PCV-13 which is designed to stimulate adolescent immune systems; and has been found to work better

than PPV-23 in patients who have an impaired immune system through age or diseases that reduce immune competence (163–165). Furthermore, there have been studies that suggest that antibody responses to pneumococcal conjugate vaccines are significantly higher than responses to PPV-23 in clinical settings where ARV therapy is available (146,166).

Further evidence to support the use of pneumococcal conjugate vaccines in HIV-infected adults has been established in a South African cohort of HIV-infected children which has shown protection against pneumonia and IPD (167).

The only randomised placebo-controlled trial of pneumococcal conjugate vaccination in HIV-infected adults demonstrated enhanced protection against recurrent pneumococcal infections in Malawi, which further supports the idea of pneumococcal conjugate vaccination (142).

From the AIR study findings, a single vaccine doses of PPV-23 against pneumococcus does not provide an immediate immunogenic response in an HIV-infected adult population. A 5 year longitudinal study following HIV-infected patients who received a SD of PPV-23 do not maintain their responses to vaccination even with ARV therapy and with low CD4 counts (168).

Mixed vaccination schedules have been performed with varying results dependent on the patient population and geographical location of the studies (136,138,144,145,169). Vaccination with two doses of PCV-7, followed by PPV-23 established immunological memory in HIV-infected children (170). Furthermore, another study found that in HIV-infected adults, revaccination with PPV-23 after 5 years on ARV therapy can establish immunological memory (147). However, it must be stressed that boosting patients with multiple doses of polysaccharide vaccines can induce hyporesponsiveness, which was first described by Felton and Bailey in 1926 (171,172). Another study in which three doses of PCV-13 were administered following a dose of PPV-23 gave an immediate rise in antibody concentration following vaccination; however, overall levels did not change following each dose (173).

It is important to highlight that there is a lack of controlled studies investigating vaccination against *S. pneumoniae* in HIV-infection. Due to the poor immune response to a single dose of PPV-23, it was decided to change the study protocol and initially give HIV-infected study

participants a single dose of PCV-13 and further boosters as required, which will be discussed later in Chapter 4.

Chapter 3. Validation of the 19-plex assay and AIR patient samples

3.1 Introduction

Previous validation work was performed during the course of this PhD project that indicated that concentrations produced by the 19-plex assay (Chapter 2; section 2.5.2.3) were not stable over time. Initially, AIR study post-vaccination concentrations were lower than pre-vaccination concentrations, which indicated that the samples needed to be re-run on the 19-plex assay. The analysis was performed using the originally recorded results from the AIR study and the results were found to be unreliable when examining longitudinal sample results.

Examining the 3 controls (in-house (HUS and CAL) and external control samples from NEQAS) gave consistent results, indicating that there was variability in the assay which varied in magnitude over time.

- *Internal controls* – HUS (normal human serum (Sigma Aldrich, UK) and CAL (normal human serum source from one healthy control donor vaccinated with PPV-23).
- *External controls* – NEQAS (human quality control serum samples from The United Kingdom National External Quality Assessment Source)
- *HIV-infected patient samples* –Pre-vaccine/baseline serum samples of the AIR cohort

To further qualify this, a sample of AIR samples were selected and retested, to determine where a larger retesting programme was required.

To further quantify the variability in the assay, a representative subset of samples were re-run, and compared to the originally reported concentrations to inform the retesting strategy.

3.2 Aims and Sample Selection

There were two main aims of the analysis:

1) To assess the consistency of unvaccinated baseline bloods over time

The assumption was that the baseline AIR patient antibody concentrations would not vary significantly over time. None of the patients had been vaccinated at this time point, so any trends in antibody concentrations over time would have implied that the level of protection in the population had changed over time. For each year 2009-2015, ten patients with pre-vaccine blood samples available were randomly selected for retesting. The resulting concentrations were tested for trends over time, as well as being compared back to the original measurements, to check for consistency of the assay.

2) To assess the variability of antibody concentrations over time on a patient basis

The ten samples from 2010/2011 from the previous aim had been stratified such that those from 2010 had PPV-23 as their initial vaccine, and those from 2011 had PCV-13, and that all patients had subsequent blood tests on at least an annual basis. All available samples for these patients were retested. This allowed the response to the initial vaccine (and any boosters) to be assessed, as well as the longevity of this response. The longitudinal values from the original and retested values could be plotted together, to see whether the changes in concentrations were reasonable (i.e. increased after a vaccine, and declined over time), and to see how much of a difference was present between the original and retested samples.

Patient selection of the validation analysis is summarised in Table 3.1.

Table 3.1. Inclusion criteria for re-testing of AIR samples for longitudinal analysis

Year	Inclusion Criteria	Samples Used for Aim
2009	• First blood sample is pre vaccine (or up to 1 week after to allow for delays)	1
2010	• Pre-vaccine sample available (Up to 4 weeks before vaccine) • Post-vaccine sample available (4-16 weeks after vaccine) • Vaccinated with PPV-23 • At least one sample available in 5 distinct years	1,2
2011	• Pre-vaccine sample available (Up to 4 weeks before vaccine) • Post-vaccine sample available (4-16 weeks after vaccine) • Vaccinated with PCV-13 • At least one sample available in 5 distinct years	1,2
2012	• First blood sample is pre vaccine (or up to 1 week after to allow for delays)	1
2013	• First blood sample is pre vaccine (or up to 1 week after to allow for delays)	1
2014	• First blood sample is pre vaccine (or up to 1 week after to allow for delays)	1
2015	• First blood sample is pre vaccine (or up to 1 week after to allow for delays)	1

The final list of samples contained 50 solely for Aim 1 (10 per year in 2009, 2012-2015), and 181 for Aim 2 (all follow up for 20 patients), of which 20 samples were also used in Aim 1. This left a total of 231 samples for retesting.

3.3 Materials and Methods

3.3.1 Validation of the 19-plex assay

As with any functional assay, there is a certain amount of intra- and inter-assay variability. Standard QC control (S1, S8, CAL, and HUS) data were collected from each 19-plex assay (described in Chapter 2, section 2.5.2.3) since 2009. Observed concentrations were converted from mg/mL to µg/mL, logged, and plotted in order to compile scatter plots. Standards were checked to see if there were any differences. The scatter plots revealed that from the period of 2009-2011, the standard ranges varied considerably. This could have been due to the fact that serum samples were manually diluted into sample buffer during this time period. In 2012, the Dynex machine was introduced and the serum samples were automatically diluted, thus further standardising the dilution assay to eliminate manual control error, as this can vary considerably amongst different users. Also, in 2011, there was

another step-change in the control serum, which is most likely due to the change from the usage of the pooled standard control serum, 89-SF to 007SP. Furthermore, there is another step-change from 2013-2014; however, no cause was determined and this was further investigated by myself and the CIS team. Since the beginning of 2014, the assay standards displayed a similar range. Thus, it was decided to re-test a small subset of samples that were tested from 2009-2015 on the 19-plex assay and re-analyse the data based on those results. Baseline and post-vaccine samples were run on the same plate to reduce interassay variability.

3.4 Results

3.4.1. First-bloods over time

Previous validation analyses showed that pre-vaccine concentrations within the cohort varied over time. In order to ascertain whether this was due to a true variation in baseline immunity, or to variability in the assay, comparisons were made between the pairs of originally reported and retested concentrations for the samples included in the validation analysis. Separate analyses were produced for the each year being considered, using Kruskal-Wallis tests. The resulting p-values are in Table 3.2 below, with significant ($p<0.05$) differences reported with single asterisks (*) and highly significant ($p<0.01$) p-values with double asterisk (**):

Table 3.2. Variation between originally reported and retested concentrations for first blood samples included in the validation analysis.

Year	2009	2010	2011	2012	2013	2014	2015
Pn1	**0.002	**0.004	*0.039	**0.008	0.084	**0.006	**0.006
Pn3	*0.037	**0.008	0.426	0.977	0.084	*0.049	0.846
Pn4	0.375	*0.027	0.625	*0.049	*0.037	1	0.193
Pn5	0.375	0.164	0.16	*0.01	**0.002	0.922	0.57
Pn6B	0.098	0.074	0.275	0.164	0.203	0.846	0.77
Pn7F	*0.037	0.496	0.432	0.625	*0.027	0.105	0.131
Pn9V	0.375	1	0.375	0.105	1	0.492	0.193
Pn14	0.734	0.652	0.129	0.432	0.91	0.695	0.496
Pn18C	0.064	*0.02	0.055	0.846	0.77	*0.037	0.16
Pn19A	*0.027	0.129	*0.037	0.322	0.432	0.557	0.375
Pn19F	0.492	0.203	**0.004	0.275	0.492	0.641	0.131
Pn23F	*0.037	**0.004	0.557	0.275	**0.004	0.625	0.193
MenA	0.77	0.652	0.275	1	0.375	0.322	0.16
MenC	0.275	**0.004	*0.014	**0.002	0.922	0.232	0.77
MenW	*0.014	**0.004	*0.037	0.193	*0.02	0.193	0.375
MenY	0.77	0.82	0.557	0.131	0.432	**0.006	0.084
Tetanus	**0.002	**0.004	**0.002	0.064	0.426	0.432	**0.002
Diphtheria	0.383	0.438	0.063	0.301	0.1	0.25	*0.027
Hib	**0.004	**0.004	0.301	0.77	0.275	0.064	*0.049

As can be seen, there were many cases where the retested concentrations came out to be significantly different to those that were originally reported. The results were generally consistent with previous validation, with those serotypes where validation samples varied considerably with time (e.g. Pn1, 3, 23F) having the greatest number of significant differences, whilst those that had more stable validation samples (e.g. Pn9V, 14) having the least. One serotype that showed a lot of variability over time (Pn3) and one with low variability over time (Pn9V) are visualised in more detail in the plots in section 3.4.2 and 3.4.3.

3.4.2 Pn3

Figures 3.1 for Pn3 shows the previous validation performed on this titre. Both the internal (HUS) external (NEQAS) and HIV-infected patient samples (First Bloods – the original concentrations for pre-vaccine blood tests of the AIR cohort) samples showed a similar trend, with the titre reducing over time, before flatting out from around 2013 onwards.

Figure 3.2 shows the retested concentrations, which are much more stable over time, and do not show the previously mentioned trends. The second graph in Figure 3.2B compared the

original (blue) to the retested (green) samples in every year, which established that the original runs of the samples gave significantly higher concentrations than the re-runs in the early period, becoming closer over time.

This is further broken down in Figure 3.3; the first graph shows the fold differences between the original and retested concentrations for each sample. Again, this is consistent with other analyses, showing that the original samples gave higher concentrations until around 2012, after which there was greater consistency.

The other two graphs in Figure 3.3 compared the original (Figure 3.3B) and retested (Figure 3.3C) concentrations in 2010 and 2015. In the former, the original run gave higher concentrations for every sample being compared, whilst in 2015, the original runs and retests were more similar.

3.4.3 Pn9V

The same graphs are reported for Pn9V (Figure 3.4-3.6) , which show that this serotype was much more consistent over time, as a result of the original and retested concentrations being more consistent than in Pn3.

In conclusion, this analysis suggests that the observed variability over time in the antibody concentrations is likely a reflection of assay, rather than patient variability. As a result, comparing samples that were tested at different times, either to compare the responses to the PPV-23 and PCV-13 vaccines, or to look at the longevity of response in a single patient, is likely to give unreliable and unrepresentative results.

3.4.4 Longitudinal analysis

In order to show how the variability in the assay would impact analysis of longitudinal data, the serial antibody concentrations, both from the original run on the assay and the re-test, were plotted over time and compared. Figures 3.7-3.10 were considered for two of the AIR patients, considering Pn3 and Pn9V for the reasons discussed earlier. These are representative plots and not all of the data for every patient will be presented in this thesis.

Again, this shows large differences between the original and retested Pn3 concentrations over time (Figure 3.7 and 3.9). The retested samples (blue) show a modest increase in concentrations after both the initial and the booster vaccine, which decline with time, as would

be expected. However, the original runs of the samples (red) showed a larger increase in concentrations after the first vaccine, which continued to increase over the course of a year. Concentrations then dropped when the booster was given, and continued to fall at a rapid rate.

Comparing the shape of the original runs of the samples (red) on this graph to the plot of the HUS samples over time shows a strong correlation, implying that the drop observed after the booster vaccine is a reflection of assay variability, rather than a true reflection of the antibody concentrations of the patient.

For the longitudinal plot of Pn9V for this patient (Figures 3.8 and 3.10), both the original and retested samples gave more consistent results, as would be expected, based on the lower variability in the assay over time for this serotype.

Pn Serotype 3

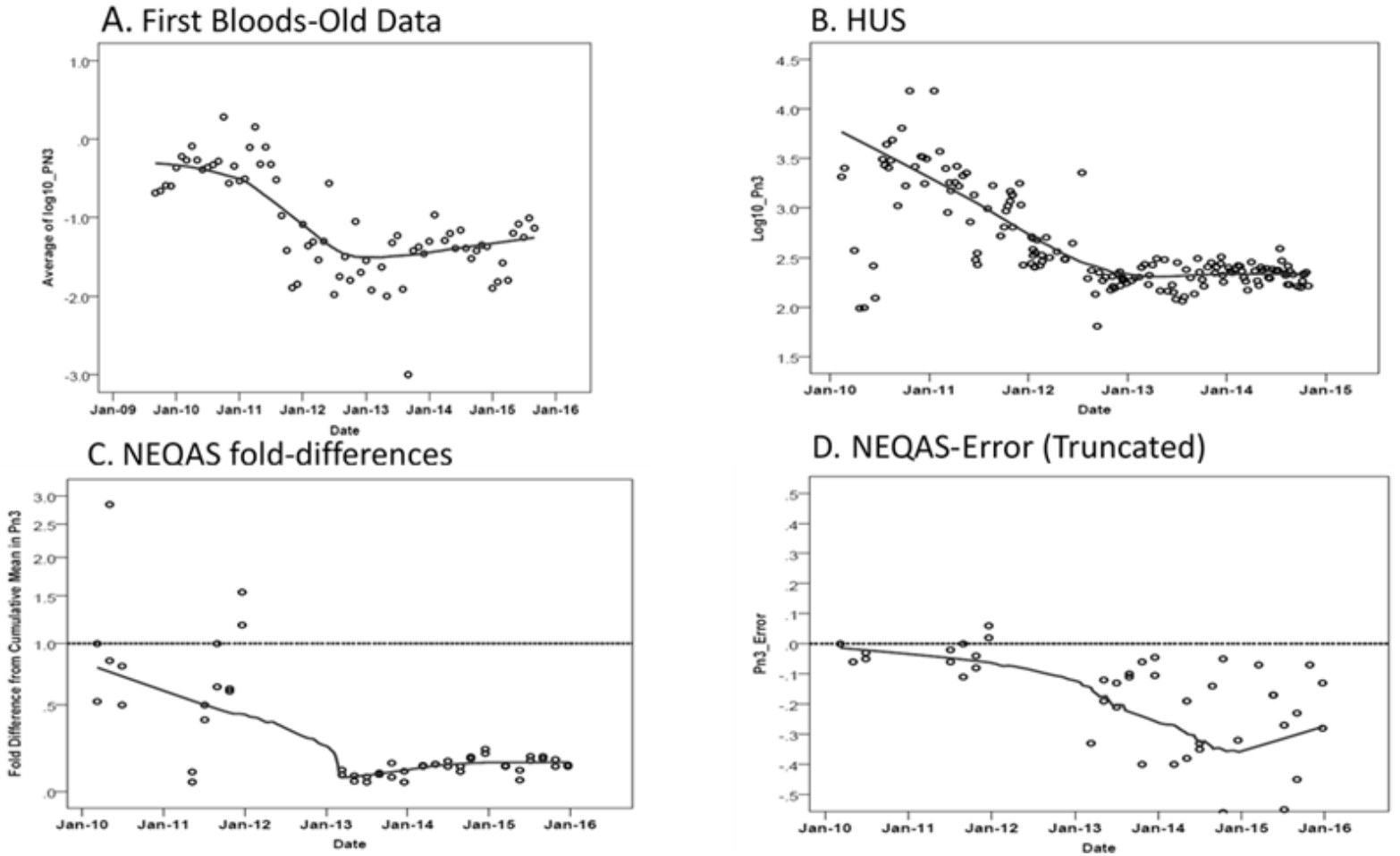


Figure 3.1 Validation of Pn Serotype 3. *A) First Bloods- Old Data-Pre-vaccine titres from originally tested serum samples B) HUS-Internal control sample showing variability over time C) NEQAS- External control sample showing fold differences over time. D) NEQAS- External control samples showing truncated error over time.*

Pn Serotype 3

A. First Bloods- Pn3 Absolute Pn-specific IgG Titres

B. First Bloods- Pn3 Yearly Pn-specific IgG Averages

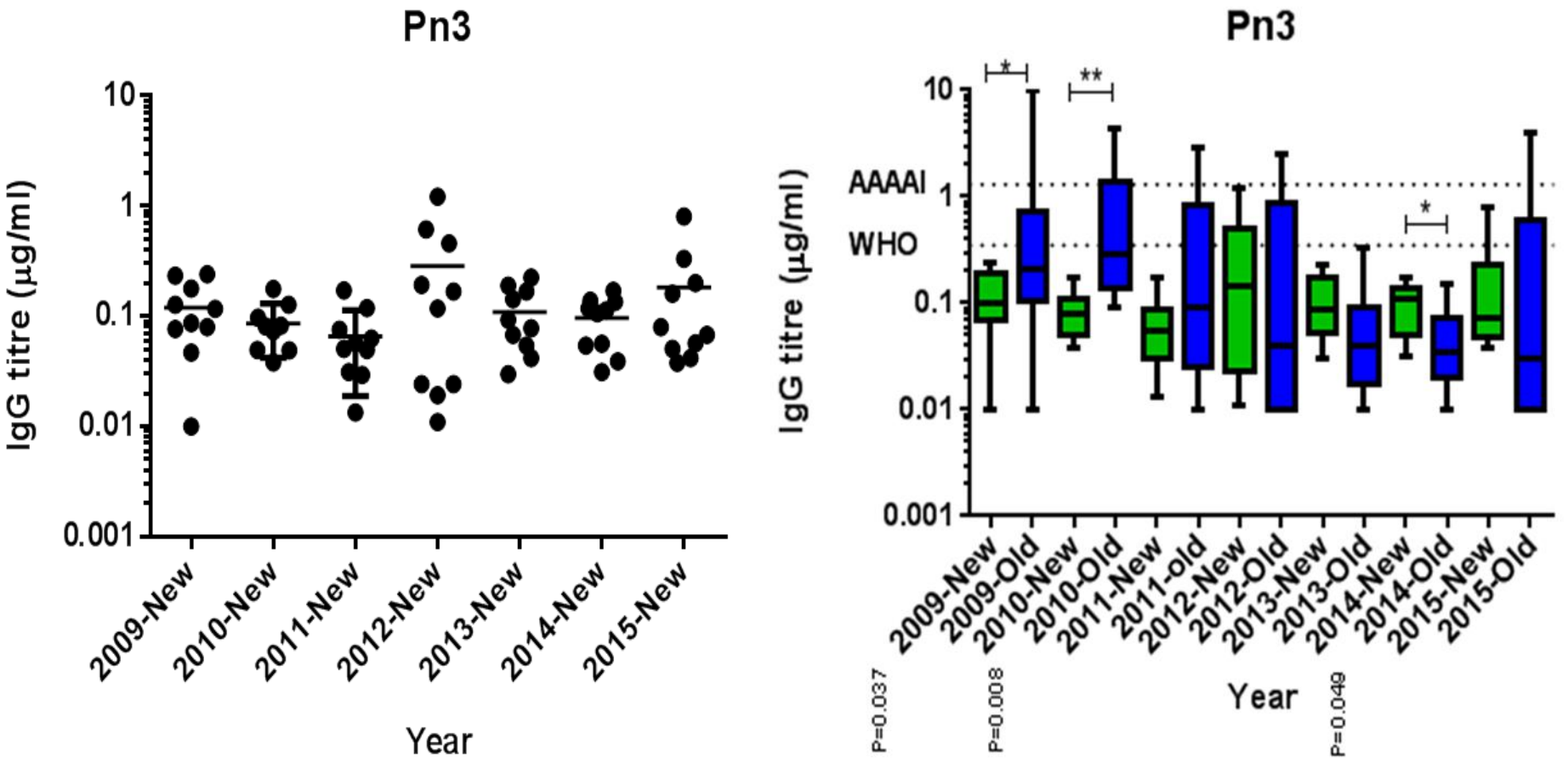


Figure 3.2 Validation of Pn Serotype 3 including first bloods analysis of absolute Pn-specific IgG concentrations and yearly averages. *A) First Bloods-Pn 3 Pn-specific IgG titres. Kruskal-Wallis Test of Pn3 ANOVA- Multiple Comparisons show non-significance between years of assay. B) First Bloods- Pn3 Yearly Specific IgG Averages. Blue=Old results; Green= New Results.Wilcoxon Matched-Pairs Signed Rank Test show significance in the years of 2009, 2010, and 2014 between the old data and new re-tested sample data.*

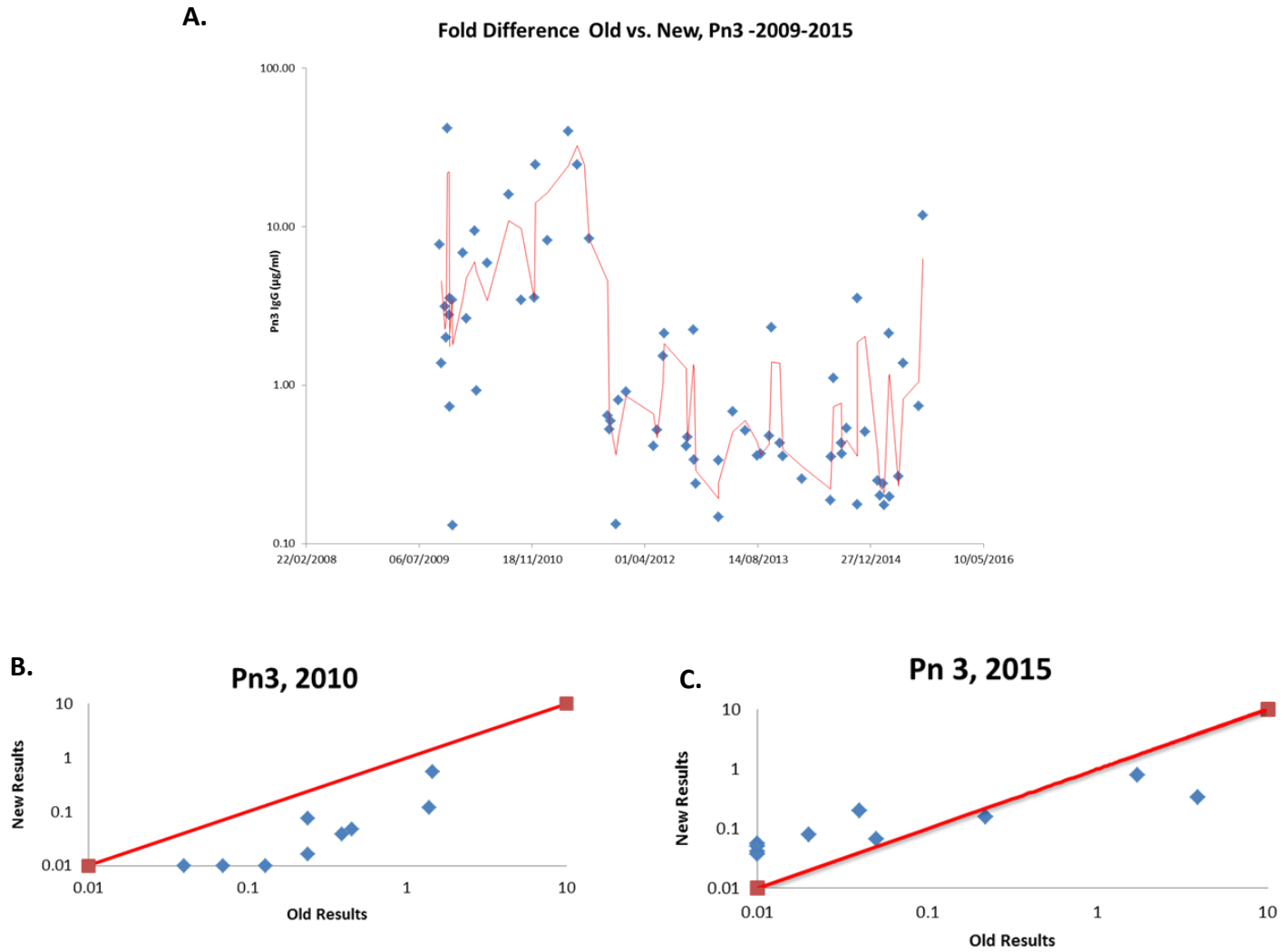
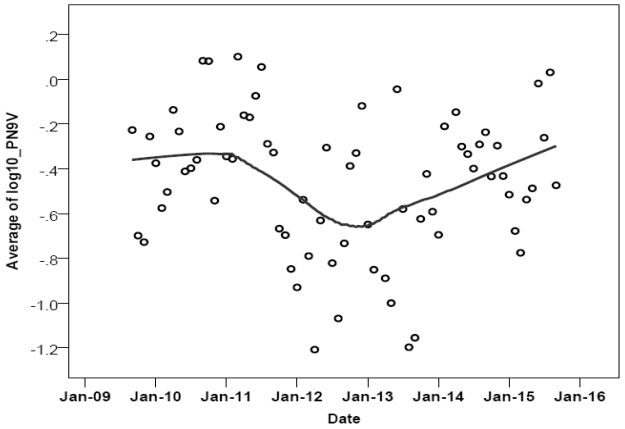


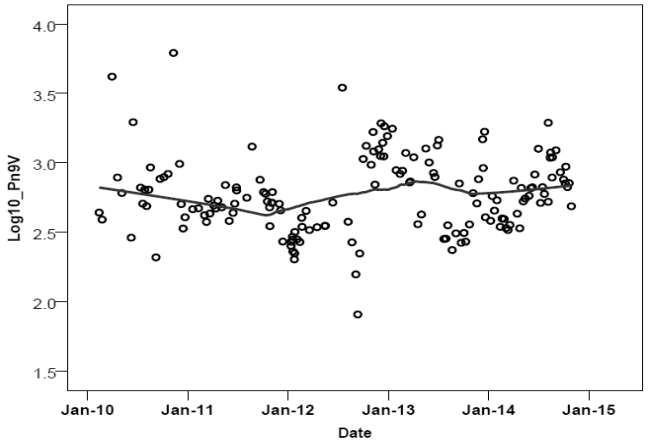
Figure 3.3 Validation of Pn Serotype 3 including fold differences between old and new analyses on first blood samples for longitudinal analysis *A) Fold differences between old versus new Pn3-IgG specific titres from 2009-2015 B) Plot of old versus new results of Pn3-IgG specific titres in 2010 C) Plot of old versus new results of Pn3-IgG specific titres in 2015.*

Pn Serotype 9V

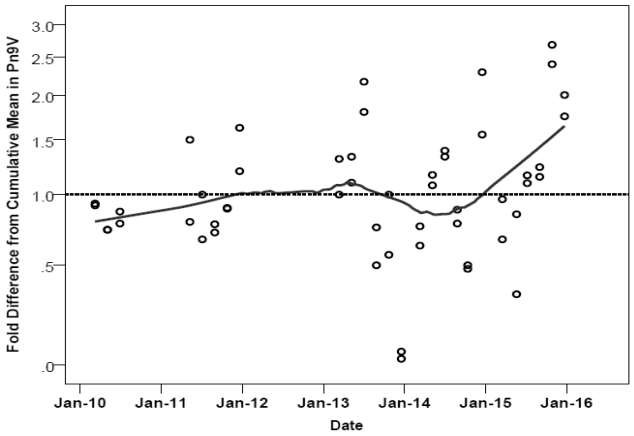
A. First Bloods –Old Data



B. HUS



C. NEQAS fold-differences



D. NEQAS-Error

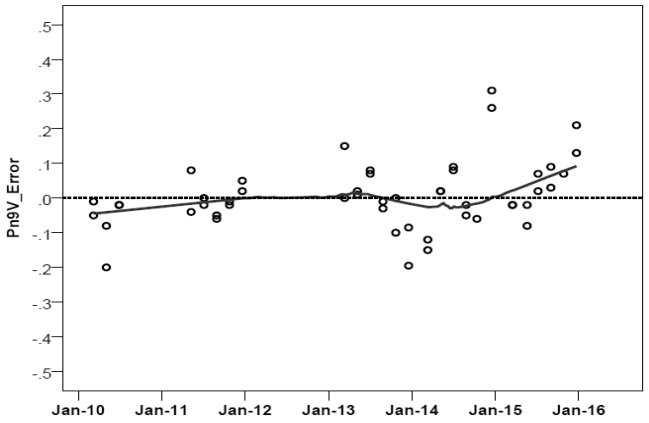


Figure 3.4. Validation of Pn Serotype 9V. *A) First Bloods-Old Data-Pre-vaccine titres from originally tested serum samples B)HUS- Internal control sample showing variability over time C) NEQAS-External control sample showing fold differences over time D) NEQAS- External control samples showing truncated error over time.*

Pn Serotype 9V

A. First Bloods- Pn9V Absolute Pn-specific IgG Titres

B. First Bloods- Pn9V Yearly Pn-specific IgG Averages

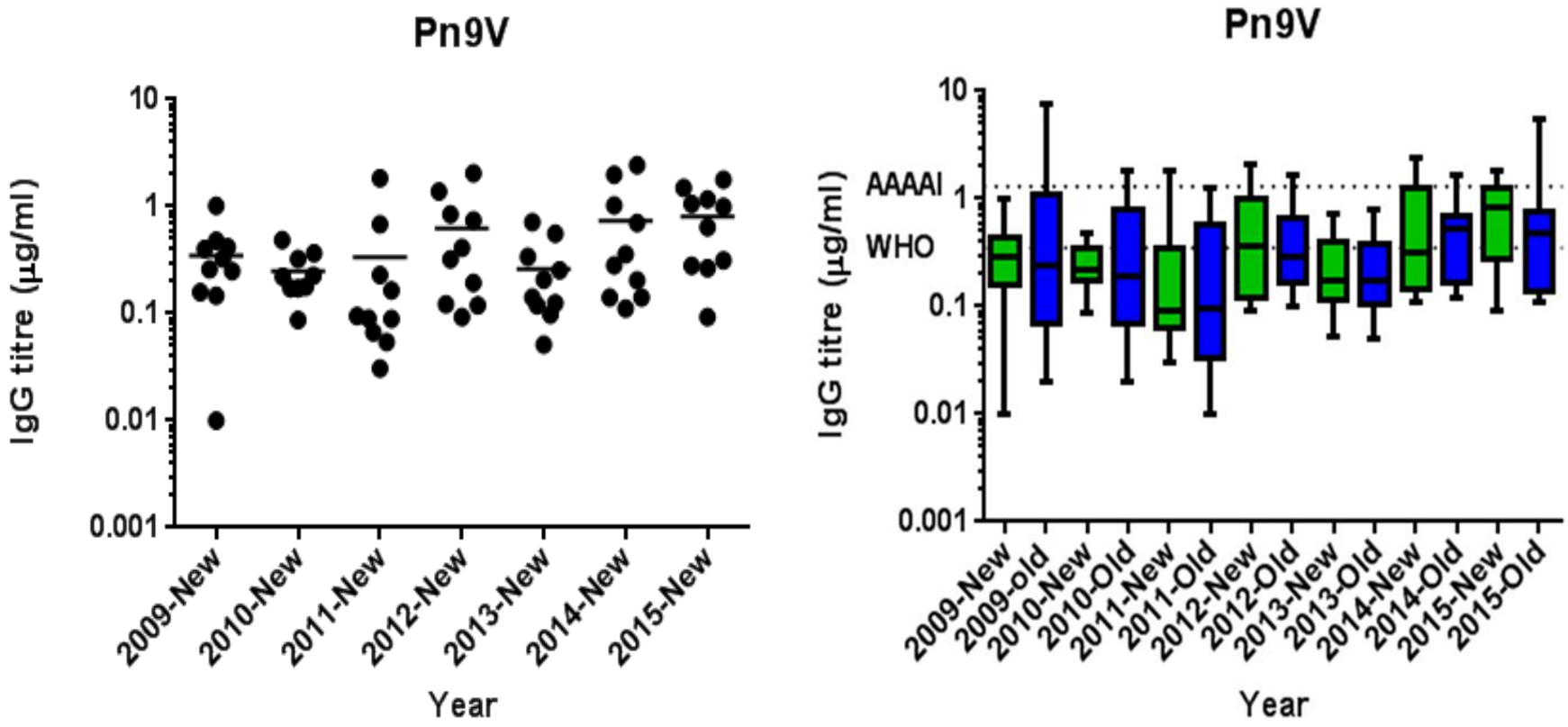


Figure 3.5 Validation of Pn Serotype 9V including first bloods analysis of absolute Pn-specific IgG concentrations and yearly averages. *A) First Bloods-Pn 9V Pn-specific IgG titres. Kruskal-Wallis Test of Pn9V ANOVA- Multiple Comparisons show non-significance between years of assay. B) First Bloods- Pn9V Yearly Specific IgG Averages.Blue=Old results; Green=New Results Wilcoxon Matched-Pairs Signed Rank Test show non-significance between the old data and new re-tested sample data.*

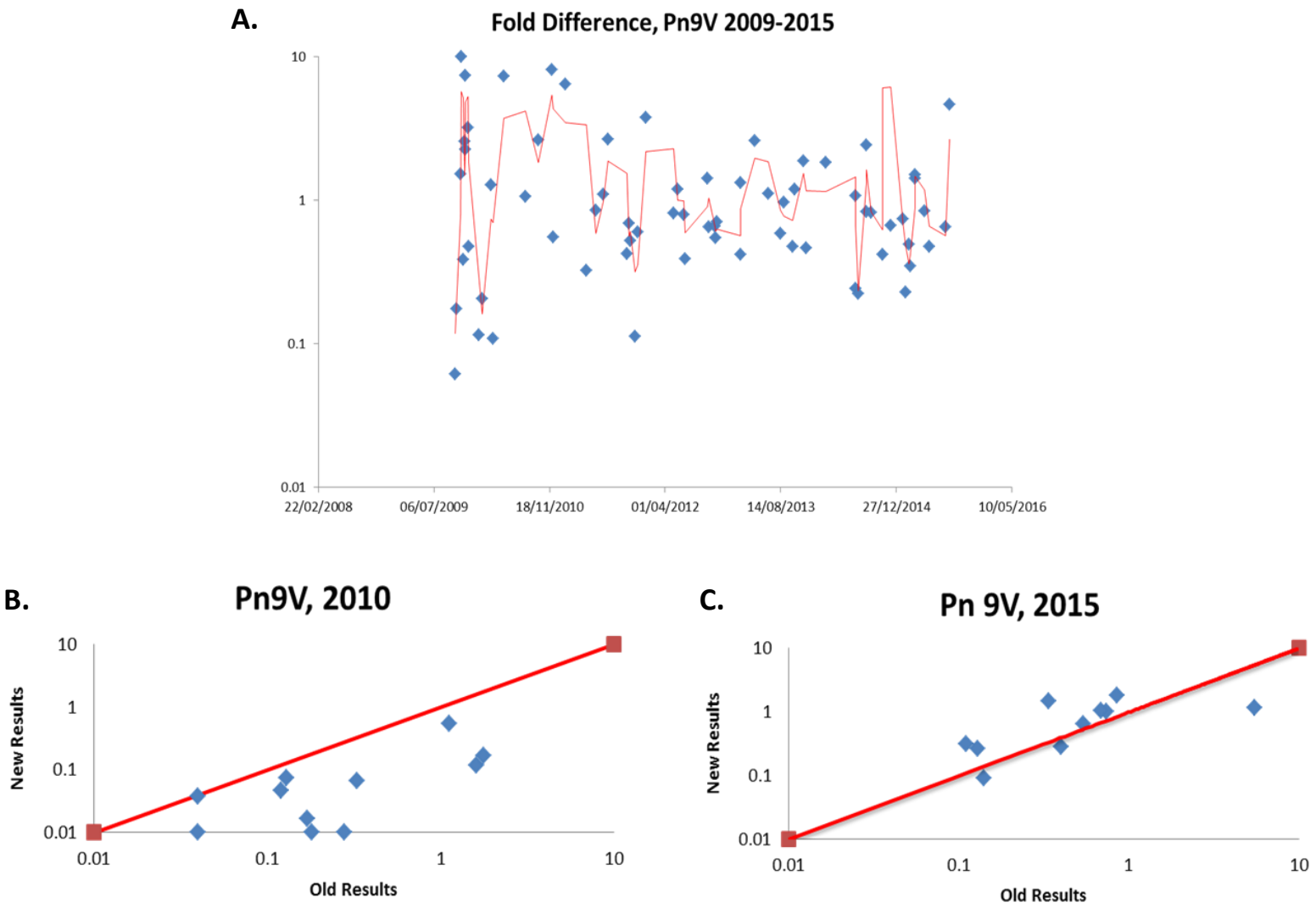


Figure 3.6 Validation of Pn Serotype 9V including fold differences between old and new analyses on first blood samples for longitudinal analysis *A) Fold differences between old versus new Pn9V-IgG specific titres from 2009-2015 B) Plot of old versus new results of Pn9V-IgG specific titres in 2010 C) Plot of old versus new results of Pn9V-IgG specific titres in 2015.*

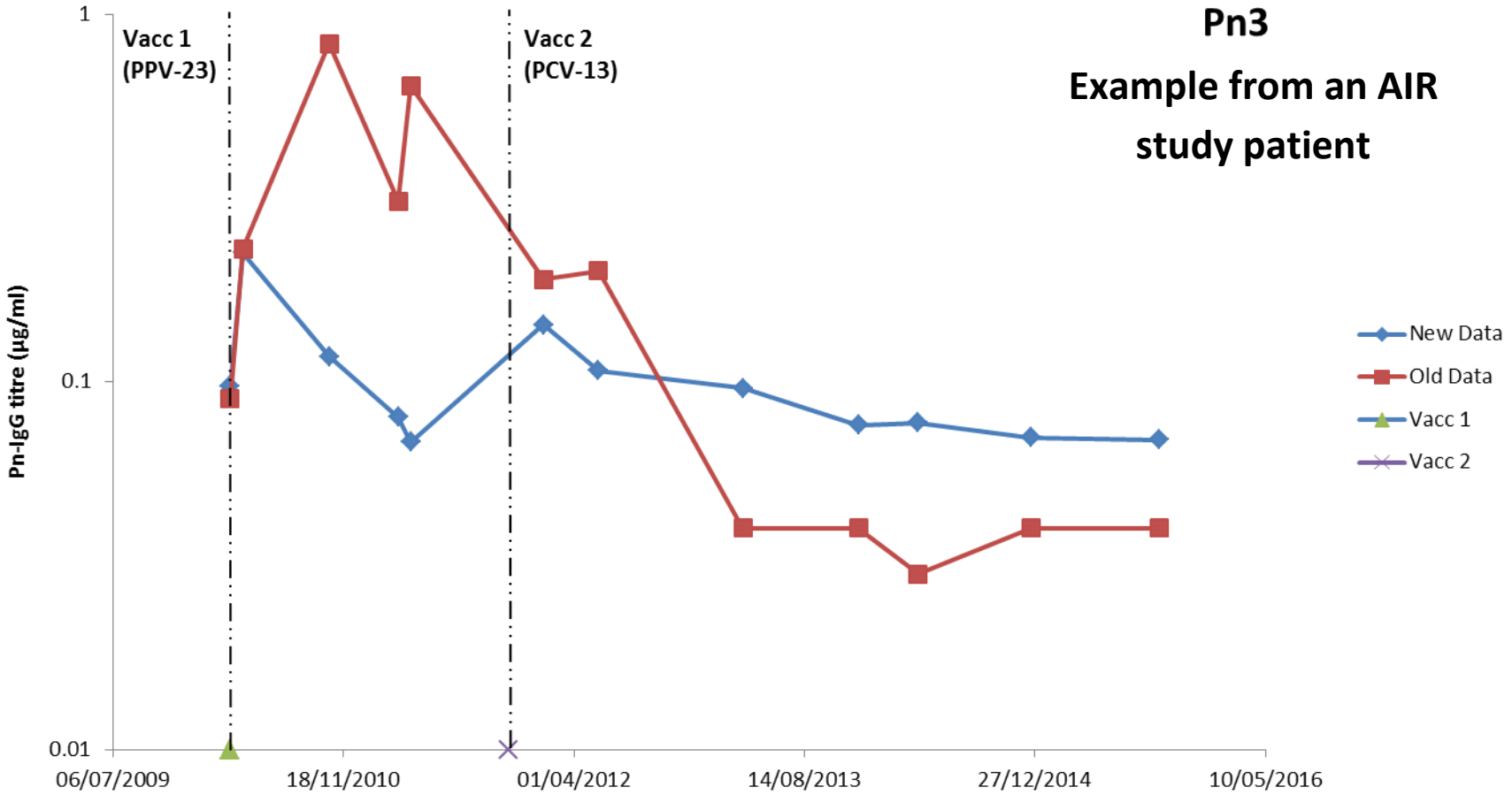


Figure 3.7. Longitudinal analysis of Pn3 IgG concentrations post-vaccination comparing old and new analyses on retested AIR samples from an AIR study patient. *Points on the graph indicate multiple time post-vaccination following an initial dose of PPV-23 (Vacc 1) and a second dose of PCV-13 (Vacc 2).*

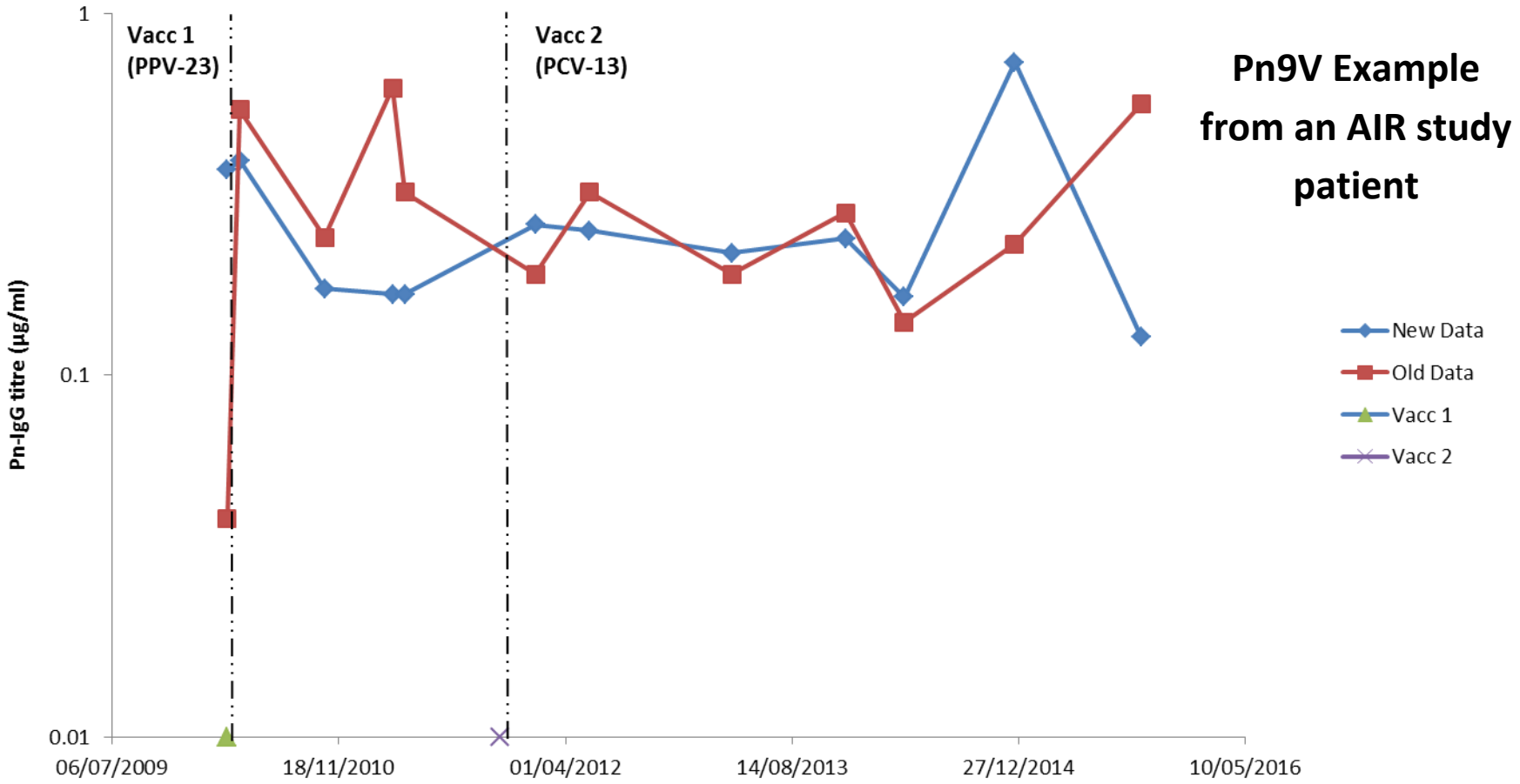


Figure 3.8 Longitudinal analysis of Pn9V IgG concentrations post-vaccination comparing old and new analyses on retested AIR samples from an AIR study patient. *Points on the graph indicate multiple time post-vaccination following an initial dose of PPV-23 (Vacc 1) and a second dose of PCV-13 (Vacc 2).*

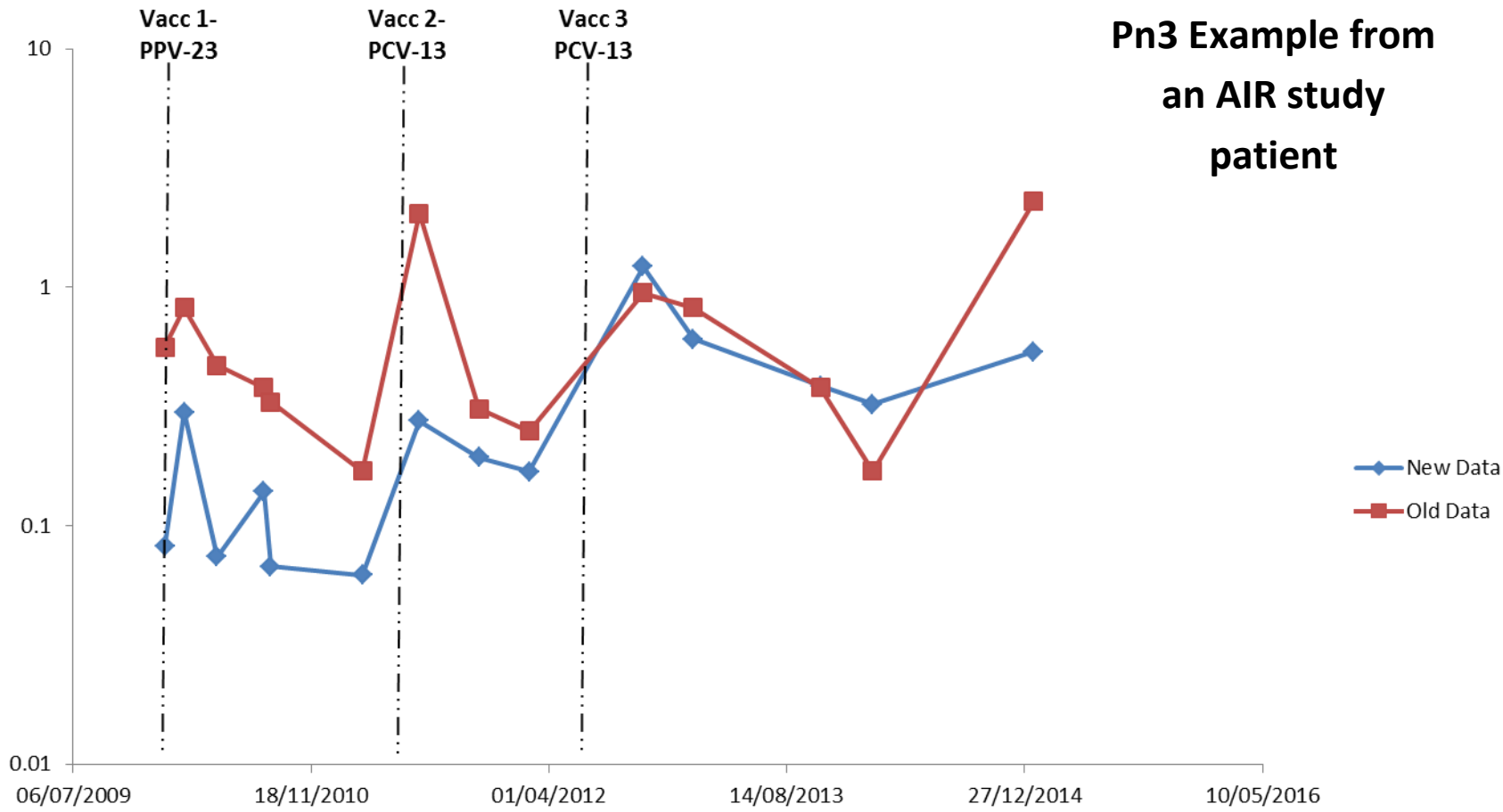


Figure 3.9 Longitudinal analysis of Pn3 IgG concentrations post-vaccination comparing old and new analyses on retested AIR samples from an AIR study patient. *Points on the graph indicate multiple time post-vaccination following an initial dose of PPV-23 (Vacc 1) , a second dose of PCV-13 (Vacc 2), and a third dose of PCV-13 (Vacc 3).*

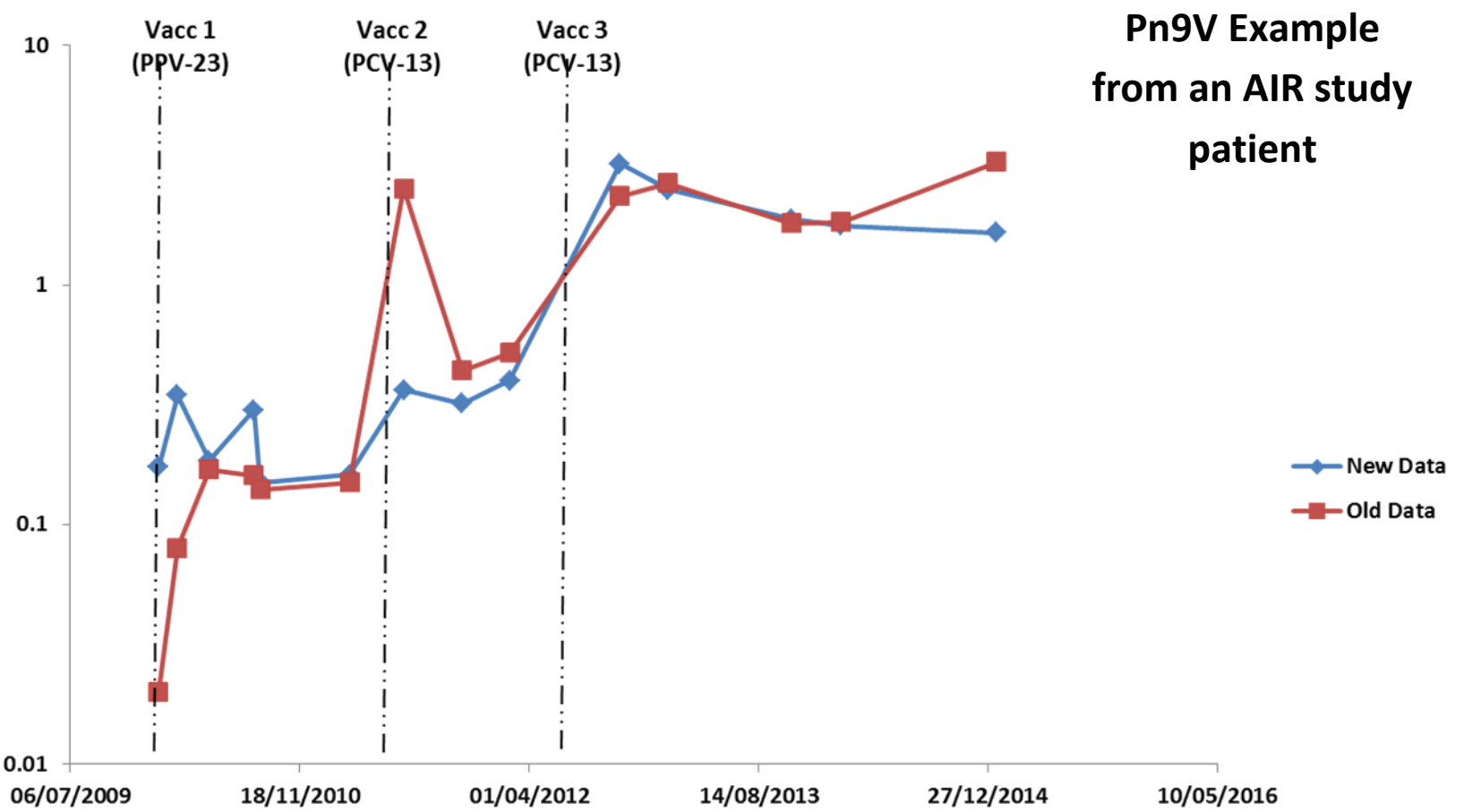


Figure 3.10 Longitudinal analysis of Pn9V IgG concentrations post-vaccination comparing old and new analyses on retested AIR samples from an AIR study patient. *Points on the graph indicate multiple time post-vaccination following an initial dose of PPV-23 (Vacc 1) , a second dose of PCV-13 (Vacc 2), and a third dose of PCV-13 (Vacc 3).*

3.5 Discussion

The original validation analyses indicated that there was variability over time in the assay, and this additional analysis has returned similar results. The early years of the study are a period where the trends over time are at the greatest magnitude, which is also the time of the change from PPV-23 to PCV-13. As such, by using the results of the original assay runs of the samples, a reliable comparison of the effectiveness of these vaccines cannot be performed.

The trend over time in the variability in the assay also has a considerable effect on the longitudinal antibody concentrations for patients. Hence, analysis of longevity of protection, or the relative effectiveness of boosters to the original vaccine could not be reliably performed.

The majority of the discussion in these analyses relates to relative measures (i.e. original vs. retested titre or longitudinal changes in patient concentrations). One of the key aspects of this study was to report protection rates, using the WHO/AAAAI thresholds. The validation analysis found that the original concentrations produced by the assay were generally highest in the early period of the study, and declined over time. Hence, if these data sets were used, the protection rates measured in the early period of the study were likely to be vast overestimates, which would decline with time, causing a considerable underestimate in longevity of protection and effectiveness of the boosters.

With so much variability over time from 2009-2015, there were many points to consider with regards to the analysis of the original and re-tested AIR study data, including whether or not a statistical adjustment factor to the pre-existing AIR data and/or running a larger subset of AIR study samples in order to obtain a more reliable data set. This would include the assignment of IgG concentrations to the re-tested samples based on the 007sp standard.

Therefore, it was decided not to use the application of a statistical adjustment factor and to re-run a larger subset of samples in order to obtain a more reliable dataset, which is discussed in Chapter 4. The percentage of HIV-infected adults who achieve threshold vaccination antibody levels with a single dose of PPV-23 and PCV-13 is investigated in Chapter 4. For patients who do not achieve threshold vaccination antibody levels with one dose of vaccination, it is important to investigate whether or not this can be achieved with

further booster vaccination. Subsequently, for those patients who do achieve threshold antibody levels with vaccination, the longevity of the threshold levels is investigated and discussed in Chapter 4.

Chapter 4. The Longevity of the Immune Response to a Single Dose of PPV-23 versus PCV-13 and booster PCV-13 Vaccinations in an HIV-infected Population in the UK

Oral presentation: 5th Network Meeting of the MIA Network in Den Bosch, The Netherlands, Luminex Corporation, May 2017.

Oral presentation: Public Health England, Vaccine Evaluation Unit, National Infection Service, Manchester Royal Infirmary, Manchester, UK, September 2017.

Manuscript in preparation.

4.1. Introduction

Immunocompromised individuals, including patients with HIV, are at increased risk of pneumococcal infection. The annual incidence of invasive pneumococcal disease is 2.5 per 1000 in the HIV population, which is fifty times higher than the general population (2) and has increased 50-fold in England and Wales from 2000-2009 (1). Bacterial pneumonia is the most common infection in HIV positive individuals and 70% of these pneumonias are caused by *Streptococcus (S.) pneumonia* (2). Despite modern antibiotics the case fatality rate for pneumococcus remains high and so vaccination has a key role to play in prevention. Perversely, those most at risk of infection are often the least able to respond to vaccination. Vaccination strategies for adults at risk of infection are often pragmatic rather than evidence based. Assessment of these strategies requires a careful chain from patient testing to national microbiological surveillance programmes and these data are difficult to systematically and successfully collate.

Pneumococcal vaccination is offered by two strategies; firstly, a pure polysaccharide vaccine, which contains 23 (out of 95) pneumococcal serotypes and secondly, a protein polysaccharide conjugate vaccine which can contain either 10 or 13 (out of 95) serotypes. The vaccines differ in their ability to recruit T-cell help, which is required for the generation of high affinity antibody and memory. Conjugate vaccines are used in the infant vaccination schedule as naïve immune systems generate an inadequate T-independent response

required by the polysaccharide vaccine. In contrast, the polysaccharide vaccine has been used for patients at risk of pneumococcus including in the UK all adults over the age of 60.

There is variation in guidelines for pneumococcal vaccination in HIV. In the UK, the British HIV Association (BHIVA) recommend a single-dose of the 13 valent conjugate vaccination Prevenar-13 (Pfizer)(PCV-13) followed by a single dose of Pneumovax-23 (PPV-23) polysaccharide vaccine, although this was not recommended at the time of the change in study protocol in 2010 (174) . The US Centre for Disease Control provides the same advice as BHIVA (175). Factors which influence these decisions include; the higher cost of PCV-13, the ability to boost PCV-13 whilst repeat vaccination in PPV-23 results in hyporesponsiveness (176), the wider coverage of serotypes in PPV-23 and the limited and short response of PPV-23, herd protection resulting in much reduced rates of PCV-13 serotype disease since introduction into the infant schedule in 2010 (177).

The Assessment of Immune Responses to Routine Immunisation in HIV-infected Adults (AIR) observational study is a collaboration under between the Clinical Immunology Service at the University of Birmingham, UK and the HIV service at University Hospitals Birmingham NHS Trust. This was the largest UK vaccination study in HIV infected adults which aimed to improve vaccine uptake in HIV and to monitor the immune response to vaccination. Between 2009-2015 the study recruited 839 HIV patients and 73 controls who attend the sexual health clinic and were confirmed to be HIV negative. At the initiation of this study, BHIVA and the JCVI recommended a single dose of PPV-23(75,82). As of November 2015, the JCVI have not changed their position with regards to the advice of adult pneumococcal vaccination and still support PPV-23 vaccination in adults (85). Patients were also offered vaccination against Influenza, Menitorix (the *Haemophilus influenzae* type b and meningococcal C glycoconjugate vaccine), hepatitis A and B and DTaP (Diphtheria, Tetanus and Pertussis) following diagnosis. Need for and response to vaccination was assessed by achieving a threshold antibody concentration as measured by multiplex immunoassay (MIA). The first results from the AIR study found that HIV infected adults had similar antibody response to Menitorix as HIV-negative controls (178) . In contrast, HIV patients had significantly lower responses to Pneumovax-23 compared with controls whose response was also limited (33% v 53%). Interim analysis at one year highlighted this lower response and a major study amendment changed the pneumococcal vaccine to Prevenar-13. This provided retrospective comparison between responses to PPV-23 and PCV-13 in HIV-infected adults and prospective monitoring of longevity of response and need for booster

vaccination which antibody levels fell below threshold (World Health Organization (WHO) protective threshold 0.35 µg/mL in ≥ 8/12 serotypes) (179).

4.2 Aims

The aim of this chapter is to investigate how HIV-1 infection affects the short-term and long-term immune response to pneumococcal plain polysaccharide (PPV-23) and conjugate (PCV-13) vaccination.

4.3 Objectives

The objective of this study was to:

1) Investigate Pn-specific IgG pre-and post- vaccination (4-weeks) serum antibody concentrations following a single dose of Pneumovax-23® (PPV-23) and Prevenar-13® (PCV-13) in HIV-infected and HIV-negative individuals.
2) Investigate the longevity of the Pn-specific IgG post-vaccine responses annually following initial pneumococcal vaccination and subsequent boosters for 6 years.
    - Assess threshold protection by pneumococcal serotype.
3) Assess the number of pneumococcal vaccine doses required to achieve threshold protective levels.

4.4 Materials and Methods

4.4.1 Ethical Approval

Ethical approval for this study is described in section 2.3.

4.4.2 19-plex multiplex Luminex assay IgG Antibody Quantification

This method is described in detail in section 2.5.2.

4.4.2.1. Preparation of Luminex Beads

4.4.2.1.1 PLL (Poly-L-lysine) conjugations

This method is described in section 2.5.2.1.

4.4.2.1.2 Bead conjugations

This method is described in section 2.5.2.2.

4.4.2.1.3 19-plex multiplex assay

This method is described in section 2.5.2.3.

4.4.3 Opsonophagocytic Killing assay (OPKA)

A 4-plexed multiplexed OPKA (for Pn serotypes 4, 6B, 14, and 23F) was developed by myself and Dr. Jenny Herbert (Post-Doctoral Fellow, Pneumococcal Research Group, Professor Tim Mitchell's laboratory, University of Birmingham, Edgbaston, UK) and used to determine the functionality of specific antibodies against pneumococcus, which is known to be impaired in some immunosuppressed groups including older patients. This assay assessed bacterial opsonophagocytosis via killing of pneumococcal serotypes using differentiated HL-60 cells both using a flow cytometric and a plate method (180,181).

4.4.3.1 HL-60 cell culture

HL-60s were used as the phagocytes in the assay. HL-60 cells were obtained from Dr. Farhat Kharim's group (University of Birmingham, U.K.). Cells were propagated in a culture medium containing RPMI 1640, 20% fetal bovine serum, FBS, and 1% L-glutamine (Gibco Invitrogen, UK). Cells were differentiated for 5 days in the above culture medium supplemented with 0.8% dimethyl formamide (DMF) (Sigma, UK). Cell morphology was assessed via cytocentrifugation and the Romanowsky method and the images were taken and displayed in Figure 4.1. Differentiated cells (Panel B, Figure 4.1) should be hypersegmented and granular in appearance.

The HL-60 cell phenotype should be determined every 4 weeks via FACS analysis. To determine if the cells are appropriately differentiated and working properly, cells should be stained for CD35 ($\geq$ 55% expression, differentiated-upregulated) and CD71($\leq$20% expression, differentiated-downregulated). (Please see Figures 4.2 and 4.3, which are

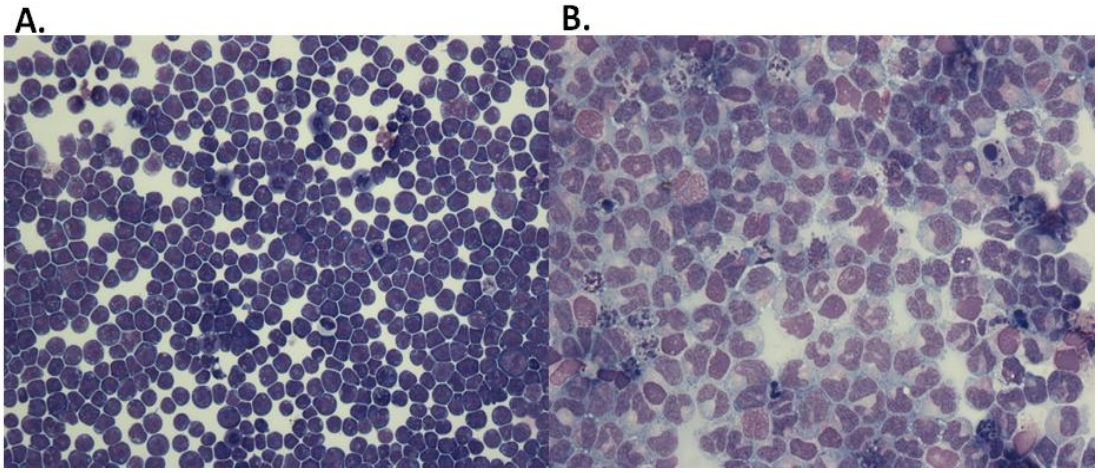


Figure 4.1. Cytocentrifugation of undifferentiated (20x dry magnification) (A) versus differentiated (40x magnification, oil immersion) (B) HL-60s using the Romanowsky staining method (Image taken by Sian E. Faustini using the Leica DM6000 (Leica Microsystems, UK).

representative flow cytometry plots that was acquired from a mOPA experiment). Also, cell viability must be above 90% (trypan-blue).

Master cell banks were prepared from the original flask of cells and were grown to a density of 5 x 10^5 cells/mL for approximately 3 weeks. Cells were prepared in freezing medium containing: 50% FBS (heat-inactivated for 30 minutes at 56°C, 0.1% DMSO, and 40% RPMI-1640. Cells were transferred to 50 mL falcons and centrifuged at ~350g (1200 rpm) for 5 minutes. The supernatant was removed and 2.5 mL of freezing medium was added to each 50 mL tube to gently re-suspend the cell pellets. Cell pellets were combined and 1 mL was aliquoted into to distribute into cryovials for freezing. Each vial contained 1 x 10^7 cells/mL. Cryovials were placed into a controlled-rate freezer to begin a freezing program and once frozen, cryovials were transferred into a liquid nitrogen storage facility. One vial was kept to test for mycoplasma contamination (MycoAlert, Lonza) and other microbial contamination.

Working cells stocks are passaged every Monday, Wednesday, and Friday if differentiating cells for up to two times per week. For example, cells that are differentiated on Wednesday will be used 5-6 days later on the following Monday or Tuesday.

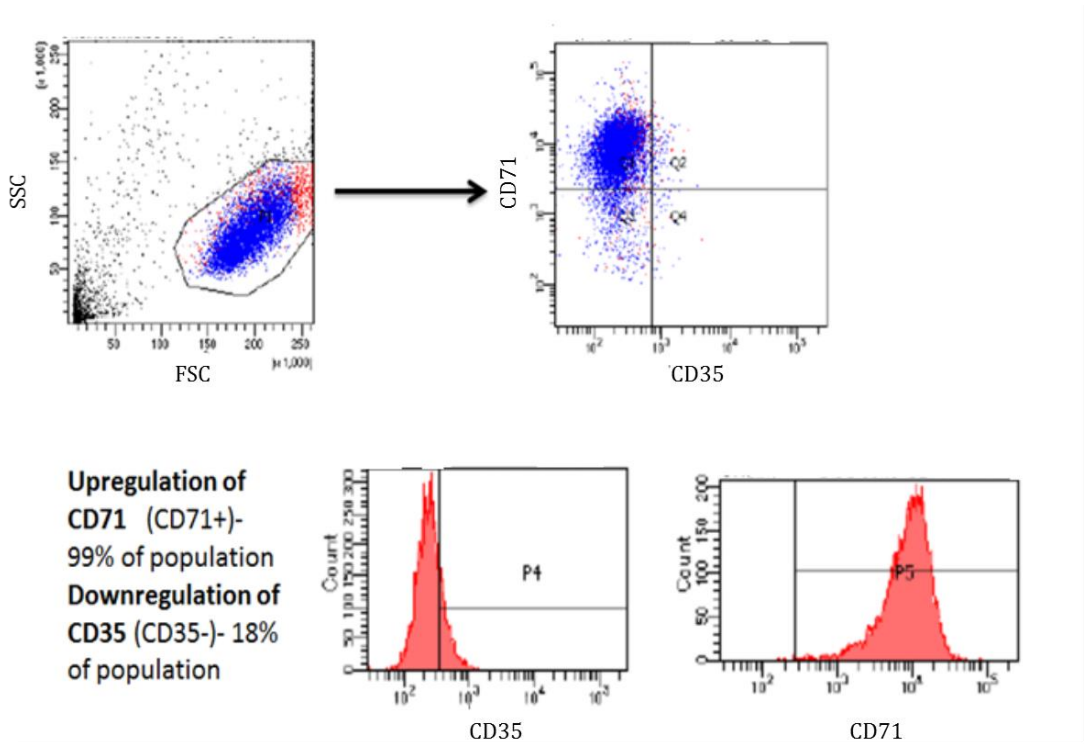


Figure 4.2 An example of a flow cytometric plot of the undifferentiated HL-60 cell population (Acquired by Sian E. Faustini on a FACSFortessa (BD Biosciences, USA)using FACSDiva software (BD Biosciences, USA) for analysis).

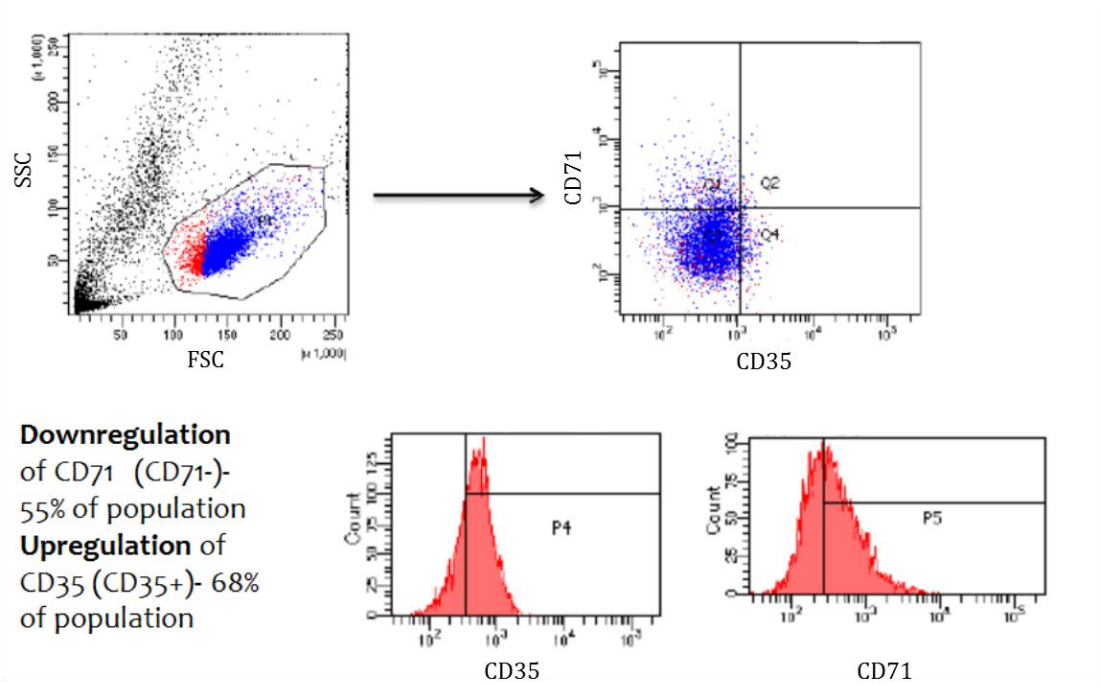


Figure 4.3 An example of a flow cytometric plot of the differentiated HL-60 cell population (Acquired by Sian E. Faustini on a FACSFortessa (BD Biosciences, USA) using FACSDiva software (BD Biosciences, USA) for analysis).

4.4.3.1.1 Cytocentrifugation of HL-60 cells

Prepare cell suspensions as needed in the relevant assay. Microscope slides (Fisher, UK) were labelled. The rough side of a filter card was placed onto (ThermoFisher,UK) onto a cytocentrifuge funnel (ThermoFisher, UK). The filter card and funnel was locked together into a stainless steel clip (ThermoFisher, UK)using the relevant key. The entire assembly was placed in the cytocentifuge rotor (ThermoFisher, UK) and 100 µl of RPMI 1640 (Sigma, UK) and 2% newborn calf serum (NCS) (Gibco, UK) was added to the funnel. 7-11 µl of the relevant sample was added to the funnel. The cover was fixed onto the rotor and placed onto the impellor. The slides were centrifuged for 3 minutes at 500 RPM. The clip was then carefully disassembled and the slide was removed and allowed to air-dry for 5 minutes before staining the slides via the Romanowsky method.

4.4.3.1.1.1 Staining of slides and cytospins by the Romanowsky method

Fresh air-dried cytospin slides were arranged in a rack. The rack was placed on the hook in the StainMate processor (R.A. Lamb (now Fisher)). The stain process was automatic and the timings were as follows:

1) Methanol (VWR, UK) fixation (5 minutes)
2) May-Grunwald (VWR, UK) stain (10 minutes)
3) Water rinse (30 seconds)
4) Giemsa stain (VWR, UK) (10 minutes)
5) Water rinse (30 seconds)

Once the slides were dry, the slides were mounted with an aliquot of DPX mounting medium (VWR, UK) and a cover glass (Fisher, UK) and allowed to dry before viewing/taking photographs on an appropriate microscope.

The staining solutions were prepared as follows:

1) Staining Buffer (Sorenson's pH 6.8) (TCS Biosciences, Ltd., UK) : 25 mL of stain buffer to 5 L of de-ionised water.
2) May-Grunwald (for staining carbohydrate-rich structures): 150 mL of staining buffer to 50 mL of May-Grunwald stain.
3) Giemsa (for staining of nucleic acids): 180 mL of staining buffer to 20 mL of Giemsa stain.

4.4.3.2 Bacterial culture

Bacterial master stocks (Pn 4, 6B, 14, and 23F) were obtained from BEI Resources (Manassas, VA, USA). Thirteen different antibiotic resistant *S. pneumoniae* strains were used for the assay and were grown from frozen glycerol stocks or blood agar base (BAB) by taking a sweep of bacteria using a sterile loop. Bacterial strains were then inoculated in Brain Heart

Infusion (BHI) broth and incubated at 37°C statically. Bacterial strains were allowed to grow to an optical density of 600 (OD_{600}) and then processed accordingly. 15% glycerol was added if making frozen bacterial stocks. The bacterial strains were natural variants resistant to specific concentrations of antibiotics derived from wild-type strains and are described in Table 4.1 (182).

Table 4.1. Bacterial Strains and their Antibiotic Resistance

Antibiotic	**C1**	**C2**	**C3**	**C4**
Optochin (Orep)	4	18C	7F	3
Spectinomycin (Spec)	6B	19F	1	6C
Streptomycin (Strep)	14	9V	5	33F
Trimethroprim (Trim)	23F	6A	19A	22F

4.4.3.2.1 Viable Counting of Bacteria

Bacteria were counted before the assay in order to account for bacterial death, which will naturally occur once a freeze-thaw occurs. A volume of 180 µl of PBS was added to 6 wells in a 96-well round-bottomed plate (Greiner, UK). 20 µl of bacterial sample was pipetted into the first well, whilst pipetting up and down (20x). A new sterile tip was used and then 20 µl of well 1 was transferred to well 2 and mixed as in the previous step. This process was repeated until the 6th well. A serial dilution results (10^{-1} to 10^{-6}) and bacteria could be plated (3 x 20 µl) onto a BAB plate (Figure 4.4) . Plates are then placed into an incubator overnight for 16 hours at 37°C. Counts are then performed the next day. Colonies should be countable (30-100 CFU per spot).

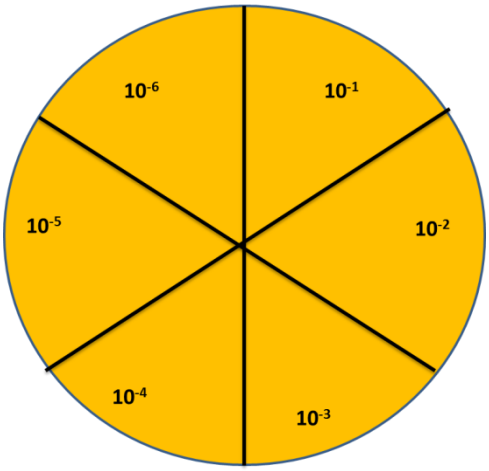


Figure 4.4. Example of a viable count plate

4.4.3.3 Human serum samples

Human serum samples were used in this assay. Serum samples were stored at -80°C until needed for testing. Samples were removed from the freezer and kept at RT until thawed completely. Once samples were thawed, test samples were heat inactivated in order to inhibit endogenous complement activity at 56°C in a water bath for 30 minutes.

4.4.3.4 Baby rabbit complement

Baby rabbit complement (Pel-freez Biologicals, Rogers, AR, USA) was used in the OPKA assay in the presence of human serum and differentiated HL-60 cells in order to opsonise the bacterial cell surface, which results in the uptake and killing via the HL-60 cells.

4.4.3.5 Multiplexed Opsonophagocytic Killing Assay (University of Alabama- mOPA procedure)

A detailed protocol of this assay can be found at www.vaccine.uab.edu (183). Prior to testing, human test serum samples and complement (control A) were heat-inactivated at 56°C for 30 minutes. Control B was normal serum. Round bottomed 96-well plates (Corning

Plate A

	1	2	3	4	5	6	7	8	9	10	11	12
A	Control A	Control B	Dilution 8	Dilution 8	Dilution 8	Dilution 8	Dilution 8	Dilution 8	Dilution 8	Dilution 8	Dilution 8	Dilution 8
B	Control A	Control B	Dilution 7	Dilution 7	Dilution 7	Dilution 7	Dilution 7	Dilution 7	Dilution 7	Dilution 7	Dilution 7	Dilution 7
C	Control A	Control B	Dilution 6	Dilution 6	Dilution 6	Dilution 6	Dilution 6	Dilution 6	Dilution 6	Dilution 6	Dilution 6	Dilution 6
D	Control A	Control B	Dilution 5	Dilution 5	Dilution 5	Dilution 5	Dilution 5	Dilution 5	Dilution 5	Dilution 5	Dilution 5	Dilution 5
E	Control A	Control B	Dilution 4	Dilution 4	Dilution 4	Dilution 4	Dilution 4	Dilution 4	Dilution 4	Dilution 4	Dilution 4	Dilution 4
F	Control A	Control B	Dilution 3	Dilution 3	Dilution 3	Dilution 3	Dilution 3	Dilution 3	Dilution 3	Dilution 3	Dilution 3	Dilution 3
G	Control A	Control B	Dilution 2	Dilution 2	Dilution 2	Dilution 2	Dilution 2	Dilution 2	Dilution 2	Dilution 2	Dilution 2	Dilution 2
H	Control A	Control B	Dilution 1	Dilution 1	Dilution 1	Dilution 1	Dilution 1	Dilution 1	Dilution 1	Dilution 1	Dilution 1	Dilution 1
			Sample 1		Sample 2		Sample 3		Sample 4		Sample 5	

Plates B, C, D, E, F, and G

	1	2	3	4	5	6	7	8	9	10	11	12
A	Dilution 8	Dilution 8	Dilution 8	Dilution 8	Dilution 8	Dilution 8	Dilution 8	Dilution 8	Dilution 8	Dilution 8	Dilution 8	Dilution 8
B	Dilution 7	Dilution 7	Dilution 7	Dilution 7	Dilution 7	Dilution 7	Dilution 7	Dilution 7	Dilution 7	Dilution 7	Dilution 7	Dilution 7
C	Dilution 6	Dilution 6	Dilution 6	Dilution 6	Dilution 6	Dilution 6	Dilution 6	Dilution 6	Dilution 6	Dilution 6	Dilution 6	Dilution 6
D	Dilution 5	Dilution 5	Dilution 5	Dilution 5	Dilution 5	Dilution 5	Dilution 5	Dilution 5	Dilution 5	Dilution 5	Dilution 5	Dilution 5
E	Dilution 4	Dilution 4	Dilution 4	Dilution 4	Dilution 4	Dilution 4	Dilution 4	Dilution 4	Dilution 4	Dilution 4	Dilution 4	Dilution 4
F	Dilution 3	Dilution 3	Dilution 3	Dilution 3	Dilution 3	Dilution 3	Dilution 3	Dilution 3	Dilution 3	Dilution 3	Dilution 3	Dilution 3
G	Dilution 2	Dilution 2	Dilution 2	Dilution 2	Dilution 2	Dilution 2	Dilution 2	Dilution 2	Dilution 2	Dilution 2	Dilution 2	Dilution 2
H	Dilution 1	Dilution 1	Dilution 1	Dilution 1	Dilution 1	Dilution 1	Dilution 1	Dilution 1	Dilution 1	Dilution 1	Dilution 1	Dilution 1
	Sample 6, 12, 18, etc		Sample 7, 13, 19, etc		Sample 8, 14, 20, etc		Sample 9, 15, 21, etc		Sample 10, 16, 22, etc		Sample 11, 17, 23, etc	

Control A	Contains bacteria + complement (HI) + HL60, t=75 minutes	Used to calculate non-specific killing.
Control B	Contains bacteria + complement + HL60, t=75 minutes	Used to calculate maximum CFU/spot in assay conditions. Used to calculate non-specific killing.

Figure 4.5 . Plate plan for mOPA (Figure taken from (183).

Inc., Corning, NY, USA) were prepared. Serum samples (30 µl) were added to the plate in duplicate in addition to 10 µl of OPA buffer (80 mL sterile water, 10 mL of 10XHBSS (with Ca^{++}/Mg^{++}), 10 mL of 1% gelatin and 5.3 mL of FBS (heat inactivated at 56°C for 30 minutes)) in each well. Test samples were diluted initially at a 4-fold dilution and then a 3-fold serial dilution by reverse pipetting.

A frozen working stock tube of each of the four bacterial strains were washed twice in OPA buffer via centrifugation at 12000g for 2 minutes and then diluted to the appropriate bacterial density (~10^5 CFU/mL for single-serotype assays and ~ 2 x 10^5 for multiplexed serotype assays). Equal volumes of the four bacterial suspensions in each cassette ie (C1, C2, C3, and C4) were combined and 10µl of the suspensions were added to each well including control wells A and B (Figure 4.5).

Test serum and bacteria were then incubated at RT on an orbital shaker at 700 rpm for 30 minutes.

10 µl of complement and 40 µl of prepared and differentiated HL-60 cells (prepared at 1 x 10^7 cells/mL) were added to each well. Prior to addition of HL-60 cells to the plate, cells were washed twice with 1X HBSS (without Ca^{++}/Mg^{++}) at ~350 g for 5 minutes at RT and then with 1X HBSS (with Ca^{++}/Mg^{++}) at ~350 g for 5 minutes.

Plates were then incubated on an orbital shaker for 45 minutes at 37°C and 5% CO_2 at 700 rpm. After the incubation period, plates were put on ice for 20 minutes to stop the phagocytic reaction. 10 µl of the reaction mixture was spotted onto four Todd-Hewitt Yeast Agar(THYA) (Becton-Dickinson) plates and tilted immediately in order to prevent the mixing of spots. Plates were left to dry at RT for 20 minutes and then a 2,3,5-triphenyltetrazolium chloride (TTC) (Sigma, UK) overlay containing one of the four antibiotics was added to each replicate plate.

Once the overlay was solidified, the plates were incubated upside down for 16-18 hours at 37°C. Colonies that grew on the THYA plates with the Orep overlay were the bacterial strains resistant to optochin and likewise for the Spec, Strep, and Trim overlays.

After the overnight incubation period, colonies were counted manually or using an automated counter. Colonies could also be enumerated using a software developed by the University of Alabama (UAB) lab in conjunction with the US National Institute of Standards and Technology (NIST) that counts colonies from a digital image. It is available via the website: ftp://ftp.nist.gov/pub/physics/mLclarke/NICE/. Opsonisation titres (OT) were then calculated using a free program available from the UAB lab called Opsotitre 3. The serum dilution that kills 50% of bacteria was classed as the OT.

4.4.3.5.1. Key Conclusions from the University of Alabama (UAB) OPKA method

After repeated unsuccessful attempts at replicating the UAB OPKA method, a new OPKA method was developed by Siân E. Faustini and Dr. Jenny Herbert (Post-Doctoral Fellow, Pneumococcal Research Group, Professor Tim Mitchell's laboratory, University of Birmingham, Edgbaston, UK) in order to obtain a working single-plex assay.

Pneumococcus does not grow under traditional UAB OPKA conditions using the shaking technique. Pneumococcal strains are not cultured whilst shaking. Instead, they grow under static, anaerobic conditions. Therefore, it is illogical to shake them vigorously for any length

of time. Additionally, the amount of bacteria is not counted using this method. Therefore, the amount of the bacteria that is put into the test well is unknown. Bacterial counts and serial dilutions are performed in order to ensure that the amount of bacteria that is put into the test well is correct.

4.4.3.6 New Optimised Method for OPKA

The key problem with the UAB-mOPA assay is as follows:

1) Pneumococcus does not grow well under traditional OPA conditions using the shaking technique. When pneumococcus is grown, they are grown under static, anaerobic conditions. They do not grow well under shaking, aerobic conditions such as the methods employed using the UAB OPKA method.

Due to inconsistent results with the UAB-mOPA method, Dr. Jenny Herbert and I developed our own opsonophagocytic killing assay as follows:

4 x 10^7 HL-60 cells were differentiated with 0.8% DMF for 5 days. Serum was heat-inactivated at 56°C for 30 minutes and then added to the plate. Serum was diluted (1:2) up to 8 wells. 2.5 x 10^5 *S. pneumoniae* were added to each well and incubated for 30 minutes at 4°C. The plate was then centrifuged for 5 minutes at 3060 g and then supernatants were removed. After this incubation period, 5 x 10^5 HL-60 cells (ATCC) and baby rabbit complement (7.5 µl) were added to the 96-well plate and then placed in the incubator at 37°C and 5% CO_2 for 40 minutes. After the second incubation period, the sample wells are serially diluted 10-fold and plated (1:10) onto a BAB plate using a tilt method. The plates are then incubated overnight (16-18 hours) at 37°C and 5% CO_2 and the colonies could be manually counted on the next day. Colonies should be countable (20-100 CFU).

Once the plates were counted and recorded, opsonophagocytic killing assay (OPKA) titres could be assigned at 50% killing in comparison to bacteria, complement, and cells only control (no serum added) (see Figure 4.6). Pn serotype 4, 6B, 14, and 23F antiserums (SSI- Statens Serum Institut) were used as internal controls.

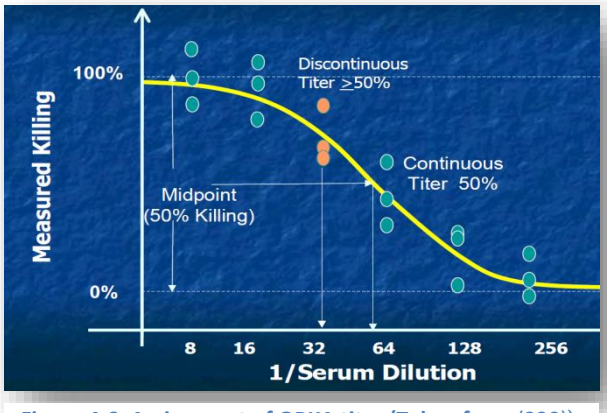


Figure 4.6. Assignment of OPKA titre (Taken from (339)).

4.4.3.6.1 Anti-serum controls show a high percentage of pneumococcal killing

Specific human anti-serums against Pn 4, 6B, 14, and 23F were used as controls in order to calculate opsonophagocytic (OPKA) titre. Titres are defined as the reciprocal of the two-fold dilution that exhibited ≥50% killing compared to the complement control wells (BCC) where no antibody source is added to the test well). An OPKA titre ≥ 1:8 is considered positive.

Serotypes 4, 14, and 23F exhibited a high percentage of killing (93-97%) in the initial test well. However, serotype 6B exhibited a lower percentage of killing in the initial test well(66%) (Table 4.2). OPKA titres were high for each anti-serum control and were as follows Pn 4 (320), 6B (80), 14 (160), and 23F (160) (Figure 4.7 A-D).

Table 4.2. Anti-serum control values (CFUs and percentage killing) in the OPKA assay.

Serum dilution	PN4 (avg. CFUs)	PN4 (% killing)	(avg. CFUs)	PN6B (% killing)	(avg. CFUs)	PN14 (% killing)	(avg. CFUs)	PN23F(% killing)
1/20	2	*94.3*	15	*65.6*	7	*92.75*	3	*97.25*
1/40	2	*94.3*	19	*55.7*	10	*90.25*	5	*94.75*
1/80	3	*90.6*	22	*49.6*	13	*87*	24	*76*
1/160	3	*88.7*	34	*21.4*	14	*86*	41	*59.25*
1/320	14	*47.2*	30	*31.3*	85	*15.5*	74	*25.75*
1/640	24	*9.4*	31	*28.2*	96	*3.75*	101	*-1*
1/1280	23	*15.1*	28	*36.6*	100	*0*	99	*1*
1/2560	23	*13.2*	42	*3.8*	100	*0*	102	*-1.75*
BCC	27	N/A	44	N/A	100	N/A	100	N/A

CFUs= Colony forming units; CFUs are calculated by averaging duplicate test well results
BCC= Bacteria + cells + complement (no antibody added)
*Percentage killing is calculated as ((BCC-test well)/(BCC))*100*

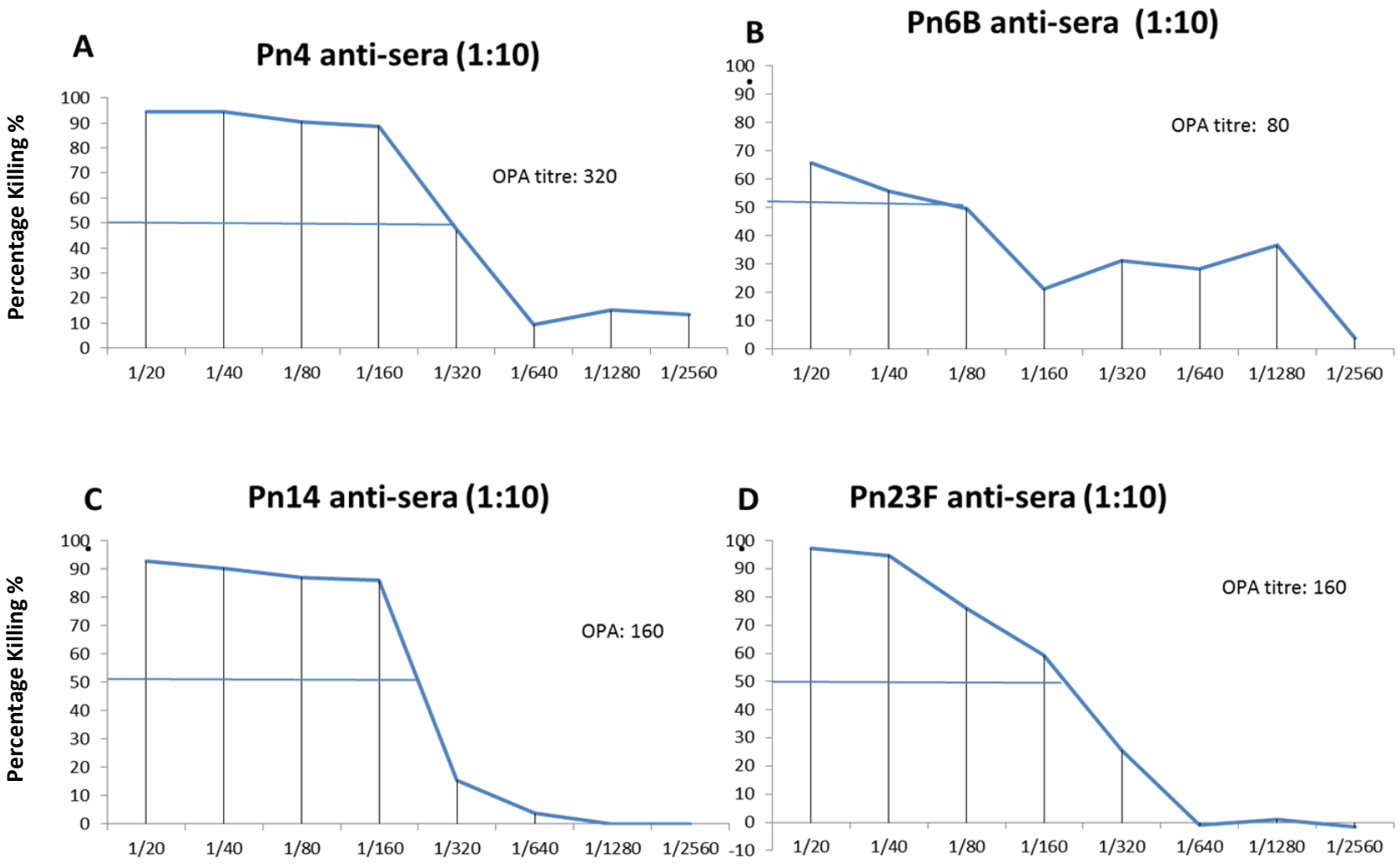


Figure 4.7. Anti-serum controls show a high percentage of killing in the opsonophagocytic killing assay (OPKA). *A. Pn4 anti-serum (1:100 dilution)(1:10 plating) B. Pn6B anti-serum (Neat dilution) (1:10 plating) C. Pn14 anti-serum (1:100 dilution) (1:10 plating). D. Pn23F anti-serum (1:100 dilution) (1:10 plating).*

4.4.4 Data collection

This study was based on a subset of the cohort of patients from the AIR study. The majority of patient samples were available; however, all samples needed to be re-run (as discussed extensively in Chapter 3), hence the available study cohort was much smaller than anticipated. Additionally, although contemporaneous measurements of Pn serotypes were available, since the samples had been tested over an extended period of time, all samples were chosen to be re-tested for the study, in order to minimise the impact of inter-plate variability. Pneumococcal vaccination was with PPV-23 until November 2010, at which point it was substituted for PCV-13. As such, in order to minimise the impact of any confounding factors that varied with time, patients were selected from those recruited prior to 2012 (N=527). From these, we included only those with available pre-vaccine (up to 4 weeks prior) and post-vaccine (4-17 weeks subsequently) blood samples taken for their initial vaccine (N=507). Since longevity was being considered, any patients that did not have at least one annual sample in the subsequent years were excluded, leaving N=152.

4.4.5 Statistical methods

Initially, patients were divided into two groups depending on the first vaccine they were administered (PCV-13 or PPV-23). Patient demographics were compared using independent samples t-tests or Fisher's exact tests, as applicable. The concentrations for each of the serotypes being considered were then compared between these groups for both the pre- and post-vaccine blood samples, as well as the fold change increase, using Mann-Whitney tests. The proportion of patients that had protective concentrations for each serotype were then identified, and compared between groups using Fisher's exact tests. To test for any associations between the concentrations considered and either VL or CD4 count, a set of correlation analyses were then performed, using Spearman's rho correlation coefficients.

The number of doses required to achieve protective levels were then compared between the groups. This used all available blood samples for a patient to identify the time at which protection was achieved, and compared the number of doses administered before this point was recorded. Patients that were lost to follow up before achieving protection were treated as censored after receiving their final vaccine. The cumulative protection rates after different numbers of vaccines were then estimated using Kaplan-Meier curves, and comparisons between the PCV-13 and PPV-23 groups were performed using a log-rank test.

Longevity of protection was also assessed using a Kaplan-Meier approach. Follow up started at the point that a patient achieved protection on at least 8 of 12 Pn serotypes, and

ended either when protection was found to be lost, or a booster vaccine was administered, with patients censored at the end of follow up. Comparisons were made by the first vaccine type (PCV-13 vs. PPV-23), as well as between those patients that required single vs. multiple doses for protection within the PCV-13 and PPV-23 subgroups.

Longevity was then further assessed on an individual serotype basis. Since the decision to vaccinate had been made on the basis of protection on at least 8 of 12 Pn serotypes, it was not possible to produce a valid Kaplan-Meier approach for separate serotypes, due to issues with informative censoring. Instead, for the subgroup of patients that were not protected at the point of their initial vaccination, and were followed up until the point that they lost overall protection, the concentrations of the individual serotypes were assessed at the point that protection was lost.

All analyses were performed using IBM SPSS 22 (IBM Corp. Armonk, NY), with $p<0.05$ deemed to be indicative of statistical significance throughout.

4.4.5.1 Sensitivity analyses

Due to the long study duration, over six years from the first to the last included blood sample, retrospective validation was performed the assay used to measure the antibody concentrations (discussed in Chapter 3). This found inconsistency over time in the measurements produced by the assay, particularly in the early years of the study. As a result, all samples used in the analysis were re-tested contemporaneously, in order to minimise the impact of inter-plate variability on the results.

Whilst this retesting should increase the reliability of the concentrations included in the analysis, the clinical decisions to vaccinate patients were still made based on the concentrations reported when the sample was originally tested. As such, there were cases where patients were vaccinated, despite their retested samples reporting that they were already protected against at least 8 of 12 Pn serotypes, and vice versa. As a result, when considering the number of doses required for protection, a proportion of patients were already found to be protected at baseline. In addition, when considering longevity, some patients were re-vaccinated, despite the fact that the retested samples identified them as still being protected at the time.

In order to assess the impact of this on the results, sensitivity analyses were performed. For the analysis of protection rates, those patients that were identified as already being protected prior to their initial vaccination were excluded. For the remainder of the cohort, the

protection rates remained significantly higher after a single dose of PCV-13 than for PPV-23, with 47% vs. 23% of patients protected on at least 8 of 12 Pn serotypes (p=0.007). The protection rates were still higher in the patient group that received PCV-13 after boosting, with 86% vs. 60% and 100% vs. 82% of patients protected after a total of two and three doses, respectively.

To investigate longevity of the immune response to pneumococcal vaccination, the analysis was repeated, with patients who were revaccinated before losing protection being censored at this point, rather than being treated as having lost protection. This increased the median longevity of protection for the cohort as a whole to 24.4 months (95% CI: 19.3 – 29.6), with the difference in longevity between those initially vaccinated with PCV-13 and PPV-23 remaining significant (median: 32.6 vs. 12.5 months, p=0.021).

4.5 Results

4.5.1 Demographics

Data were available for N=152 patients, with an average age of 41.1 ± 10.2 years at the point of vaccination, and of whom 66% were male. Of these, 89 (59%) received PPV-23 and 63 (41%) PCV-13 as their first vaccine. Comparisons between the groups (Table 5.1) found a similar gender distribution (p=0.490), with the PPV-23 group being marginally younger at the point of vaccination (mean: 39.5 vs. 43.2 years, p=0.028). Whilst the pre-vaccine CD4 count was not found to differ significantly between the groups (p=0.056), the VL was significantly higher in the patients receiving PPV-23, with 39% vs. 21% having VL≥50.

Table 4.3 – Patient Demographics

	Whole Cohort (N=152)	PCV-13 (N=63)	PPV-23 (N=89)	p-Value
Age at Vaccination (Years)	41.1 ± 10.2	43.2 ± 10.8	39.5 ± 9.5	**0.028**
Gender (N,% Male)	101 (66%)	44 (70%)	57 (64%)	0.490
Pre-Vaccine CD4	506 ± 215	547± 229	477 ± 200	0.056
Pre-Vaccine VL≥50	48 (32%)	13 (21%)	35 (39%)	**0.021**

Data reported as mean±SD, with p-values from independent t-tests, or N (%), with p-values from Fisher's exact tests, unless stated otherwise.
Pre-vaccine VL and CD4 counts were unavailable for one patient, who was excluded from these analyses.
Bold p-values are significant at p<0.05

4.5.2 Single dose of PPV-23 vs. PCV-13

The Pn concentrations for the included patients are reported in Table 4.4. Prior to vaccination, no significant differences were detected between the PCV-13 and PPV-23 groups in the concentrations of any of the 12 Pn serotypes considered. After vaccination, the patients receiving PCV-13 had significantly greater fold change increases compared to PPV-23 to 8 out of 12 Pn serotypes (Pn1, 3, 4, 5, 6B, 7F, 9V and 18C).

The data were also analysed based on the proportion of patients with protective levels on each serotype (Table 4.5). Prior to the initial vaccination, the numbers of patients protected (≥0.35 µg/mL) to at least 8 of the 12 Pn serotypes was similar in the two groups (PCV-13=16%, PPV-23=17%, p=1.000). After vaccination, 54% of patients treated with PCV-13 achieved protection, which was significantly greater than the 33% of patients receiving PPV-23 (p=0.012) giving a relative risk for protection of 1.66 (95% CI: 1.10 – 2.46).

Table 4.4 – Comparisons of concentrations between vaccines

	Pre-Vaccine			Post-Vaccine			Fold Change		
Serotype	***PCV-13 (N=63)***	***PPV-23 (N=89)***	***p-Value***	***PCV-13 (N=63)***	***PPV-23 (N=89)***	***p-Value***	***PCV-13 (N=63)***	***PPV-23 (N=89)***	***p-Value***
Pn1	0.07 (0.04 - 0.15)	0.07 (0.02 - 0.16)	0.599	0.59 (0.23 - 3.65)	0.35 (0.12 - 0.81)	**0.003**	8.53 (2.76 - 30.29)	4.97 (1.84 - 11.68)	**0.006**
Pn3	0.07 (0.05 - 0.14)	0.10 (0.06 - 0.15)	0.147	0.19 (0.09 - 0.51)	0.13 (0.08 - 0.26)	**0.042**	2.14 (1.37 - 4.19)	1.38 (0.82 - 2.80)	**<0.001**
Pn4	0.08 (0.05 - 0.18)	0.08 (0.05 - 0.18)	0.827	0.36 (0.17 - 1.41)	0.15 (0.09 - 0.36)	**<0.001**	3.28 (1.47 - 15.64)	1.75 (1.07 - 2.64)	**<0.001**
Pn5	0.21 (0.07 - 0.44)	0.13 (0.08 - 0.29)	0.281	0.67 (0.29 - 2.31)	0.27 (0.11 - 0.54)	**<0.001**	2.82 (1.47 - 10.26)	1.80 (1.03 - 3.23)	**<0.001**
Pn6B	0.12 (0.05 - 0.62)	0.19 (0.08 - 0.52)	0.282	0.57 (0.21 - 4.33)	0.29 (0.11 - 1.56)	**0.046**	3.45 (1.26 - 11.46)	1.64 (0.88 - 3.91)	**0.002**
Pn7f	0.50 (0.28 - 1.05)	0.76 (0.41 - 1.19)	0.103	0.85 (0.43 - 2.34)	0.80 (0.42 - 1.51)	0.483	1.41 (1.00 - 2.02)	1.22 (0.73 - 1.71)	**0.045**
Pn9V	0.32 (0.20 - 0.76)	0.48 (0.25 - 0.82)	0.155	0.94 (0.37 - 2.51)	0.52 (0.28 - 1.06)	**0.014**	1.58 (1.09 - 4.94)	1.12 (0.70 - 1.85)	**<0.001**
Pn14	2.03 (0.56 - 8.14)	1.82 (0.71 - 4.61)	0.891	8.22 (1.65 - 10.00)	3.53 (1.03 - 10.00)	0.100	1.57 (1.00 - 3.89)	1.45 (1.00 - 2.87)	0.221
Pn18C	0.17 (0.07 - 0.95)	0.17 (0.05 - 0.66)	0.692	1.45 (0.39 - 6.75)	0.61 (0.15 - 3.23)	**0.012**	5.71 (2.26 - 17.24)	3.53 (1.33 - 9.24)	**0.011**
Pn19A	0.32 (0.10 - 1.13)	0.30 (0.13 - 0.95)	0.963	1.24 (0.27 - 4.96)	0.48 (0.18 - 3.74)	0.178	1.99 (1.13 - 5.84)	1.77 (1.01 - 4.37)	0.353
Pn19F	0.29 (0.08 - 1.46)	0.28 (0.11 - 1.07)	0.918	1.53 (0.24 - 5.37)	0.82 (0.23 - 3.98)	0.333	1.92 (1.16 - 6.78)	2.58 (1.04 - 6.31)	0.821
Pn23F	0.22 (0.05 - 0.52)	0.12 (0.06 - 0.41)	0.380	0.84 (0.14 - 3.99)	0.37 (0.10 - 1.26)	**0.026**	3.11 (1.21 - 10.46)	2.28 (1.23 - 5.67)	0.101
Men C	0.14 (0.06 - 0.55)	0.38 (0.18 - 0.75)	**0.002**	1.84 (0.82 - 4.83)	1.89 (0.48 - 3.54)	0.354	10.39 (3.42 - 32.56)	4.11 (1.44 - 9.41)	**<0.001**
Tetanus	0.34 (0.06 - 0.62)	0.28 (0.06 - 0.66)	0.847	1.55 (0.28 - 3.03)	0.96 (0.23 - 2.46)	0.062	5.38 (3.16 - 9.09)	2.91 (1.28 - 7.97)	**0.013**
Diphtheria	0.02 (0.01 - 0.04)	0.03 (0.02 - 0.05)	**0.008**	0.08 (0.03 - 0.20)	0.02 (0.01 - 0.04)	**<0.001**	3.43 (1.84 - 7.26)	0.80 (0.59 - 1.14)	**<0.001**
Hib	1.25 (0.48 - 2.90)	1.54 (0.87 - 2.84)	0.124	7.32 (2.27 - 20.00)	2.90 (1.59 - 10.22)	**0.005**	4.18 (1.90 - 9.64)	1.84 (1.10 - 4.29)	**<0.001**

Data are reported as medians and interquartile ranges, with p-values from Mann-Whitney tests
Bold p-values are significant at p<0.05

Table 4.5. Concentrations for individual serotypes at the point that overall protection was achieved and lost

	At Overall Protection (N=100)*		At Loss of Overall Protection (N=63)**	
	Median Titre (IQR)	***% > 0.35µg/mL***	***Median Titre (IQR)***	***% > 0.35µg/mL***
Pn1	0.56 (0.27 - 1.85)	69 (69%)	0.22 (0.13 - 0.46)	20 (32%)
Pn3	0.24 (0.15 - 0.57)	39 (39%)	0.12 (0.08 - 0.16)	7 (11%)
Pn4	0.54 (0.20 - 1.21)	64 (64%)	0.19 (0.08 - 0.37)	16 (25%)
Pn5	0.56 (0.32 - 2.15)	71 (71%)	0.27 (0.15 - 0.48)	20 (32%)
Pn6B	1.14 (0.40 - 4.22)	80 (80%)	0.37 (0.18 - 1.39)	33 (52%)
Pn7F	1.25 (0.70 - 2.29)	96 (96%)	0.49 (0.31 - 1.15)	40 (63%)
Pn9V	1.24 (0.61 - 2.49)	95 (95%)	0.43 (0.28 - 0.79)	38 (60%)
Pn14	6.39 (2.29 - 10.00)	99 (99%)	2.39 (0.82 - 6.53)	56 (89%)
Pn18C	1.41 (0.53 - 4.93)	87 (87%)	0.46 (0.23 - 1.72)	36 (57%)
Pn19A	1.35 (0.50 - 4.97)	87 (87%)	0.43 (0.19 - 2.88)	36 (57%)
Pn19F	1.79 (0.75 - 5.22)	90 (90%)	0.64 (0.20 - 2.64)	40 (63%)
Pn23F	1.13 (0.46 - 3.89)	84 (84%)	0.37 (0.17 - 0.78)	33 (52%)

**Values reported are from the first post-vaccine blood test which showed protective levels (>0.35 µg/mL) on at least 8/12 serotypes. Patients already protected at the point of their first vaccine were excluded.*

***Values reported are from the first blood test after achieving protection where patients had lost overall protection, i.e. less than 8/12 serotypes having levels >0.35 µg/mL. Patients who were already protected at the point of their first vaccine were excluded, or who were revaccinated before losing protection were exclud*

4.5.2.1 Comparative data on a selection of study participant serum investigating the quantitative and qualitative responses to a single dose of PPV-23 or PCV-13.

From Pn-specific IgG serum studies (section 4.5.2), it was evidenced that a single dose of PCV-13 appears to be more immunogenic than a single dose of PPV-23; however, the functionality of the Pn-specific IgG was not assessed in multiplexed Luminex studies.

Furthermore, there is no protective correlate for OPA GMTs in the OPKA, so it hasn't been assessed whether or not patients are protected post-vaccination (151).

Evidence in the elderly suggests that an individual can have antigen-specific antibodies present; however, they may not necessarily be functional with regards to opsonising pneumococcal strains (56,184,185). Thus, Pn-specific IgG concentrations and opsonophagocytic killing assay titres were assigned in post-vaccination serum samples so that a relationship could be established between the amount and functionality of Pn-specific IgG.

There was no clear correlation found between Pn-specific IgG and OPKA titres (Pn 4,6B, 14, and 23F) as evidenced by regression analyses in Figure 4.8/Table 4.6.

Table 4.6. Comparison of quantitative and qualitative Pn-specific IgG against Pn-serotypes (Pn 4, 6B, 14, and 23F)

Vaccine Type	*OPKA Pn4*	*Pn-specific IgG Pn4*	*OPKA Pn6B*	*Pn-specific IgG Pn6B*	*OPKA Pn14*	*Pn-specific IgG Pn14*	*OPKA Pn23F*	*Pn-specific IgG Pn23F*
SD PCV	11	0.07	893	0.05	437	1.23	19	0.02
SD PCV	10	0.12	16	0.3	1564	9.5	838	0.1
SD PCV	10	0.14	19	0.11	11	1.83	718	4.19
SD PCV	792	0.19	1321	1.15	8	5.29	2819	8.46
SD PCV	1548	4.53	700	0.35	2961	8.62	3921	2.85
SD PCV	10	0.01	19	0.02	13	0.45	19	0.01
SD PPV	10	0.06	28	0.02	8	0.57	1288	0.05
SD PPV	10	0.02	21	0.02	702	3.08	1231	0.84
SD PPV	10	0.3	16	4.12	267	9.4	U-shaped curve	1.7
SD PPV	1245	0.13	N-shaped curve	0.07	470	1.7	847	0.2

OPKA titres are reported as OPA GMTs (opsonic phagocytic assay geometric mean titre) and Pn-specific IgG is reported as µg/mL. An OPKA titre above 8 is considered protective although there have been no correlates of protection established for PCV-13 vaccination, particularly in an HIV-infected cohort. A Pn-specific IgG titre of 0.35 µg/mL (WHO threshold) is considered the protective threshold,

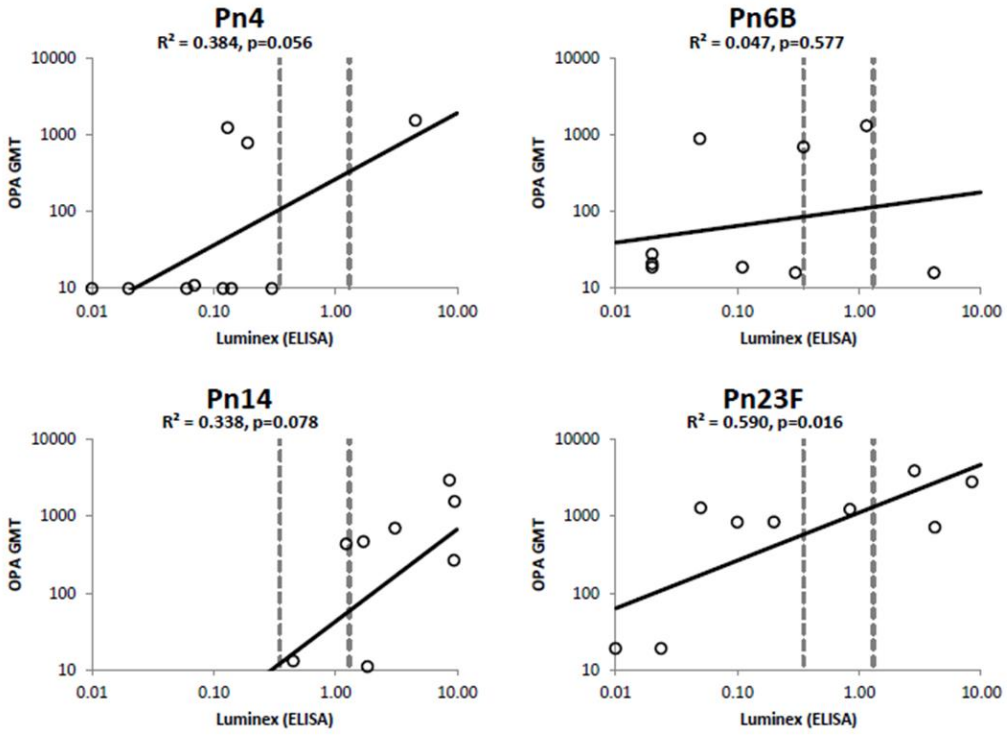


Figure 4.8. Comparison of Pn serotypes 4, 6B, 14, and 23F OPA GMT titres and Luminex (ELISA) Pn-specific IgG antibody titres from post-vaccination (4-weeks) serum. *Regression analyses were performed. Data are reported as logged values of OPA GMTs (opsonic phagocytic assay geometric mean titre) and Pn-specific IgG is reported as µg/mL. R^2 values are reported as the coefficients of determination. P values < 0.05 are considered significant.*

4.5.3 Correlations between concentrations and CD4/VL

On account of the significant difference in VL between the two groups at point of vaccination, a set of correlation analyses were performed to assess whether either VL or CD4 impacted on vaccine response (Table 4.7). Neither CD4 nor VL were found to be significantly correlated with the concentrations of any of the Pn serotypes in either the pre- and post-vaccine periods. Additionally, neither PPV-23 or PCV-13 vaccine responses were affected by VL or CD4 counts.

Table 4.7 - Correlations between CD4/VL and concentrations

	CD4			VL		
	Pre-Vaccine	**Post-Vaccine**	**Fold Change**	**Pre-Vaccine**	**Post-Vaccine**	**Fold Change**
Pn1	0.037 (p=0.651)	0.074 (p=0.366)	0.027 (p=0.738)	0.021 (p=0.800)	-0.052 (p=0.528)	0.001 (p=0.989)
Pn3	-0.090 (p=0.273)	-0.069 (p=0.397)	-0.001 (p=0.986)	0.113 (p=0.167)	0.031 (p=0.703)	-0.056 (p=0.497)
Pn4	0.044 (p=0.594)	0.052 (p=0.524)	-0.009 (p=0.916)	-0.001 (p=0.991)	-0.012 (p=0.882)	0.011 (p=0.891)
Pn5	-0.012 (p=0.885)	0.072 (p=0.377)	0.048 (p=0.555)	0.134 (p=0.102)	0.042 (p=0.610)	-0.005 (p=0.956)
Pn6B	-0.043 (p=0.601)	-0.003 (p=0.968)	0.066 (p=0.421)	0.028 (p=0.730)	0.002 (p=0.983)	0.009 (p=0.911)
Pn7f	-0.048 (p=0.560)	-0.018 (p=0.830)	0.086 (p=0.294)	0.155 (p=0.057)	0.086 (p=0.295)	-0.082 (p=0.318)
Pn9V	-0.096 (p=0.243)	0.002 (p=0.985)	0.091 (p=0.267)	0.126 (p=0.123)	0.078 (p=0.340)	-0.015 (p=0.853)
Pn14	0.037 (p=0.648)	0.051 (p=0.535)	0.000 (p=0.999)	0.011 (p=0.895)	0.019 (p=0.816)	0.023 (p=0.775)
Pn18C	-0.085 (p=0.302)	-0.025 (p=0.760)	0.027 (p=0.745)	0.102 (p=0.213)	0.039 (p=0.638)	-0.097 (p=0.234)
Pn19A	0.057 (p=0.485)	0.069 (p=0.400)	0.029 (p=0.722)	0.033 (p=0.686)	0.041 (p=0.617)	0.012 (p=0.883)
Pn19F	0.018 (p=0.825)	0.074 (p=0.363)	0.057 (p=0.485)	-0.012 (p=0.887)	0.018 (p=0.827)	0.022 (p=0.787)
Pn23F	-0.094 (p=0.250)	-0.012 (p=0.885)	0.074 (p=0.366)	0.029 (p=0.725)	0.076 (p=0.351)	0.061 (p=0.454)
MenC	-0.144 (p=0.077)	0.012 (p=0.883)	0.147 (p=0.071)	0.133 (p=0.104)	-0.074 (p=0.365)	**-0.182 (p=0.025)**
Tetanus	-0.016 (p=0.846)	0.104 (p=0.205)	**0.171 (p=0.036)**	0.134 (p=0.101)	-0.028 (p=0.733)	-0.145 (p=0.076)
Diphtheria	-0.155 (p=0.057)	-0.028 (p=0.736)	0.067 (p=0.414)	0.151 (p=0.065)	0.066 (p=0.423)	-0.024 (p=0.770)
Hib	**-0.194 (p=0.017)**	0.015 (p=0.859)	**0.167 (p=0.040)**	**0.190 (p=0.020)**	0.053 (p=0.520)	-0.086 (p=0.296)

Data are reported as Spearman's rho correlation coefficients and p-values. Correlations that were significant at p<0.05 are in bold. One patient had no recorded CD4 or VL prior to the vaccine, and so was excluded from the analysis.

4.5.4 Number of vaccine doses required for protection

An analysis was then performed to consider the number of vaccines received by patients in order to achieve protection on 8 of 12 Pn serotypes (Figure 4.9). This found that patients receiving PCV-13 as their initial vaccine required significantly less doses to achieve Pn protection ($p<0.001$). After their initial vaccine, 39% of patients receiving PPV-23 and 70% receiving PCV-13 were protected. It must be noted that the percent protected reported after a single vaccine in the previous analysis (Table 4.8) differs from that found in this analysis (Figure 4.9). This is since the former only considers the immediate post-vaccine sample (4-17 weeks after the vaccine) for each patient, whilst the latter includes all available blood samples taken after the first vaccine, and before any subsequent vaccines. Hence, patients that developed protection after an extended period will be classified as protected in the latter, but not the former.

Patients receiving PPV-23 followed by a boost with PCV-13 had a similar rate of protection to those receiving a single dose of PCV-13 (67% vs. 70%), and those receiving PPV-23 followed two doses of PCV-13 had similar rates of protection to those receiving two doses of PCV-13 (85% vs. 88%). After receiving three doses of PCV-13, the cumulative protection rate in the cohort was 100%.

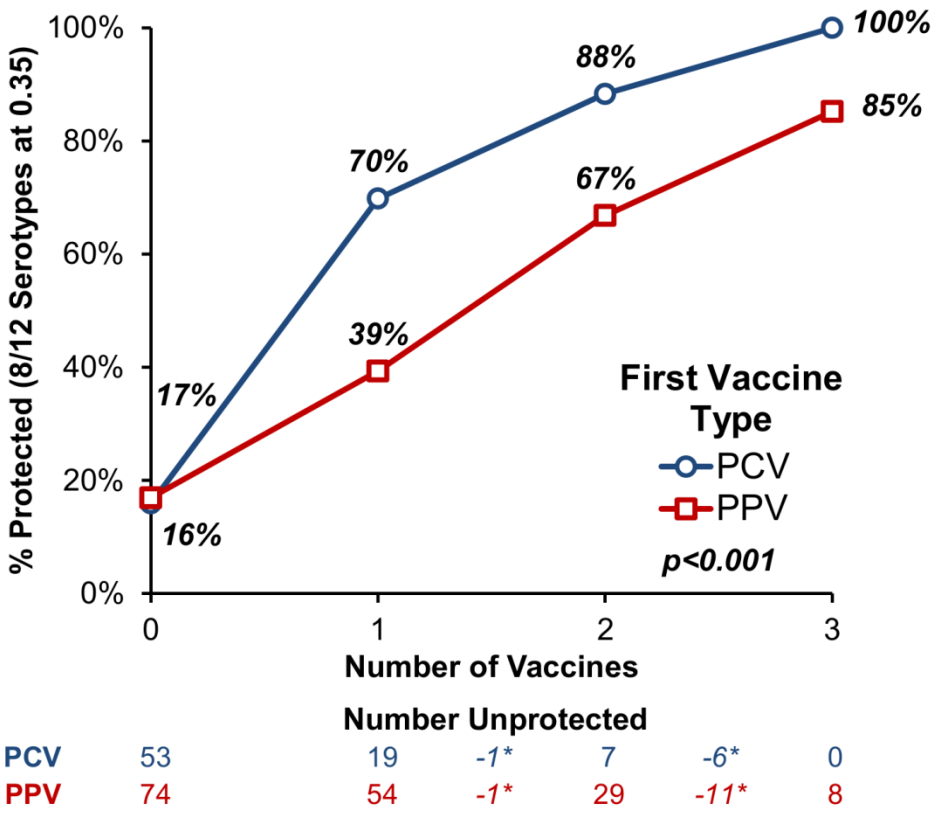


Figure 4.9. Protection rates by number of vaccines. *Rates are Kaplan-Meier estimates, and the p-value is from a log-rank test. The "number unprotected" is the number at risk after the stated vaccine, hence represents the number of patients that remain unprotected after the specified number of vaccines. *The number of patients who were censored between the stated vaccine numbers, due to being lost to follow up without achieving protective levels.*

Table 4.8– Comparison of protection rates between vaccines

	Pre-Vaccine			Post-Vaccine		
	PCV	***PPV***	***p-Value***	***PCV***	***PPV***	***p-Value***
Pn1	9 (14%)	10 (11%)	0.624	39 (62%)	45 (51%)	0.188
Pn3	7 (11%)	3 (3%)	0.094	20 (32%)	15 (17%)	**0.050**
Pn4	6 (10%)	7 (8%)	0.773	32 (51%)	23 (26%)	**0.002**
Pn5	20 (32%)	19 (21%)	0.187	42 (67%)	36 (40%)	**0.002**
Pn6B	19 (30%)	30 (34%)	0.726	41 (65%)	42 (47%)	**0.033**
Pn7F	39 (62%)	72 (81%)	**0.015**	50 (79%)	74 (83%)	0.672
Pn9V	29 (46%)	59 (66%)	**0.019**	48 (76%)	58 (65%)	0.156
Pn14	52 (83%)	79 (89%)	0.341	59 (94%)	82 (92%)	1.000
Pn18C	23 (37%)	31 (35%)	0.865	48 (76%)	53 (60%)	**0.037**
Pn19A	31 (49%)	38 (43%)	0.509	42 (67%)	48 (54%)	0.133
Pn19F	29 (46%)	42 (47%)	1.000	44 (70%)	58 (65%)	0.601
Pn23F	21 (33%)	25 (28%)	0.591	40 (63%)	45 (51%)	0.136
8 of 12 Pns*	10 (16%)	15 (17%)	1.000	34 (54%)	29 (33%)	**0.012**
Men C	5 (8%)	12 (13%	0.312	30 (48%)	43 (48%)	1.000
Tetanus	44 (70%)	63 (71%)	1.000	58 (92%)	75 (84%)	0.214
Diphtheria	6 (10%)	11 (12%)	0.795	28 (44%)	11 (12%)	**<0.001**
Hib	35 (56%)	63 (71%)	0.060	57 (90%)	76 (85%)	0.458

Data are reported as the N (%) of patients that had protective levels for individual serotypes
Protective levels were: 0.35 for Pns, 2 ug/mL for Men C, 1 ug/mL for Hib and 0.1 IU/mL for Dip. and Tet.
**Protective levels on 8 or more serotypes.*
p-Values are from Fisher's exact tests, and bold values are significant at p<0.05

4.5.5 Longevity of protection

After excluding those 25 patients that were already protected at the time of their first vaccination, a total of 100 patients achieved protective levels on at least 8 of 12 Pn serotypes at some point during the follow up. This protection was found to last for a median of 17.4 months (95% CI: 9.4 – 25.4), with 56%, 37% and 23% retaining protective levels for at least 1,2 and 3 years, respectively.

Patients whose initial vaccination was with PCV-13 were found to retain their protection for significantly longer than those whose first vaccine was PPV-23 (p=0.010, Figure 4.10), with median longevities of 23.5 (95% CI: 19.2 – 27.8) vs. 11.1 (9.1 – 13.2) months, and a hazard ratio of 1.80 (95% CI: 1.14 – 2.84). Subgroup analyses were then performed within these two groups to assess whether the longevity of protection differed between those patients who only needed a single vaccine to achieve protection, and those that required a booster (Figure 4.11A/4.11B). No significant difference was detected between these groups in either those patients that received PCV-13 (p=0.830) or PPV-23 (p=0.472) as their initial vaccine.

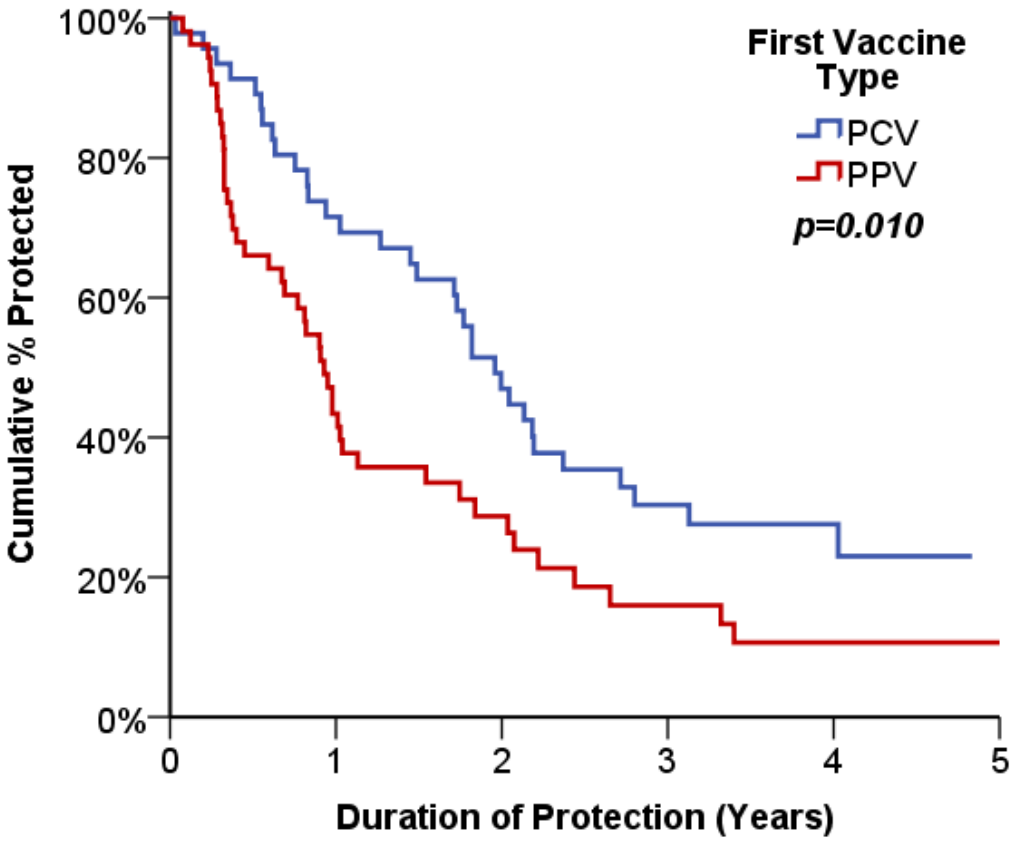


Figure 4.10. Longevity of Pn protection by first vaccine type. *Follow up starts at the point that protection (8/12 Pns>0.35) was achieved, and ends at the first blood test showing a loss of protection, or at the time that a booster vaccine was given, with patients being censored at the end of follow up. Those patients who did not achieve protective levels during the study period (N=27), or were already protected at the point of their first vaccine (N=25) were excluded from the analysis.*

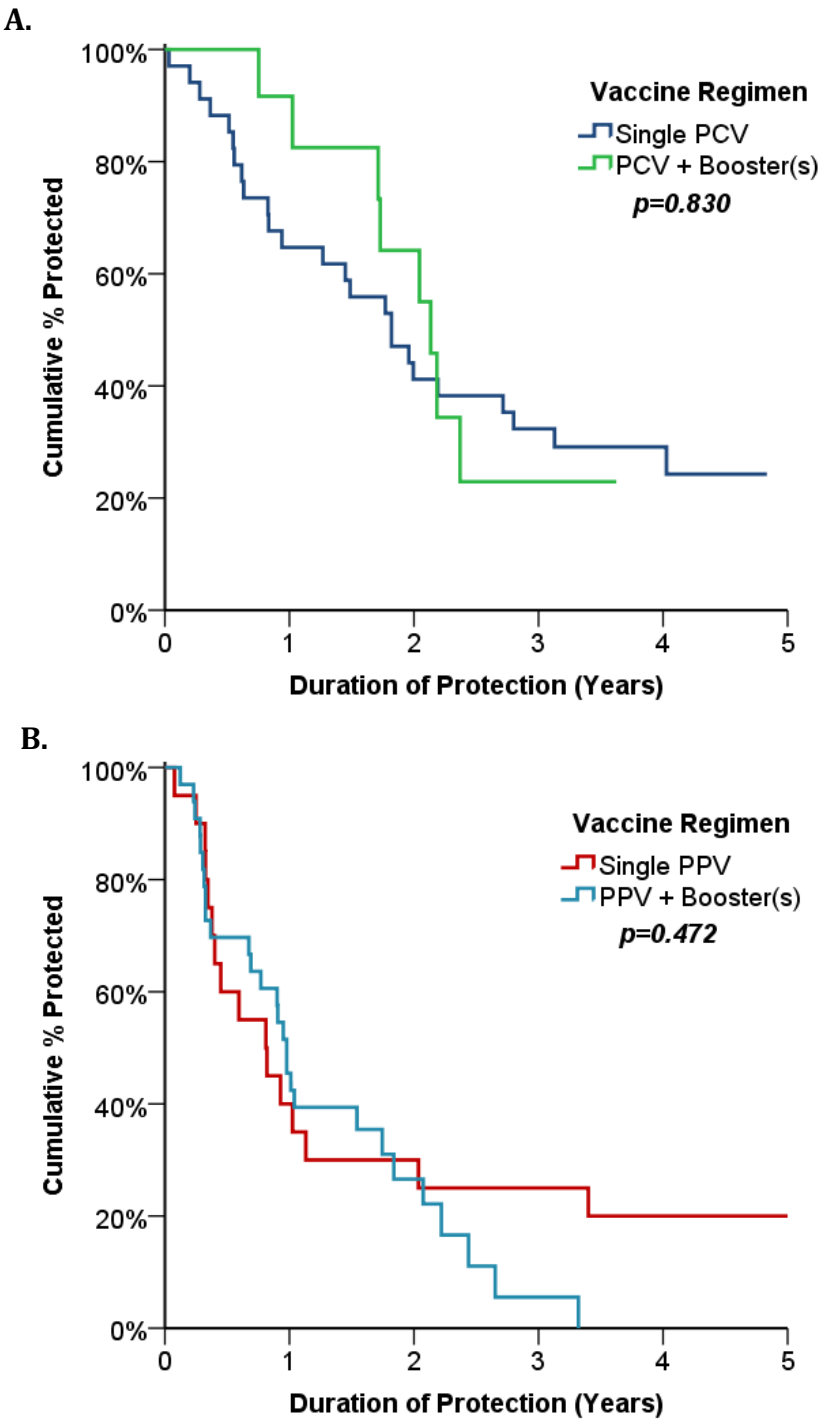


Figure 4.11. Longevity of Pn protection by vaccine regimen for patients *A) Vaccine regimen for patients that receive PCV-13 as their first Pn vaccine B) Vaccine regimen for patients that receive PPV-23 as their first Pn vaccine. Follow up starts at the point that protection (8/12 Pns>0.35) was achieved, and ends at the first blood test showing a loss of protection, or at the time that a booster vaccine was given, with patients being censored at the end of follow up. Those patients who did not achieve protective levels during the study period (N=7 PCV, 20 PPV), or were already protected at the point of their first vaccine (N=10, 15) were excluded from the analysis.*

4.5.6 Protection by serotype

The concentrations of the individual serotypes were then further assessed for the 100 patients that achieved protective levels on at least 8 of 12 Pn serotypes at some point during the follow up (Table 4.5). At the point that overall protection was achieved, concentrations were highest for Pn14, with a median of 6.39µg/mL, and 99% of patients having concentrations >0.35 µg/mL for this serotype. In contrast, Pn3 had the lowest median concentrations at the point of overall protection, at 0.24µg/mL, with only 39% of patients having concentrations ≥0.35 µg/mL.

For the subgroup of 63 patients who subsequently lost protection on at least 8 of 12 Pn serotypes, the concentrations were similarly assessed at this point. Despite losing overall protection, levels of Pn14 remained high, with median concentrations of 2.39µg/mL, and 89% of patients retaining levels ≥0.35µg/mL.

4.6. Discussion

PCV-13 covers approximately 61% of the serotype distribution amongst people co-infected with invasive pneumococcal disease (IPD) and HIV (1). In fact, three out of every 5 IPD episodes among HIV-positive adults could be prevented by successful vaccination with PCV-13 (1).

The AIR study provided an opportunity to directly compare polysaccharide and conjugate vaccinations in HIV infected adults due to a deliberate change in the study schedule pneumococcal vaccination that occurred after 1 year of AIR study.

This study provides further evidence that pneumococcal conjugate vaccination appears a more successful strategy than pure polysaccharide vaccination and has the added advantage that patients who receive PCV-13 as an initial dose can be boosted. A randomised placebo-controlled trial of PCV-7 in HIV-infected adults was carried out in Malawi and demonstrated enhanced immunogenic protection against recurrent pneumococcal disease (142). Protein conjugate vaccination works by eliciting T-cell help rather than a T-independent response traditionally associated with pure polysaccharide vaccine. HIV has multiple effects on the immune system but the hallmark association is a low CD4-T cell count. It seems counter intuitive that a vaccine that requires T-cell help for immunogenicity would be more successful in a disease where CD4-T cells are deficient or dysregulated, however, we found no association between response to conjugate vaccination and CD4

count, viral load or anti-retroviral (ARV) therapy. This is consistent with our group's previous findings (178), as well as other studies which found no correlation between the pneumococcal-specific antibody response and CD4-T cell count (156,158). This suggests that the B-cell compartment may be responsible for successful responses to pneumococcal conjugate vaccination. However, some studies have reported inadequate vaccine responses with a low CD4-T cell count (159,160). Furthermore, the lack of effect of ARV therapy on pneumococcal –specific antibody response has been shown in other studies (160). Conversely, it has been shown that antibody responses to PCV-13 are greater than with PPV-23 in patients that have received ARV therapy (146,166).

Vaccination regimes including both conjugate and polysaccharide vaccines have shown conflicting results, which suggests that there is potential variability in study design and patient settings (136,138,144,145,186). It was clear that neither a single dose of polysaccharide or conjugate vaccination was sufficient to provide adequate coverage for the HIV- population and that booster regimens may be more effective.

Additionally, no clear correlations were found between Pn-specific IgG quantities and OPKA titres from a preliminary analysis on a subset of single dose vaccine samples. This finding has also been evidenced in one additional study in patients with multiple myeloma and other B-cell disorders (187). Thus, these results highlight the need for the assignment of OPKA titres because it reflects the functionality of the Pn-specific antibody response to pneumococcal vaccination. Used in conjunction with the Pn-specific IgG data, which establishes the breadth and granularity of the Pn-specific IgG response to pneumococcal vaccination, OPKA assays can be utilised to assess opsonic capacity. Thus, a larger sample size should be considered in future studies in order to further assess the opsonic capacity of Pn-specific IgG in single dose and booster dose samples.

Given that there were no surrogate markers that could predict response to vaccination, this suggests that either schedules have to include vaccine response testing or the whole cohort could be routinely vaccinated with at least 2 conjugate vaccines. Our study only vaccinated when threshold levels drops so an appropriate booster schedule would have to be elucidated in adults.

This study describes for the first time longevity of the antibody response to different pneumococcal vaccines. Following successful vaccination, the conjugate vaccine had a significantly longer effect than the polysaccharide vaccine. However, the median response

even for the conjugate vaccine was short lived providing evidence that a booster schedule is necessary. We know from childhood pneumococcal vaccine studies that a 3 dose schedule is most effective in preventing invasive pneumococcal disease and it is likely that at least a 2 dose schedule is required for HIV infected adults (77,164,165). A study that gave three doses of PCV-13 in HIV-infected adults who were previously vaccinated with PPV-23 found an initial increase in pneumococcal-specific antibodies, but had an overall similar level after each vaccine (173). It is interesting that both the UK and US, HIV associations recommend conjugate followed by polysaccharide vaccination to enable broader serotype coverage. However, we have evidence from this study that the polysaccharide response is very short lived at only 11 months and jeopardises response to further booster conjugate vaccination. It is also currently recommended (The Green Book, Chapter 25) not to use polysaccharide pneumococcal vaccination within 5 years because of hyporesponsiveness (188). In patients that remain at risk of pneumococcal disease long term, there is a real need for newer conjugate vaccinations with wider serotype coverage that can be used in boosting regimens.

It is important to highlight that there is a lack of controlled studies investigating vaccination against *S. pneumoniae* in HIV-infection. Although the AIR study aimed to improve patient care in the context of HIV-infection through the implementation of BHIVA vaccination guidelines, it had its potential weaknesses including the use of fixed vaccine doses without specified booster vaccine dates. Patients were only given a booster vaccination if they failed to mount an immune response to 8 out of 12 pneumococcal (Pn) serotypes.

Limitations of our study include lack of understanding appropriate correlates of protection for pneumococcal plain polysaccharide and conjugate vaccines in HIV-infected adults, suggesting that the WHO threshold of 0.35 μg/mL may not be representative of this cohort and a higher correlate for protection (0.81 μg/mL) may be more appropriate (151) .

Chapter 5. Pn-specific Subclass Assignment and Investigation of HIV-infected Serum Samples

Oral presentation: xMAP Connect 2017, Amsterdam, The Netherlands, November 2017
Abstract submitted: ISPPD-11, The 11th International Symposium on Pneumococci and Pneumococcal Diseases (ISPPD), Melbourne, Australia, April 2018.

5.1 Introduction

IgG comprises about 10-20% of plasma protein in human serum and it is the most abundant class of the five immunoglobulins in humans. Each of these immunoglobulin classes are closely related; however, they differ in their effector functions and heavy chain structure (189). The IgG protein can be divided into four subclasses (discovered in the 1960s): IgG1, IgG2, IgG3, and IgG4, which are named in numerical order for their decreasing abundance in human serum (190). The generation of human monoclonal antibodies by Köhler and Millstein in 1975 has allowed for the investigation of antibody-antigen specificity (191). Furthermore, each IgG subclass has a unique function with regards to the binding of antigens, activation of complement proteins, formation of immune complexes, and activation of effector cells (189). Therefore, IgG responses to different antigens will activate the production of specific IgG subclasses (Figure 5.1).

Although the structures of the IgG subclasses are very similar, there is still a great deal of variation within their hinge regions and CH2/CH3 domains (of the Fc portion of the antibody), particularly, an N-linked glycosylation site at position 297 of IgG, which can affect the quaternary structure of the Fc portion, thus exposing a larger area for FcγR (IgG-Fc receptors) to bind to (Figure 5.1) (189).

The hinge region is the area of the IgG antibody that forms a bridge between the Fab and Fc portion. It has varying degrees of length and flexibility amongst the IgG subclasses and therefore, it affects active binding to FcγR and complement (C1q), binding of antigens, and formation of immune complexes. The hinge region is also an important structural difference that can impact the effector function of antibodies, most notably, the C1q binding site and FcγR on innate immune cells (189). Most notably, variation in flexibility of the hinge region

will contribute to the orientation and movement of the Fab and Fc portion of the antibody. Thus, Fab flexibility with respect to the length of the hinge region differs vastly across the IgG subclasses in this decreasing order: IgG3 > IgG1 > IgG4 > IgG2 (192).

Protein antigens typically result in activation of B-cells by T-helper cells through MHC-class II binding, which results in an IgG1, IgG3, or IgG4 response (189). Non-protein or polysaccharide antigens results in a T-cell independent response, which involves class-switching to IgG2.

IgG1 is the most abundant subclass. IgG1 responses are produced as a result of antibody responses to soluble protein antigens and membrane proteins. Furthermore, a lack of IgG1 can result in low total IgG concentrations (hypogammaglobulinemia) and recurrent infections (193,194).

IgG2 is the second most abundant subclass, which is almost entirely due to IgG responses to encapsulated bacteria surrounded by polysaccharide (193,195–197). Furthermore, a lack of IgG2 can result in the complete loss of IgG anti-carbohydrate antibodies, although, it has been established that IgG1 and IgG3 will compensate for the loss of IgG2 (198,199).In IgG2 deficiencies, patients have an increased susceptibility to bacterial infections, which suggests that IgG2 is integral in the bacterial immune response (200).

Furthermore, it has been evidenced that total IgG1 and IgG3 responses are common immune responses to second-generation pneumococcal vaccines (including protein-conjugated polysaccharides such as those in PCV-13) in normal healthy individuals, which is contrary to wide spread belief that total IgG2 is the most common immune response (201).

IgG3 is a pro-inflammatory antibody with a short half-life (limits excessive inflammatory responses) (189). Interestingly, IgG3 and IgG1 subclass antibodies tend to appear in the primary course of viral infections (193). IgG4 antibodies are produced following chronic exposure to an antigen in a non-infectious environment, which have the possibility of becoming a dominant subclass i.e. long-term bee keepers (189). Additionally, IgG4, IgG1, and IgE responses are produced in response to allergens, such as bee-stings and allergic individuals (202–204). Furthermore, helminth and filarial parasite infections induce a robust IgG4 response (205,206).

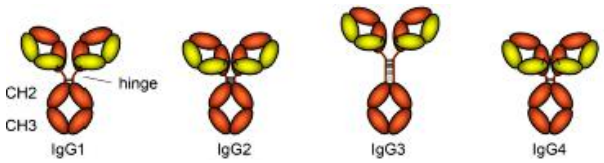


Figure 5.1. Diagram of the IgG subclasses: IgG1, IgG2, IgG3, and IgG4 *(Adapted from* (189)*). This diagram depicts the structure of each IgG subclass in addition to their CH2, CH3, and hinge regions.*

Traditionally, Pn-specific IgG has been used to quantify patient immune responses to pneumococcal vaccination. However, it is known that in older children and adults, serotype-specific antibodies following plain polysaccharide vaccination and/or natural exposure to pneumococcus in the environment tend to be IgG2-subclass specific as a result of a T-cell independent response (195,196,207–210). However, serotype-specific antibodies following protein glycoconjugate vaccination against the non-toxic mutant of diphtheria toxin (CRM_{197}) and the tetanus toxoid tend to be IgG1-subclass specific as a result of a T-cell dependent response (211–213).

As the patients within the AIR study were vaccinated with both plain polysaccharide pneumococcal vaccination (PPV-23) and pneumococcal polysaccharide conjugate vaccination (PCV-13), it seemed very appropriate to assign Pn-specific IgG1 and IgG2 antibody concentrations to the standard lot 007sp for the multiplex Luminex assays in addition to the Pn-specific IgG that was also assessed. Assessing and quantifying these Pn-specific subclasses is essential for understanding the immune response to different vaccines in this patient cohort.

Other reasons for quantifying Pn-specific IgG subclasses include a study in adults that found Pn-specific IgG2 naturally acquired as a result of previous exposure to pneumococcus (207). Furthermore, other studies have shown that both IgG1 and IgG2 Pn-specific antibodies are produced in response to pneumococcal vaccination (207,214). IgG subclasses should also be quantified and measured in response to pneumococcal vaccination as there are known functional differences including avidity, neutralisation, opsonic activity between subclasses found from previous studies investigating immune responses to other encapsulated bacteria (215–217).

Furthermore, not much is understood with regards to which IgG subclass is responsible for immune recognition and defence against pneumococcus and other encapsulated bacteria

(218–220). Also, with so much chronic immune inflammation and humoral dysregulation in HIV-infected adults, there could be an inhibitory effect of a particular IgG subclass, which inhibits opsonisation, successful phagocytosis, and clearance of pneumococcus and particular encapsulated bacteria as seen in three studies investigating immune responses to *Salmonella typhimurium* and *Pseudomonas aeruginosa* (221–223).

Two separate weight-based antibody assignments were performed in order to assign values from the pneumococcal reference serum standard lot 89-SF for IgG1 and IgG2 (224,225). However, in recent years, the pneumococcal reference serum was switched to 007sp and there have not been any bridging experiments performed to assign IgG1 and IgG2 antibody concentrations to 007sp. Therefore, we have performed a bridging experiment using a multiplex Luminex method in order to assign IgG1 and IgG2 antibody values from 89-SF to 007sp. We have also tested a subset of HIV-infected serum samples in order to assess if there is a predominant IgG subclass produced in response to pneumococcal vaccination and whether there is a difference in subclass produced depending on the type of pneumococcal vaccination administered.

Furthermore, whole PCP-IgG and PCP-IgG2 ELISA Binding Site assays were compared to Pn-specific IgG and IgG2 multiplex assays in order to assess whether or not the Binding Site assays predicted protection against 8 out 12 Pn serotypes. The pitfalls of the whole PCP-Binding Site Assays include misdiagnosing protection against pneumococcus or 8 out of 12 Pn serotypes because Pn serotype-specific IgG levels are not determined in these assays. Thus, high Pn-specific IgG concentrations against a specific serotype such as Pn14, may mask protection against other Pn serotypes in the Binding Site Assays investigated in this chapter.

5.2 Materials and Methods

5.2.1. Ethical Approval

Ethical approval for the serum samples used in this investigation is outlined in section 2.3.

5.2.2. 18-plex Luminex assay IgG subclass Antibody Quantification and Assignment from human reference standard serum 89-SF to 007sp

The same methods (described in section 2.5.2) to assess Pn-specific IgG subclasses (IgG1-4) were performed in order to assign and quantify Pn-specific IgG subclass antibody concentrations in HIV-infected human serum. *S. pneumoniae* antigens (Pn

1,3,4,5,6B,7F,9V,14,18C,19A,19F, and 23F) in addition to PPV-23 and PCV-13 vaccine beads were assessed. However, Pn19A, PPV-23, and PCV-13 IgG1 and IgG2 antibody concentrations were not assigned from standard lot 89-SF to 007sp as there were no available antibody concentrations assigned to lot 89-SF from the original experiments performed by Soininen et al (224) and Sikkema et al (225). Furthermore, Pn-specific IgG3 and IgG4 antibody concentrations are expressed as MFIs as opposed to concentrations as these assignments have not been performed because previous studies have found these concentrations to be below the limit of detection in 89-SF (225).

5.2.2.1. Conjugation of whole PPV-23 and PCV-13 vaccines to Luminex beads for use in the Luminex IgG assay

5.2.2.1.1 Conjugation of whole PPV-23 vaccines to Luminex beads

Whole PPV-23 (Total concentration of each polysaccharide is 50 µg/mL) was conjugated to PLL (described previously in section 2.5.2.1.) except two 0.5 mL vials (575 µg polysaccharide per vaccine vial) were combined on G25 PD-10 Sephadex desalting columns to double the concentration of each pneumococcal polysaccharide in the PPV-23-PLL eluate (Estimated concentration was 328.6 µg/mL).

Furthermore, we found that 65 µg of PPV-23-PLL conjugated to 1 x 10^6 Luminex beads (as described previously in section 2.5.2.2) achieved optimal MFIs in the Luminex Pn-IgG assay.

6.2.2.1.2 Conjugation of whole PCV-13 vaccines to Luminex beads

The whole PCV-13 vaccine was conjugated directly to Luminex beads (as described previously in section 2.1.2) via the CRM_{197} protein. The total concentration of the Pn-polysaccharides in the vaccine (0.5 mL) was 30.8 µg (2.2 µg of Pn 1,3, 4, 5, 6A, 7F, 9V, 13, 18C, 19A, 19F, and 23F; 4.4 µg of Pn 6B). The total amount of the CRM_{197} protein was 32µg per 0.5 mL of vaccine.

Furthermore, we found that 12 µg of CRM_{197} (0.8 µg of each Pn polysaccharide except 1.6 µg Pn 6B in the PCV-13 vaccine) conjugated to 1 x 10^6 Luminex beads (as described previously in section 2.5.2.2) achieved optimal MFIs in the Luminex Pn-IgG assay. Previous experiments found that pre-absorption with the CRM_{197} protein had a minimal effect on 007sp standard reference serum curves; therefore, it was decided not to pre-absorb the patient serum samples with the CRM_{197} in subsequent experiments.

5.2.3 Anti-PCP IgG Antibody Quantification using the Binding Site Assay Kit

Anti-PCP IgG antibody concentrations were measured using commercially available VaccZyme™ PCP IgG ELISAs (The Binding Site Group Limited, Birmingham, UK). Methods were described as per manufacturer's guidelines (226). The cut-off for protection required for an adequate immune response to pneumococcal vaccination was ≥50 mg/L, as identified by Chua and colleagues (227). 88 HIV-infected patient serum samples were randomly selected from four quartiles (low, low medium, high medium, high) based on MFI titres to the whole PPV-23 Luminex bead investigation. Of the 88 patient serum samples, 11 patients received a single dose of PPV-23 and 11 patients received a single dose of PCV-13 as part of the AIR study.

5.2.4. Anti-PCP IgG2 subclass Antibody Quantification using the Binding Site Assay Kit

PCP IgG2 subclass antibody concentrations were measured using commercially available VaccZyme™ PCP IgG2 ELISAs (The Binding Site Group Limited, Birmingham, UK). Methods were described as per manufacturer's guidelines (210). The cut-off for protection required for an adequate immune response to pneumococcal vaccination was 12 mg/L, as identified by Parker and colleagues (210). 88 HIV-infected patient serum samples (pre- and post-vaccination) were used. 22 patients received a single dose of PPV-23 and 22 patients received a single dose of PCV-13 as part of the AIR study.

5.2.5. Serum

Human anti-pneumococcal standard reference serum lot 89-SF (1:20 dilution) and 007sp (1:10 dilution) were used in order to quantitate Pn-specific IgG1 and IgG2 antibody concentrations.

HIV-infected serum (1:100 dilution) from 50 patients (pre- and post-pneumococcal vaccination) was used in order to quantify Pn-specific IgG1 and IgG2 antibody concentrations. IgG1 and IgG2 antibody MFIs were reported for antigen Pn19A. Furthermore, HIV-infected serum from a subset of 17 patients was used in order to quantify Pn-specific IgG3 and IgG4 antibody MFIs for all of the antigens mentioned in section 5.2.2.

5.2.6. Monoclonal Antibodies against Human IgG Subclasses

Murine monoclonal anti-human IgG1 (MG6.41 #200113/2), anti-human IgG2 (HP-6200 #050513/1), anti-human IgG3 (MG5.161 #260914/3), and anti-human IgG4 (RJ4 #210816/P) were used to detect the binding of Pn-specific subclass antibodies in the 18-plex Luminex assay (Available from Sera Science, Abingdon Health, Dr. Margaret Goodall). The

specificities of these anti-human IgG subclass antibodies have been established in a collaboration with the WHO and the International Union of Immunological Societies (228–230).

5.2.6.1. Conjugation of anti-human monoclonal antibodies against human IgG subclasses using Lightning-Link RPE technology

Anti-human monoclonal antibodies as mentioned in section 5.2.5 were conjugated to R-Phycoerythrin (RPE) (Lightning Link R-PE Conjugation Kit; Innova Biosciences, Cambridge, UK) in order to be used as secondary antibodies in the 18-plex Luminex assay. Antibodies were conjugated at 1 mg/mL. A volume of 1 µl of LL-modifier reagent (Lightning Link R-PE Conjugation Kit; Innova Biosciences, Cambridge, UK) was added for every 10 µl of antibody conjugated to RPE. The antibody and LL-modifier reagent mixture was then added to the Lightning-Link lyophilised mixture in a glass vial (Lightning Link R-PE Conjugation Kit; Innova Biosciences, Cambridge, UK). Once added, the vial was left to incubate in the dark at RT overnight. Then, 1 µl of LL-quencher (Lightning Link R-PE Conjugation Kit; Innova Biosciences, Cambridge, UK) was added for every 10 µl of antibody conjugated to RPE. The conjugates can be stored safely in the dark at 4°C for up to 18 months.

5.2.6.2. Titration of anti-human RPE-conjugated monoclonal antibodies

Anti-human monoclonal antibodies as mentioned in section 5.2.5 were titrated at various concentrations (5 µg/mL, 2 µg/mL , 0.5 µg/mL, and 0.25 µg/mL) using standard lot 007sp (1:10 dilution in standard buffer as mentioned in section 5.2.2.) against the antigens mentioned in section 5.2.2. in the 18-plex Luminex assay. Samples were tested in duplicate and average MFIs were generated. Optimal MFIs were generated for IgG1-RPE at 0.5 µg/mL, IgG2-RPE at 5 µg/mL, and IgG3-RPE and IgG4-RPE at 2 µg/mL. A volume of 100 µl of the secondary-antibody conjugates was added to the Luminex sample wells as described in section 5.2.2.

5.2.6.3. Equivalence-of-absorbance method for assigning serotype-specific IgG1 and IgG2 quantities

The Pn-specific quantities assigned to IgG1 and IgG2 assigned previously for human reference standard serum 89-SF (224,225) to serotypes 1,3,4,5,6B,7F,9V,14, 18C, 19F, and 23F were used to extrapolate serotype-specific IgG1 and IgG2 values in the human reference standard serum 007sp using an equivalence of absorption method (153). Not all MFIs reached 1000, as used monoclonal antibodies which will only bind one epitope, not multiple epitopes with regards to polyclonal antibodies. Independent standard curves were generated for each serotype-specific subclass reference by plotting the log of optical density

versus the log of serum dilution using a validated curve fitting data analysis software (MasterPlex ReaderFit; Hitachi Solutions, Irvine,CA,USA) (224,225). The concentration of lot 007sp (µg/mL) for a given MFI was calculated using either a four or five-parameter logistic curve fitting algorithm (Figure 6.3 and 6.5) that were suitable for all 11 Pn serotypes. Lots 89-SF and 007sp were tested in duplicate and run three times for 16 (IgG1) and (IgG2) standard points (in order to establish the dynamic range of the assay) and an average of the MFIs (Figure 5.2 and 5.4) were calculated for each serotype-specific IgG1 and IgG2 subclass and used to assign a new subclass value for each serotype in lot 007sp (Table 5.1).

The dynamic range of the Pn-IgG1 assay for each of the 11 Pn serotypes tested was established as 0.01-3 µg/mL. Pn-IgG2 assay for each of the 11 Pn serotypes tested was established as 0.01-6 µg/mL. For any patient sample results that were below or above these limits, the cut-off values were applied.

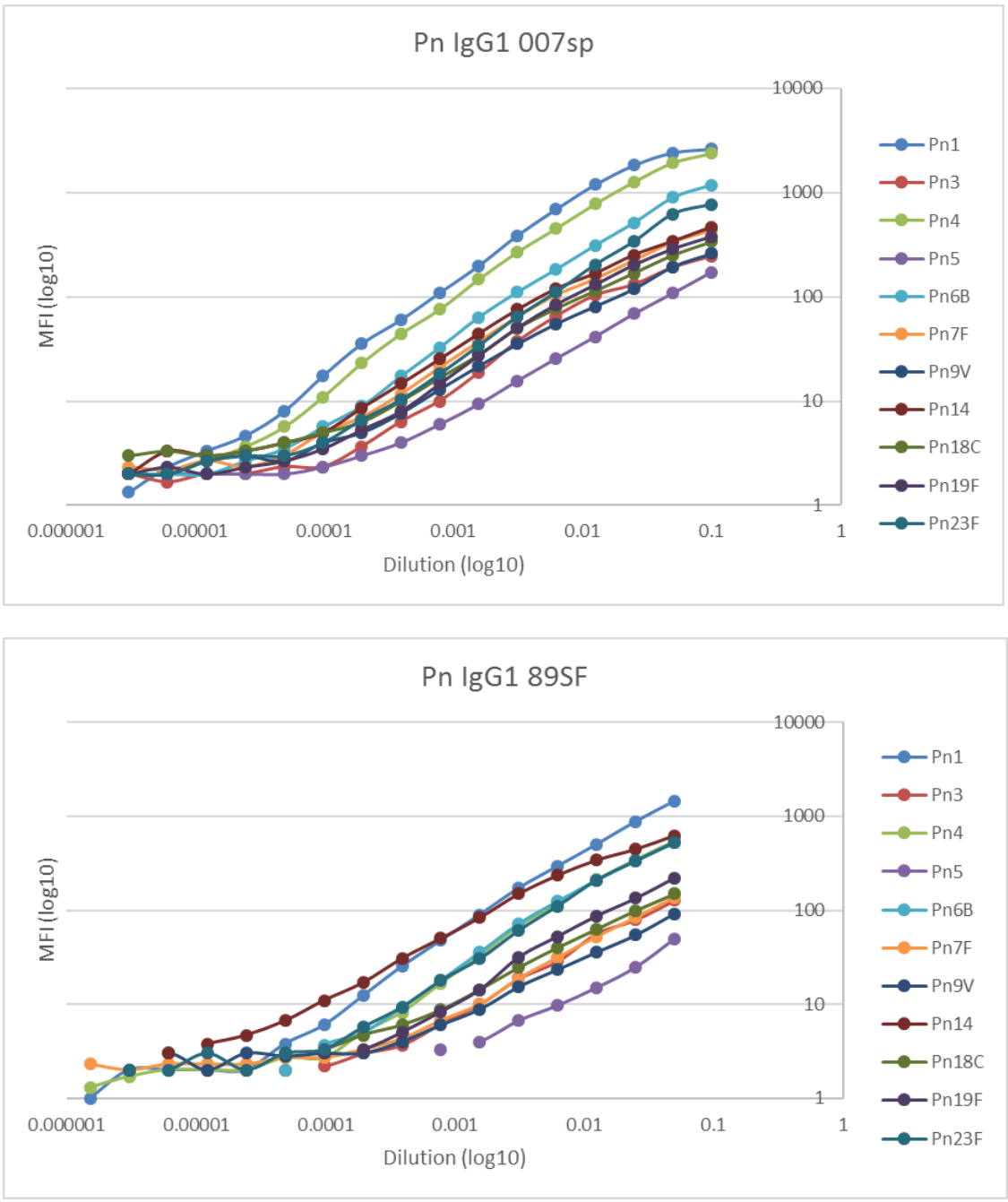
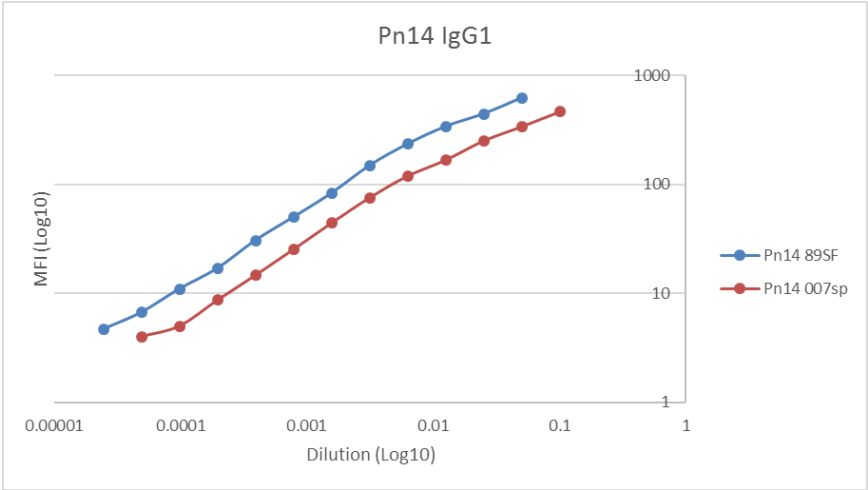


Figure 5.2. Pn serotype-specific IgG1 MFIs in standard reference lot 007sp and 89-SF. *These graphs show 16-point standard curves that were used in order to establish the dynamic ranges of the Pn-specific IgG1 assays using 89-SF and 007sp. Furthermore, the MFIs from 007so were used to assign a new subclass value for each 11 serotypes in 007sp.*

A.



B.

Standard	*1 in 20*	*Pn14 89-SF MFI*	*1 in10*	*Pn14 007sp MFI*
Type	**Dilution**	**MFI**	**Dilution**	**MFI**
S1	0.05	623.3	0.1	467
S2	0.025	445.2	0.05	342
S3	0.0125	341	0.025	253
S4	0.00625	235.7	0.0125	168
S5	0.003125	149.2	0.00625	120
S6	0.0015625	83.2	0.003125	76
S7	0.00078125	50.2	0.001563	44
S8	0.000390625	30.7	0.000781	25
S9	0.000195313	17	0.000391	15
S10	9.76563E-05	11	0.000195	9
S11	4.88281E-05	6.7	9.77E-05	5
S12	2.44141E-05	4.7	4.88E-05	4

C.

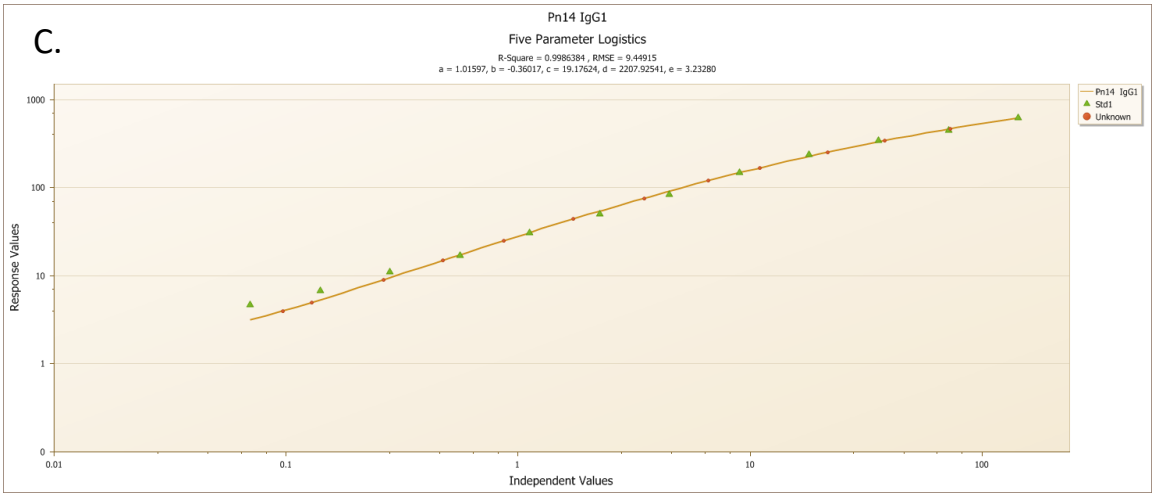
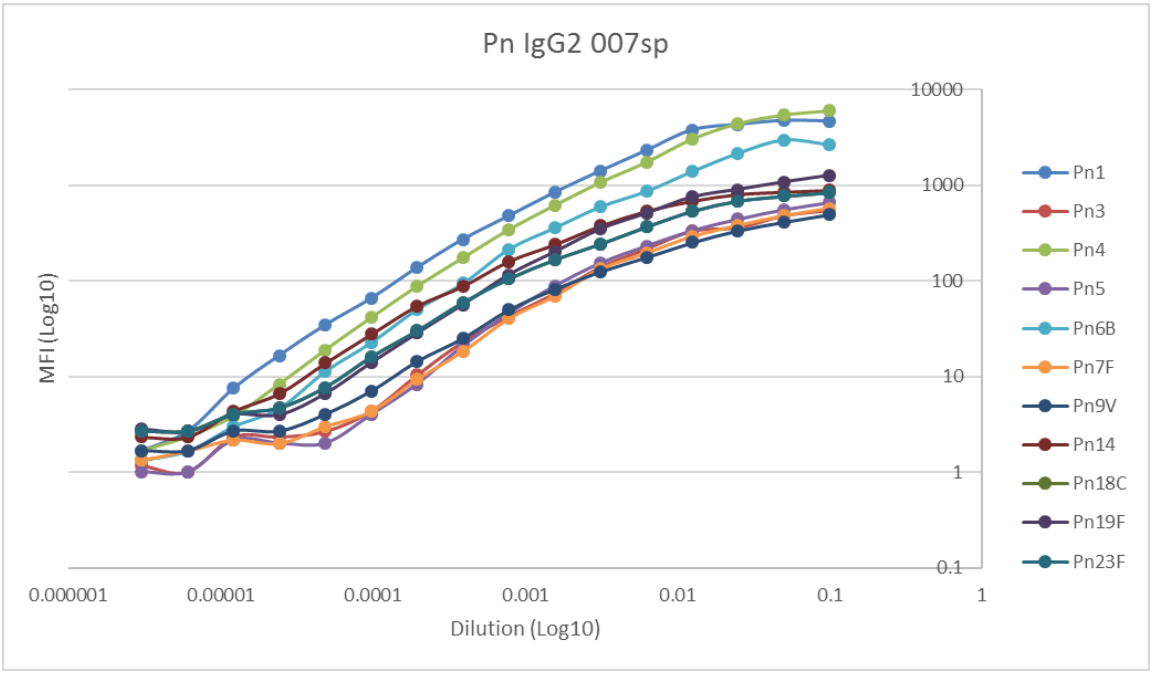


Figure 5.3. Representative schematic of the equivalence-of-absorbance method for assigning serotype-specific IgG1 using ReaderFit Curve fitting software for assignment of Pn14 IgG1-specific antibody titres from 89-SF to 007sp. *A) This graph shows averaged MFIs (Log10) for Standards 1- 12 dilutions of both Pn-14 IgG1 in 89-SF and 007sp. B) This table shows each averaged MFI recorded for both the 89-SF and 007sp curves shown in Panel A. C) This graph depicts a 5-parameter logistics calculation in the ReaderFit program that has been applied to both the 89-SF and 007sp standard curves in order to assign a Pn14-IgG1 antibody titre to 007sp.*



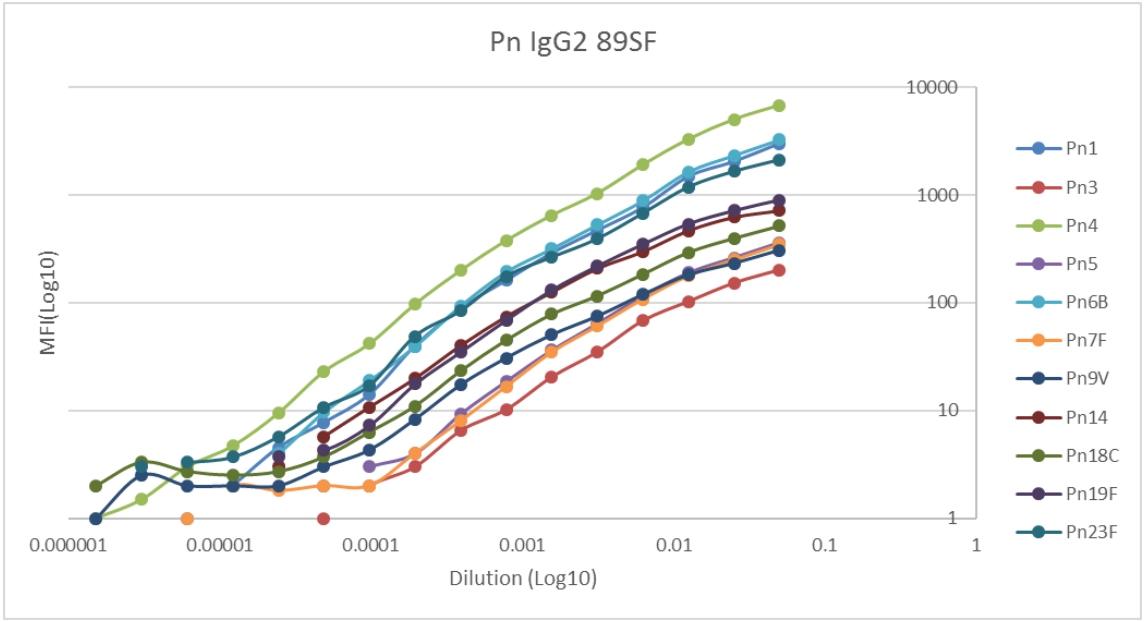
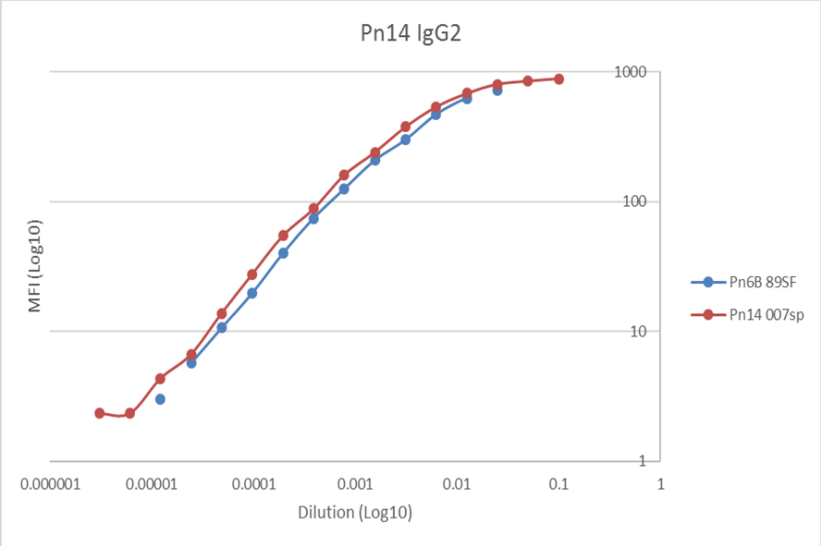


Figure 5.4. Pn serotype-specific IgG2 MFIs in standard reference lot 007sp and 89-SF. *These graphs show 16-point standard curves that were used in order to establish the dynamic ranges of the Pn-specific IgG1 assays using 89-SF and 007sp. Furthermore, the MFIs from 007so were used to assign a new subclass value for each 11 serotypes in 007sp.*

A.



B.

Standard Type	*1 in 20 Dilution*	*Pn14 89-SF MFI MFI*	*1 in10 Dilution*	*Pn14 007sp MFI MFI*
S1	0.05	---	0.1	887.3
S2	0.025	718.7	0.05	851.0
S3	0.0125	624	0.025	803.2
S4	0.00625	469	0.0125	683.3
S5	0.003125	298.7	0.00625	536.5
S6	0.0015625	208	0.003125	376.2
S7	0.00078125	125.3	0.0015625	240.0
S8	0.000390625	74.5	0.00078125	160.2
S9	0.000195313	40	0.000390625	88.0
S10	9.76563E-05	19.8	0.000195313	54.7
S11	4.88281E-05	10.7	9.76563E-05	27.7
S12	2.44141E-05	5.7	4.88281E-05	13.8
S13	1.2207E-05	3	2.44141E-05	6.7
S14	6.10352E-06	---	1.2207E-05	4.3
S15	3.05176E-06	---	6.10352E-06	2.3
S16	1.52588E-06	---	3.05176E-06	2.3

C.

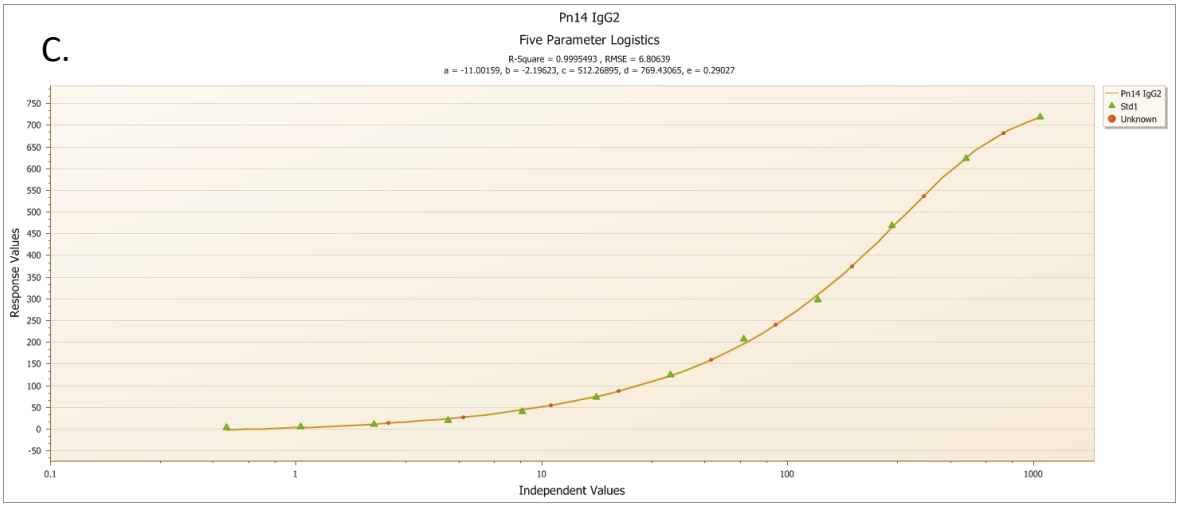


Figure 5.5. Representative schematic of the equivalence-of-absorbance method for assigning serotype-specific IgG2 using ReaderFit Curve fitting software for assignment of Pn14 IgG2-specific antibody titres from 89-SF to 007sp. *A) This graph shows averaged MFIs (Log10) for Standards 1- 16 dilutions of both Pn-14 IgG2 in 89-SF and 007sp. B) This table shows each averaged MFI recorded for both the 89-SF and 007sp curves shown in Panel A. C) This graph depicts a 5-parameter logistics calculation in the ReaderFit program that has been applied to both the 89-SF and 007sp standard curves in order to assign a Pn14-IgG2 antibody titre to 007sp.*

Table 5.1. Novel assigned Pn-specific subclass antibody values from reference standard lot 007sp.

	Concentration (ug/ml)			
Pn Serotype	***IgG1***	***IgG2***	***IgG1 + IgG2***	***Assigned Total IgG in 007sp***
Pn1	0.21	1.85	2.06	8.50
Pn3	0.16	0.20	0.36	1.45
Pn4	1.14	0.28	1.42	3.33
Pn5	0.09	6.03	6.12	7.51
Pn6B	0.41	0.85	1.25	9.05
Pn7F	0.29	2.40	2.68	8.30
Pn9V	0.39	5.99	6.37	6.44
Pn14	0.15	2.13	2.28	37.99
Pn18C	0.41	6.40	6.81	7.30
Pn19F	0.26	20.48	20.74	14.61
Pn23F	0.14	0.11	0.25	5.95

Pn-specific IgG1 and IgG2 are represented in µg/mL. Pn-specific IgG1 and IgG2 antibody titres were assigned from standard reference lot 89-SF to 007sp.

5.3 Results: Examination of the IgG Subclass Response to Pneumococcal Vaccination

5.3.1 Whole IgG versus IgG subclass response to vaccination

The initial analysis was to examine MFIs pre- and post-vaccination for IgG and IgG1, IgG2, IgG3, and IgG4 (Figure 5.6/Table 5.2)). We found that the PPV-23 vaccine serotype response for IgG2 was much greater than the response to IgG1, IgG3, and IgG4; however, there was still a strong IgG1 response for all 11 Pn serotypes tested (Figure 5.6/Table 5.2). Furthermore, the PCV-13 vaccine serotype response for IgG1 and IgG2 was equivalent, which was more robust than the response to IgG3 and IgG4 (Figure 5.6/Table 5.2). However, the IgG3 response was higher in the PCV-13 vaccine cohort than the PPV-23 vaccine cohort (Figure 5.6/Table 5.2). We also found that the IgG4 response was limited for both the PPV-23 and PCV-13 vaccine cohorts (Figure 5.6/Table 5.2).

Table 5.2. Number of Pn vaccine antigens with a significant difference pre-to-post vaccination

	Number of Pn vaccine antigens with a significant difference pre-to post-vaccination	
	PPV-23 (1x)	PCV-13 (1x)
IgG	7/11	11/11
IgG1	9/11	11/11
IgG2	10/11	11/11
IgG3	3/11	8/11
IgG4	2/11	3/11

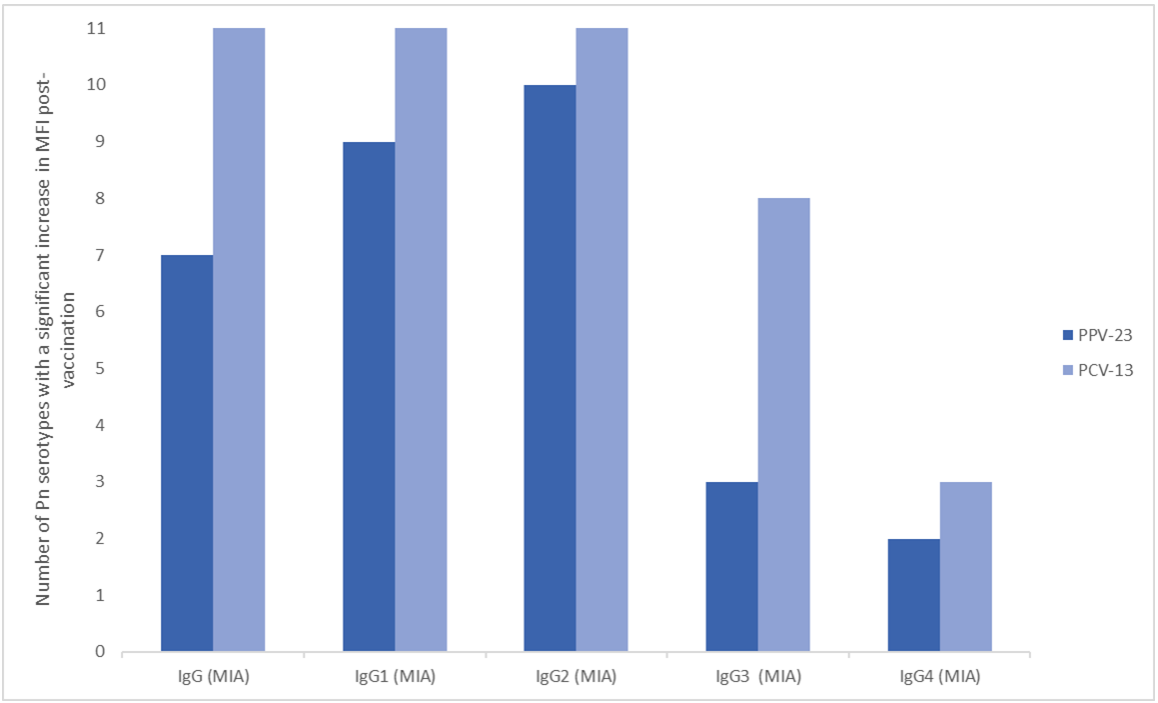


Figure 5.6. Whole IgG versus IgG subclass response to pneumococcal vaccination in a cohort of HIV-infected patients in the UK. *For pre-post vaccination: Wilcoxon Matched Pair t-tests, non-parametric. For pre and post-vaccination between PPV and PCV groups: Mann-Whitney U t-tests, unpaired, non-parametric tests were performed.*

5.3.2. Examination of Fold Changes

We then went on to examine fold changes for Pn-specific IgG (Figure 5.7) and Pn-specific IgG subclasses (Figure 5.8). The IgG1-4 responses including the absolute antibody concentrations and fold changes differed across all of the 11 Pn serotypes. Furthermore, this was vastly different between the PPV-23 and PCV-13 cohorts. We then ranked fold changes to see which Pn serotypes had the greatest/lowest fold change for Pn-specific IgG and the IgG subclasses and then analysed these changes into vaccine cohorts (Figure 5.8/Table 5.3).

We found that Pn 1 has the highest rank across the Pn-specific IgG and Pn-specific IgG1-4 for both the PPV-23 and PCV-13 cohorts. This could be due to the fact that it is an invasive serotype, so therefore, patients mount a more robust immune response to this serotype as it's not naturally acquired through the environment. Pn 1 is ranked as one of the most prevalent serotypes that causes IPD on a global scale even though it is not normally detected in nasopharyngeal carriage (231,232). It has been established that Pn 1 is carried for a relatively short period of approximately 9 days post-exposure in humans (233). Therefore, it has been hypothesized that Pn 1 may undergo a lower rate of genetic recombination due to lesser opportunities for genetic exchange during nasopharyngeal carriage (234,235). Furthermore, the Pn1 capsular polysaccharide is a zwitterionic structure that induces a T-cell dependent response, which could explain the robust Pn-1 specific IgG response seen in Figure 5.7 (235). However, Pn 14 can be naturally acquired through the environment, which is evidenced by the high antibody concentrations seen in most patient samples . The fold change ranks across the subclasses for this serotype are relatively low, which reflects the idea that this strain is naturally acquired through the environment.

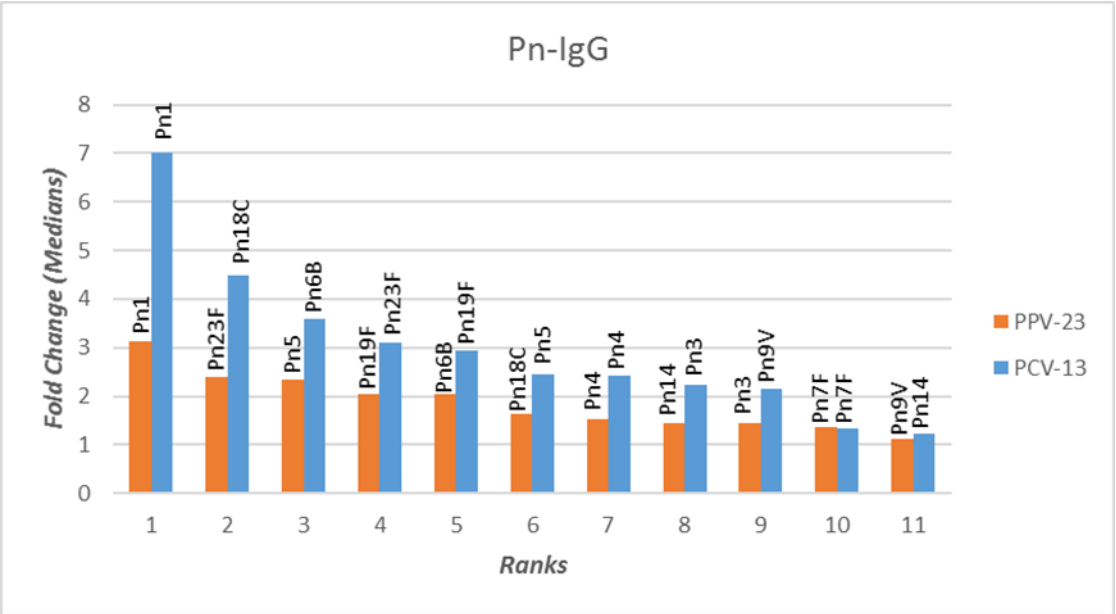


Figure 5.7. Pn-specific IgG fold change and ranks for 11 Pn serotypes according to PPV-23 or PCV-13 vaccination.
Fold changes are represented as medians.

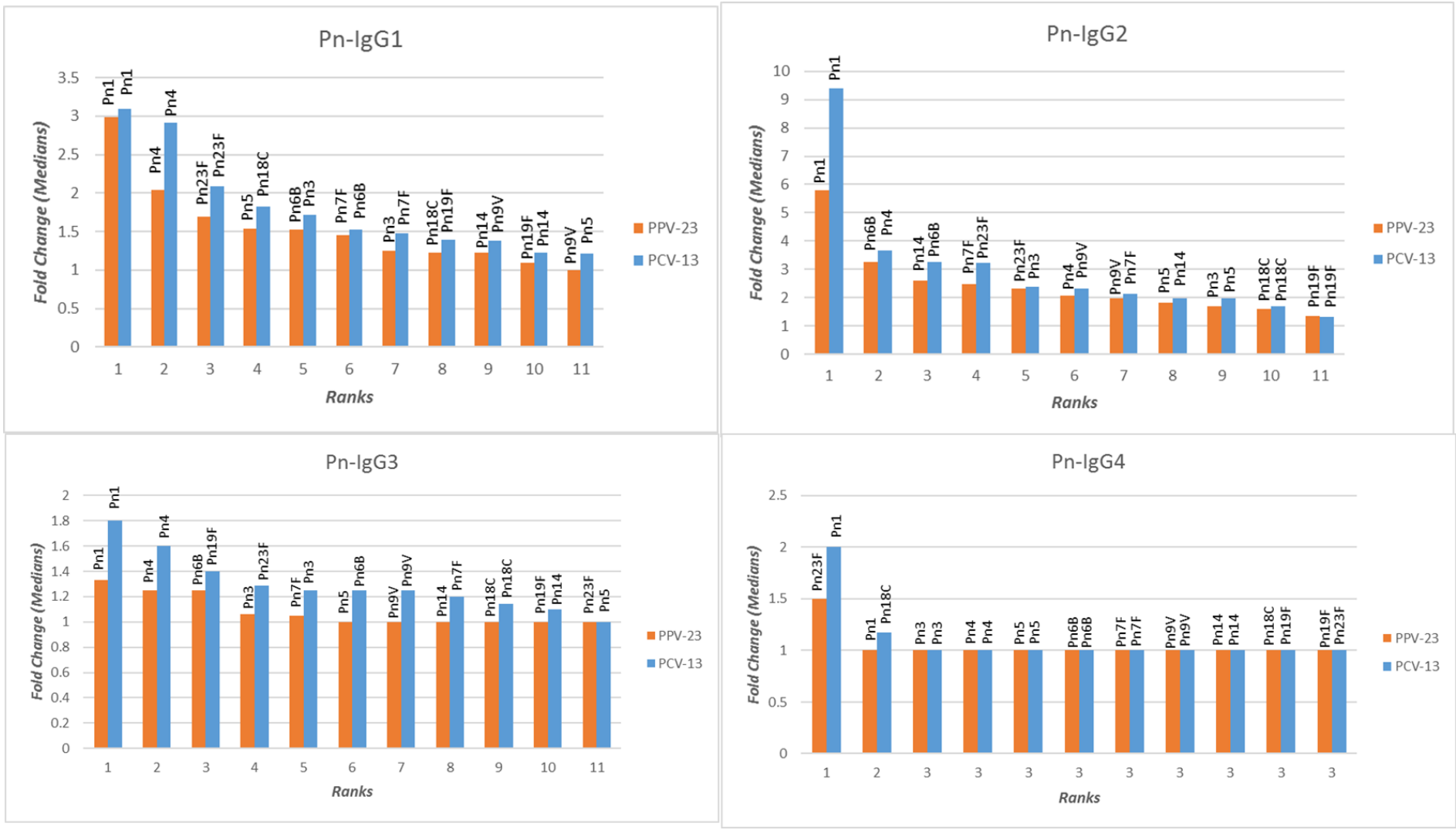


Figure 5.8. Pn-specific IgG1-4 fold change and ranks for 11 Pn serotypes according to PPV-23 or PCV-13 vaccination. *Fold changes are represented as medians.*

Table 5.3. Pn-specific IgG and IgG subclasses- Examination of fold change ranks for 11 Pn serotypes according to PPV-23 or PCV-13.

Ranks	PPV-23 IgG	PCV-13 IgG	PPV-23 IgG1	PCV-13 IgG1	PPV-23 IgG2	PCV-13 IgG2	PCV-13 IgG3
1	1	1	1	1	1	1	1
2	23F	18C	4	4	6B	4	4
3	5	6B	23F	23F	14	6B	19F
4	19F	23F	5	18C	7F	23F	23F
5	6B	19F	6B	3	23F	3	3
6	18C	5	7F	6B	4	9V	6B
7	4	4	3	7F	9V	7F	9V
8	14	3	18C	19F	5	14	7F
9	3	9V	14	9V	3	5	18C
10	7F	7F	19F	14	18C	18C	14
11	9V	14	9V	5	19F	19F	5

Rank	PPV-23 IgG3
1	1
2	4
3	6B
4	3
5	7F
5	5
5	9V
5	14
5	18C
5	19F
5	23F

Rank	PPV-23 IgG4	PCV-13 IgG4
1	23F	1
2	1	18C
3	3	3
3	4	4
3	5	5
3	6B	6B
3	7F	7F
3	9V	9V
3	14	14
3	18C	19F
3	19F	23F

5.3.3. Quality of the Pn-specific IgG response in an individual

The proportional amount of Pn-specific IgG1-4 produced is different for each Pn serotype. Therefore, we examined absolute values, fold change, and IgG: IgG subclass ratios (Figures 5.9- 5.19/Tables 5.4-5.9). The significant increase in whole IgG concentrations differ to examining IgG subclasses. Furthermore, IgG subclass analysis offers further understanding of the IgG response to different vaccines.

5.3.3.1. Pn Serotype 1

For Pn-serotype 1 (Figure 5.9), there was more IgG and IgG2 produced in the PCV-13 cohort. Significant differences were detected from baseline to post-vaccination for all of the IgG-IgG4 assays in each cohort; however, there were no significant differences detected post-vaccination between the PPV-23 and PCV-13 cohorts for the Pn-IgG1, IgG3, and IgG4 assays. Furthermore, there were no significant differences detected for fold changes between vaccine cohorts; however, it is important to note the vast differences in fold change for each Pn-IgG-IgG4 assay (Figure 5.8/Tables 5.4-5.9). A difference was seen in the amount of IgG:IgG1 produced for Pn1 between the PPV-23 and PCV-13 vaccine cohorts ($p=0.0170$). This suggests that the proportion of IgG to IgG1 produced in the PCV-13 cohort was greater in contrast to the amount of IgG produced in the PPV-23 cohort.

5.3.3.2. Pn Serotype 3

For Pn-serotype 3 (Figure 5.10), there were no significant differences found post-vaccination between PPV-23 and PCV-13 cohorts for absolute Pn3-IgG, Pn3-IgG3, and Pn3-IgG4 values. However, there was a significant difference ($p=0.0445$) seen pre-vaccination between the two cohorts. Furthermore, there was a significant difference seen from baseline to post-vaccination in the PCV-13 cohort ($p<0.0001$). Significant differences were also seen from baseline to post-vaccination in both cohorts (PPV-23 ($p=0.0098$) and PCV-13 ($p=0.0002$) for Pn3-IgG1. However, only a significant difference was seen for the PCV-13 cohort for Pn3-IgG2 ($p<0.0001$). Pn3-IgG3 and IgG4 MFIs were also quite low in both vaccine cohorts. A greater fold change was seen in the amount of Pn3-IgG produced for the PCV-13 cohort ($p=0.0052$, 2.22 median fold-change (1.32-3.89)) (Figure 5.10/Table 5.4).). No other differences in fold change were seen in the Pn-IgG subclass assays for both vaccine cohorts; however, it is worthwhile to note that the fold changes varied widely. A difference was seen in the amount of IgG:IgG1 produced for Pn3 between the PPV-23 and PCV-13 vaccine cohorts ($p=0.0028$). This also suggests that the proportion of IgG to IgG1 produced in the PCV-13 cohort was greater in contrast to the amount of IgG produced in the PPV-23 cohort. There were no significant differences between the vaccine cohorts for the IgG:IgG2-4 ratios.

5.3.3.3 Pn Serotype 4

For Pn-serotype 4 (Figure 5.11), there was a greater amount of Pn3-IgG (p=0.0009), Pn3-IgG2 (p=0.0140), and Pn3-IgG3 (0.0230) produced post-vaccination for the PCV-13 cohort. Additionally, significant differences were seen from baseline to post-vaccination for both vaccine cohorts for Pn4-IgG (PPV-23 (p=0.0255) and PCV-13 (p<0.0001)), Pn4-IgG1 (PPV-23 (p=0.0004) and PCV-13 (p<0.0001)), Pn4-IgG2 (PPV-23 (p=0.0003) and PCV-13 (p<0.0001)), and Pn4-IgG3 (PPV-23 (p=0.0204) and PCV-13 (p=0.0024)). Pn-IgG4 MFIs were low for both vaccine cohorts and no significant differences were detected. Furthermore, no significant differences were detected with regards to fold change for the Pn4-IgG-IgG4 assays; however, the PCV-13 cohort produced the greatest fold change in Pn4-IgG2 produced (median 3.68) compared to the PPV-23 cohort (median 2.06), although, this observation was non-significant (p=0.2776). Differences were seen in the amount of IgG:IgG1 (p=0.0011) and IgG:IgG4 (p=0.0216) produced for Pn4 between the PPV-23 and PCV-13 vaccine cohorts. This also suggests that the proportion of IgG to IgG1 and IgG4 produced in the PCV-13 cohort was greater in contrast to the amount of IgG produced in the PPV-23 cohort. There were no significant differences between the vaccine cohorts for the IgG:IgG2-3 ratios.

5.3.3.4 Pn Serotype 5

For Pn-serotype 5 (Figure 5.12), there were no significant differences detected between the vaccine cohorts, however, there were differences seen from baseline to post-vaccination for Pn5-IgG (PPV-23 (p=0.0088) and PCV-13 (p<0.0001)), Pn5-IgG1 (PPV-23 (p=0.0229) and PCV-13 (p<0.0060)),and Pn5-IgG2 (PPV-23 (p<0.0001) and PCV-13 (p=0.0001)). There was a significant difference detected between baselines between the PPV-23 and PCV-13 cohort (p=0.0237). Pn5-IgG3 and Pn5-IgG4 MFI levels were quite low with no significant differences detected between the vaccine cohorts or from baseline to post-vaccination within the vaccine cohorts. Additionally, no significant differences were seen in fold-change for Pn5-IgG-IgG4. However, Pn5-IgG1-4 all produced median fold changes below 2. The greatest fold changes for Pn5 were detected for IgG (PPV-23 (median 2.34) and PCV-13 (median 2.45); ns)). Differences were seen in the amount of IgG:IgG1 (p=0.0014), IgG:IgG2 (p=0.0059), IgG:IgG3 (p=0.0410)) produced for Pn5 between the PPV-23 and PCV-13 vaccine cohorts. This also suggests that the proportion of IgG to IgG1-IgG3 produced in the PCV-13 cohort was greater in contrast to the amount of IgG produced in the PPV-23 cohort. There were no significant differences between the vaccine cohorts for the IgG:IgG4 ratio.

5.3.3.5 Pn Serotype 6B

For Pn-serotype 6B (Figure 5.13), there were no significant differences detected between the vaccine cohorts, however, there were differences seen from baseline to post-vaccination for Pn6B-IgG (PPV-23 ($p=0.0315$) and PCV-13 ($p<0.0001$)), Pn6B-IgG1 (PPV-23 ($p=0.0004$) and PCV-13 ($p<0.0007$)),Pn6B-IgG2 (PPV-23 ($p<0.0001$) and PCV-13 ($p<0.0001$)), Pn6B-IgG3 (PPV-23 ($p=0.0317$) and PCV-13 ($p=0.0002$)). There were no significant differences seen for Pn6B-IgG4 and MFIs were also low in both vaccine cohorts. The PCV-13 cohort had the greatest fold change (median 3.58) for Pn6B-IgG post-vaccination compared to the PPV-23 cohort (median 2.03). However, this observation was non-significant ($p=0.0642$). Both PPV-23 and PCV-13 cohorts had equivalent fold changes (medians 3.25) for Pn6B-IgG2 (ns; $p=0.6472$). There were no significant differences seen between the vaccine cohorts for the IgG:IgG1-4 ratios.

5.3.3.6 Pn Serotype 7F

For Pn-serotype 7F (Figure 5.14), there were no significant differences detected between the vaccine cohorts, however, there were differences seen from baseline to post-vaccination for Pn7F-IgG (PPV-23 ($p=0.0450$) and PCV-13 ($p=0.0025$)), Pn7F-IgG1 (PPV-23 ($p=0.0067$) and PCV-13 ($p=0.0001$)),Pn7F-IgG2 (PPV-23 ($p<0.0001$) and PCV-13 ($p=0.0002$)), Pn7F-IgG3 (PCV-13 ($p=0.0038$). There were no significant differences detected for Pn7F-IgG4 and the MFIS were also low in both vaccine cohorts. The fold changes for both vaccine cohorts were below a median of 2 for Pn7F-IgG, Pn7F-IgG1, Pn7F-IgG3, and Pn7F-IgG4 and were also non-significant. However, both PPV-23 and PCV-13 vaccine cohorts produced median fold changes greater than 2 (PPV-23 (median 2.47) and PCV-13 (median 2.12)), although, these fold changes were non-significant ($p=0.2629$). There were no significant differences produced for Pn IgG:IgG1-4 for both vaccine cohorts (Table 5.9).

5.3.3.7 Pn Serotype 9V

For Pn-serotype 9V (Figure 5.15), there was a greater amount of Pn9V-IgG2 produced for the PCV-13 than the PPV-23 cohort ($p=0.0217$). Additionally, significant differences were only seen from baseline to post-vaccination in the PCV-13 cohort for Pn9V-IgG ($p<0.0001$), Pn9V-IgG1 ($p=0.0042$), and Pn9V-IgG3 ($p=0.0232$). However, significant differences were detected for both vaccine cohorts for Pn9V-IgG2 (PPV-23 ($p=0.0003$) and PCV-13 ($p\sim<0.0001$)). Both vaccine cohorts did not produce greater than a 2-fold change for Pn9V-IgG1, Pn9V-IgG3, and Pn9V-IgG4. However, the PCV-13 cohort produced 2-fold changes for Pn9V-IgG (median 2.16) and Pn9V-IgG2 (median 2.32). The PCV-13 cohort produced a 2-fold change in

Pn9V-IgG, which was greater than the PPV-23 cohort (median 1.12) (p=0.0045). There were no significant differences produced for Pn IgG:IgG1-4 for both vaccine cohorts (Table 5.9).

5.3.3.8. Pn Serotype 14

For Pn-serotype 14 (Figure 5.16), there were significant differences seen from baseline to post-vaccination for Pn14-IgG (PPV-23 (p=0.0229) and PCV-13 (p=0.0010)), Pn14-IgG1 (PPV-23 (0.0055) and PCV-13 (p=0.0192)), Pn14-IgG2 (PPV-23 (0.0007) and PCV-13 (p=0.0007)), and Pn14-IgG4 (PCV-13 (p=0.0352)). No significant differences were detected between vaccine cohorts. Furthermore, Pn14-IgG, Pn14-IgG1, Pn14-IgG3, and Pn14-IgG4 had fold changes below 2 for both vaccine cohorts. However, Pn14-IgG2 had a median fold change above 2 for the PPV-23 cohort (median 2.59) compared to the PCV-13 cohort (median 1.98), although there was no significant difference detected between the two vaccine cohorts (p=0.3316). There were no significant differences produced for Pn IgG:IgG1-4 for both vaccine cohorts (Table 5.9).

5.3.3.9. Pn Serotype 18C

For Pn-serotype 18C (Figure 5.17), the only significant different seen between vaccine cohorts was Pn18C-IgG (p=0.0350). Furthermore, the PCV-13 cohort only had a significant difference from baseline to post-vaccination (p<0.0001). Additionally, there were significant differences seen from baseline to post-vaccination for Pn18C-IgG1 ((PPV-23 (p=0.0167) and PCV-13 (p=0.0011)) , Pn18C-IgG2 (PPV-23 (p=0.0009) and PCV-13 (p<0.0001)), Pn18C-IgG3 (PCV-13 (p=0.0459)), and Pn18C-IgG4 (PCV-13 (p=0.0193)). No significant differences were found between vaccine cohorts for fold changes for Pn18C-IgG1-4 and they were all well below a median of 2. However, the fold change for Pn18C-IgG for the PCV-13 vaccine cohort had a median of 4.48, which was significantly different than the PPV-23 vaccine cohort (median 1.63) (p=0.0028). There were no significant differences produced for Pn18C IgG:IgG1-4 for both vaccine cohorts (Table 5.9).

5.3.3.10 Pn Serotype 19F

For Pn-serotype 19F (Figure 5.18), there were no significant differences seen between vaccine cohorts; however, significant differences were found between baseline and post-vaccination for Pn19F-IgG (PPV-23 (p=0.0483) and PCV-13 (p<0.0001)), Pn19F-IgG1 (PCV-13 (p=0.0003)), Pn19F-IgG2 (PPV-23 (p=0.0004) and PCV-13 (p<0.0001)), and Pn19F-IgG3 (PCV-13 (p=0.0007)). Pn19F-IgG4 MFIs were particularly low and no significant differences were found between vaccine cohorts. No significant differences were found between vaccine cohorts for fold changes for Pn19F-IgG1-4 and they were all well below a median of 2.

However, the fold change for Pn19F-IgG for the PCV-13 vaccine cohort (median of 2.93) was greater than the PPV-23 vaccine cohort (median 2.04) (p=0.3938). There were no significant differences produced for Pn19F IgG:IgG1-4 for both vaccine cohorts (Table 5.9).

5.3.3.11 Pn Serotype 23F

For Pn-serotype 23F (Figure 5.19), there were no significant difference seen between vaccine cohorts; however, significant differences were found between baseline and post-vaccination for Pn23F-IgG (PPV-23 (p=0.0367) and PCV-13 (p<0.0001)), Pn23F-IgG1 (PPV-23 (p=0.0031) and PCV-13 (p=0.0001)), Pn23F-IgG2 (PPV-23 (p<0.0001) and PCV-13 (p<0.0001)), Pn23F-IgG3 (PCV-13 (p=0.0035)), and Pn23F-IgG4 (PPV-23 (p=0.0166)). Furthermore, no additional significant differences were found for fold changes between vaccine cohorts; however, Pn23F-IgG1, Pn23F-IgG3, and Pn23F-IgG4 all had median fold changes below 2 (ns). Additionally, Pn23F-IgG had median fold changes above 2-fold (PPV-23 (2.39) and PCV-13 (3.11) (ns; p=0.1623)) and Pn23F-IgG2 had median fold changes (PPV-23 (2.31) and PCV-13 (3.23) (ns; p=0.2638)).

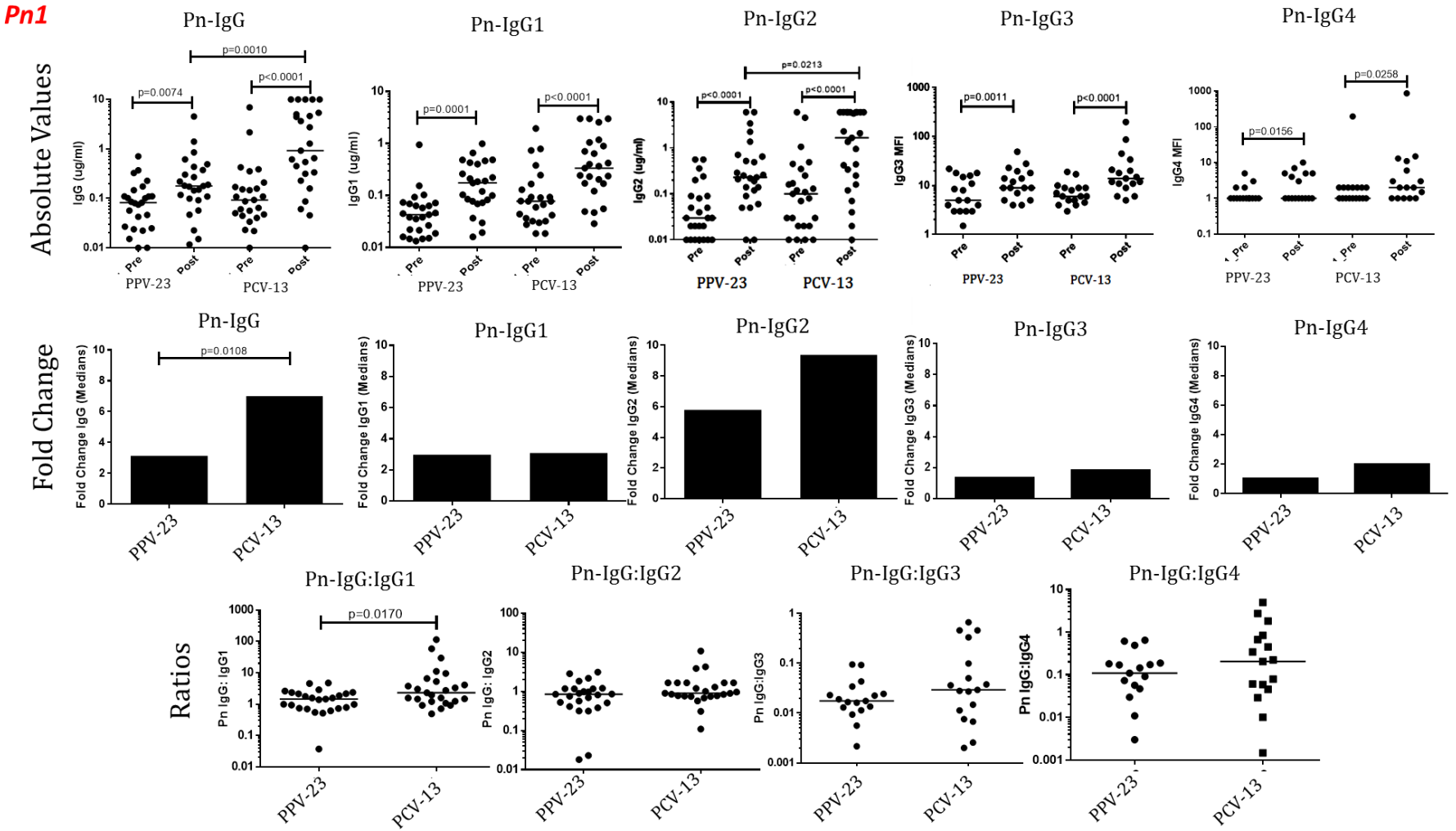


Figure 5.9. Pn-specific IgG, IgG1-4 responses to pneumococcal vaccination- Serotype 1. *Each point represents one study participant. Solid horizontal lines indicate medians. Absolute values are represented as µg/mL. Fold changes are represented as medians. Ratios are represented as medians. P-values are significant at p<0.05.*

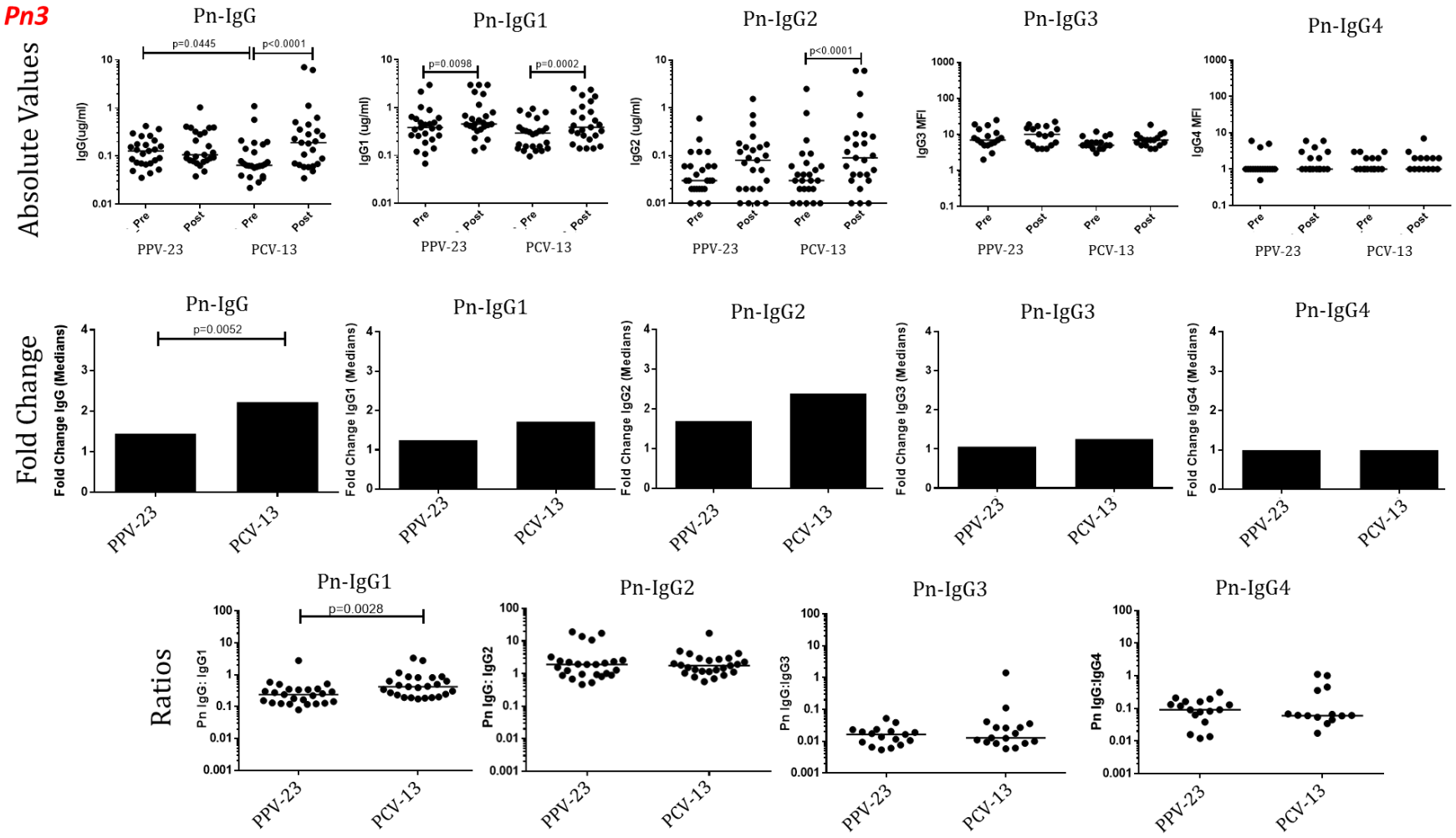


Figure 5.10. Pn-specific IgG, IgG1-4 responses to pneumococcal vaccination- Serotype 3. *Each point represents one study participant. Solid horizontal lines indicate medians. Absolute values are represented as µg/mL. Fold changes are represented as medians. Ratios are represented as medians. P-values are significant at p<0.05.*

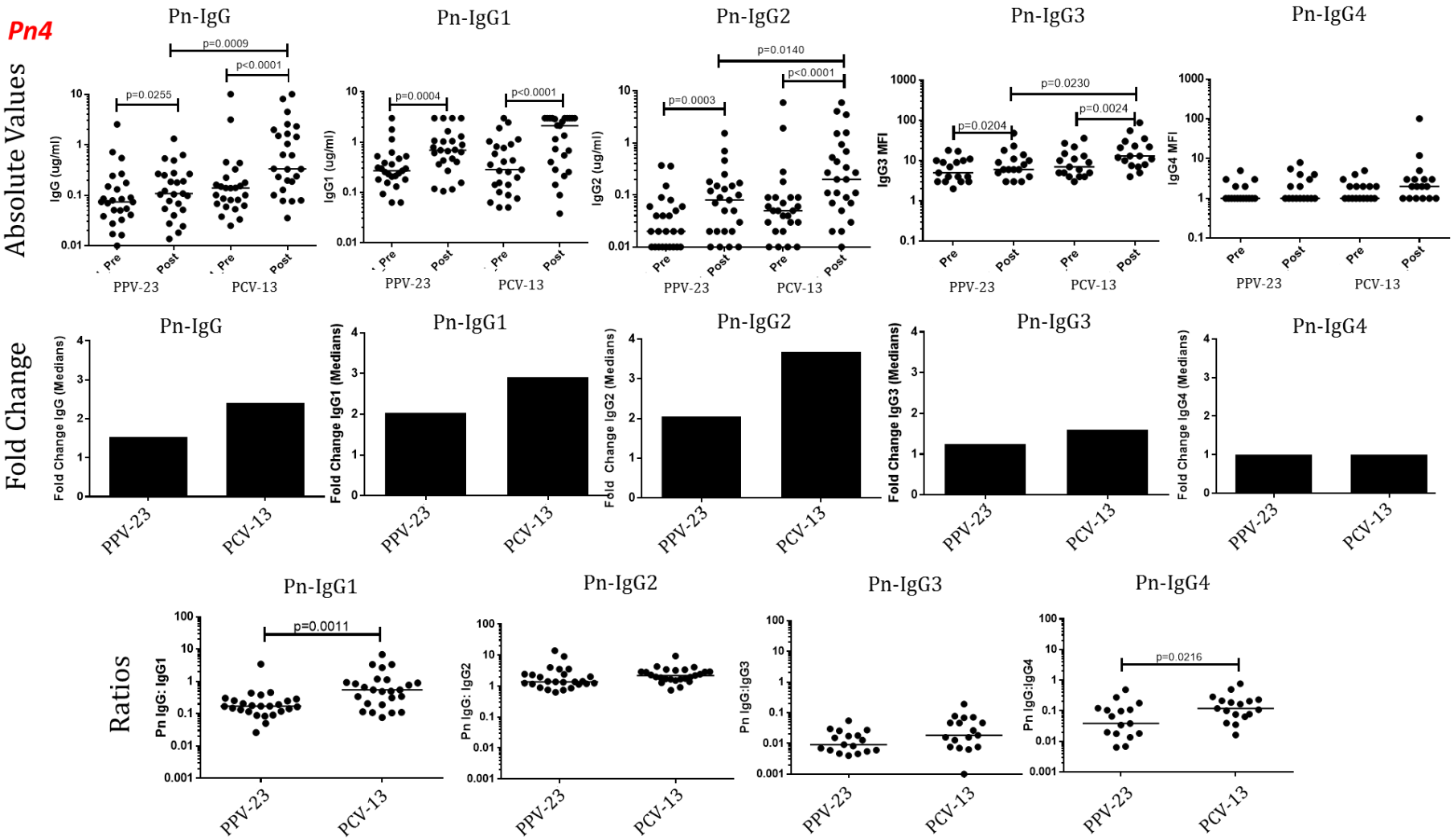


Figure 5.11. Pn-specific IgG, IgG1-4 responses to pneumococcal vaccination- Serotype 4. *Each point represents one study participant. Solid horizontal lines indicate medians. Absolute values are represented as µg/mL. Fold changes are represented as medians. Ratios are represented as medians. P-values are significant at p<0.05.*

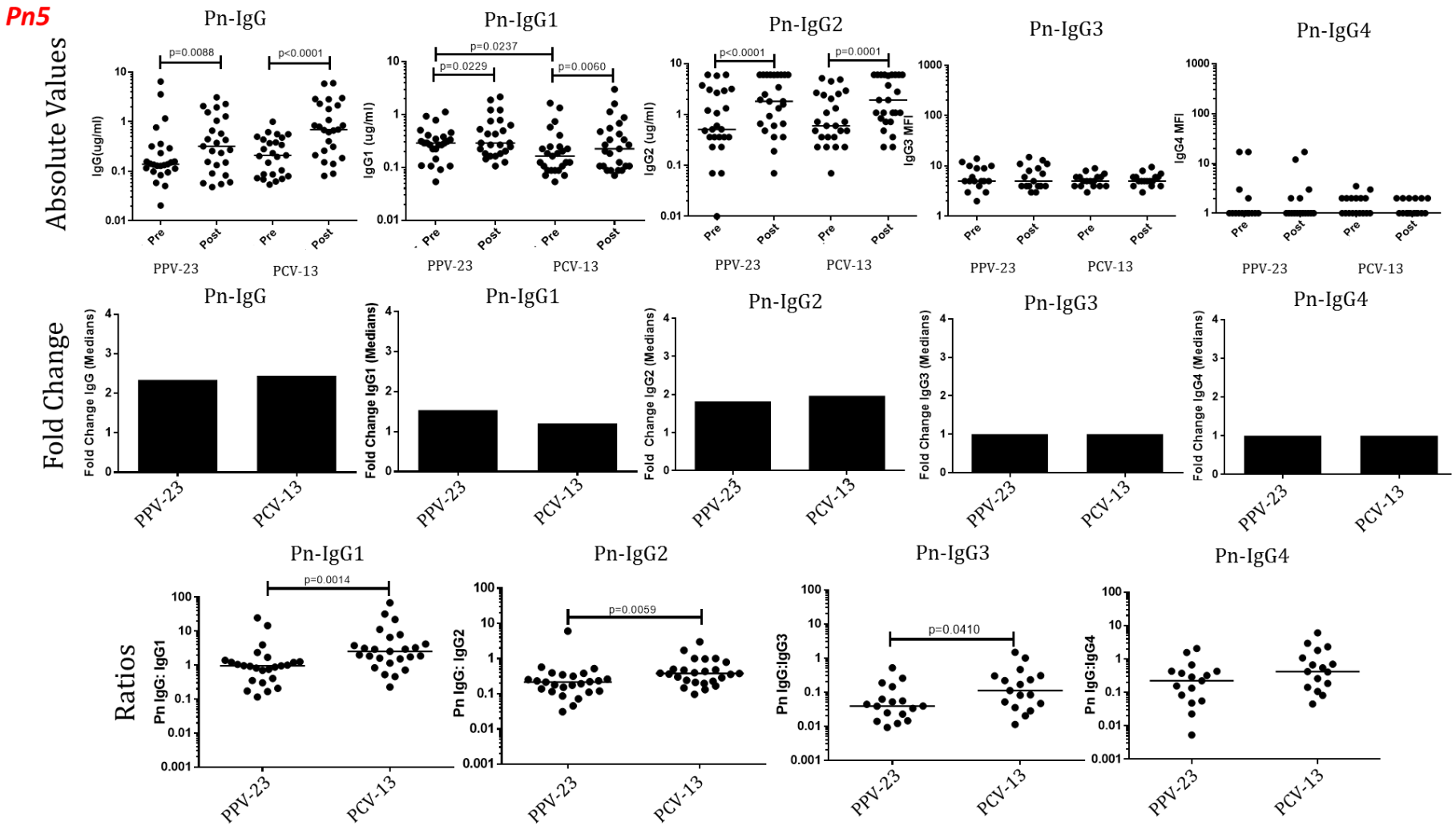


Figure 5.12. Pn-specific IgG, IgG1-4 responses to pneumococcal vaccination- Serotype 5. *Each point represents one study participant. Solid horizontal lines indicate medians. Absolute values are represented as µg/mL. Fold changes are represented as medians. Ratios are represented as medians. P-values are significant at p<0.05.*

Pn6B

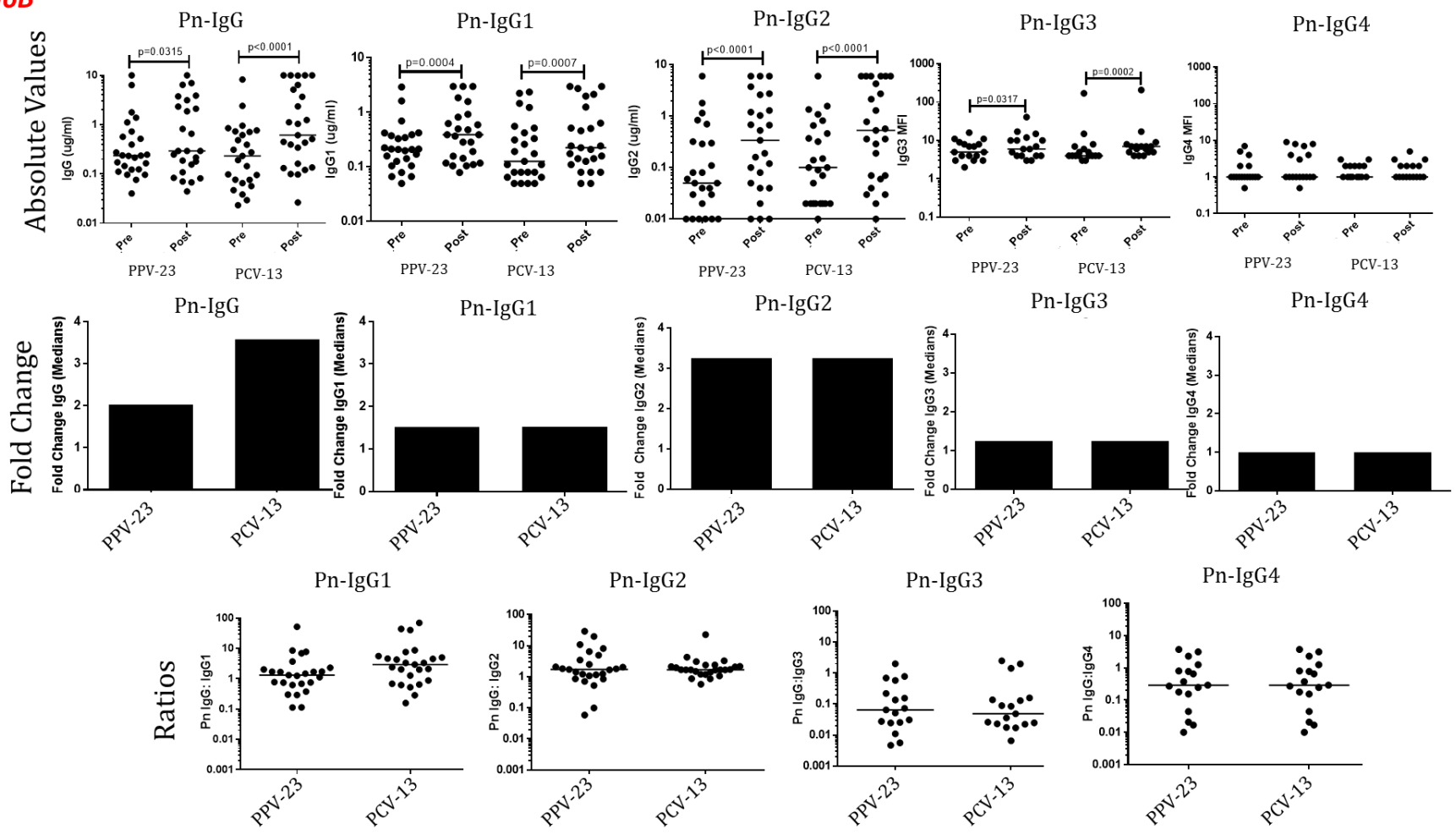


Figure 5.13. Pn-specific IgG, IgG1-4 responses to pneumococcal vaccination- Serotype 6B. *Each point represents one study participant. Solid horizontal lines indicate medians. Absolute values are represented as µg/mL. Fold changes are represented as medians. Ratios are represented as medians. P-values are significant at p<0.05.*

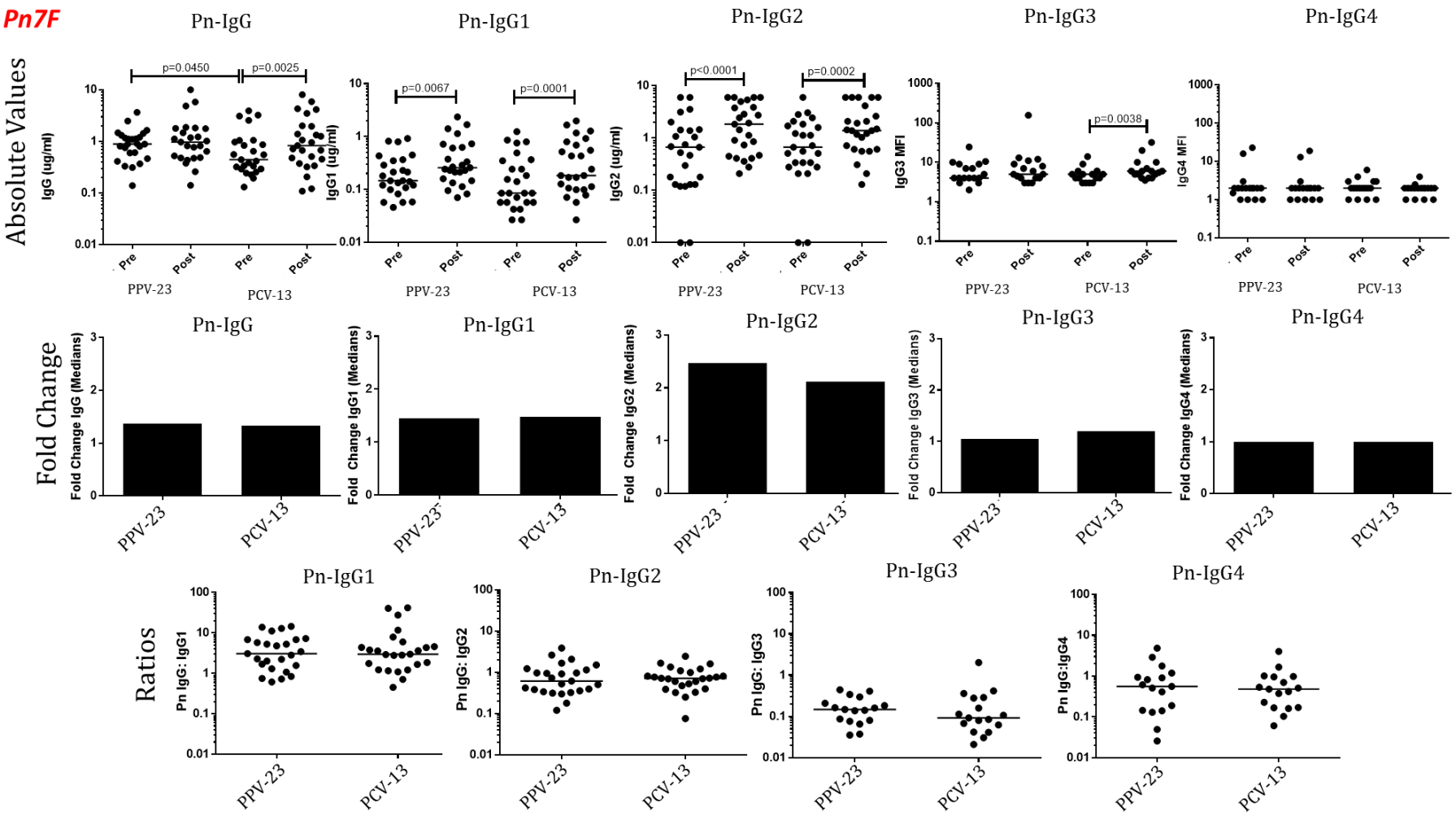


Figure 5.14. Pn-specific IgG, IgG1-4 responses to pneumococcal vaccination- Serotype 7F. *Each point represents one study participant. Solid horizontal lines indicate medians. Absolute values are represented as µg/mL. Fold changes are represented as medians. Ratios are represented as medians. P-values are significant at p<0.05.*

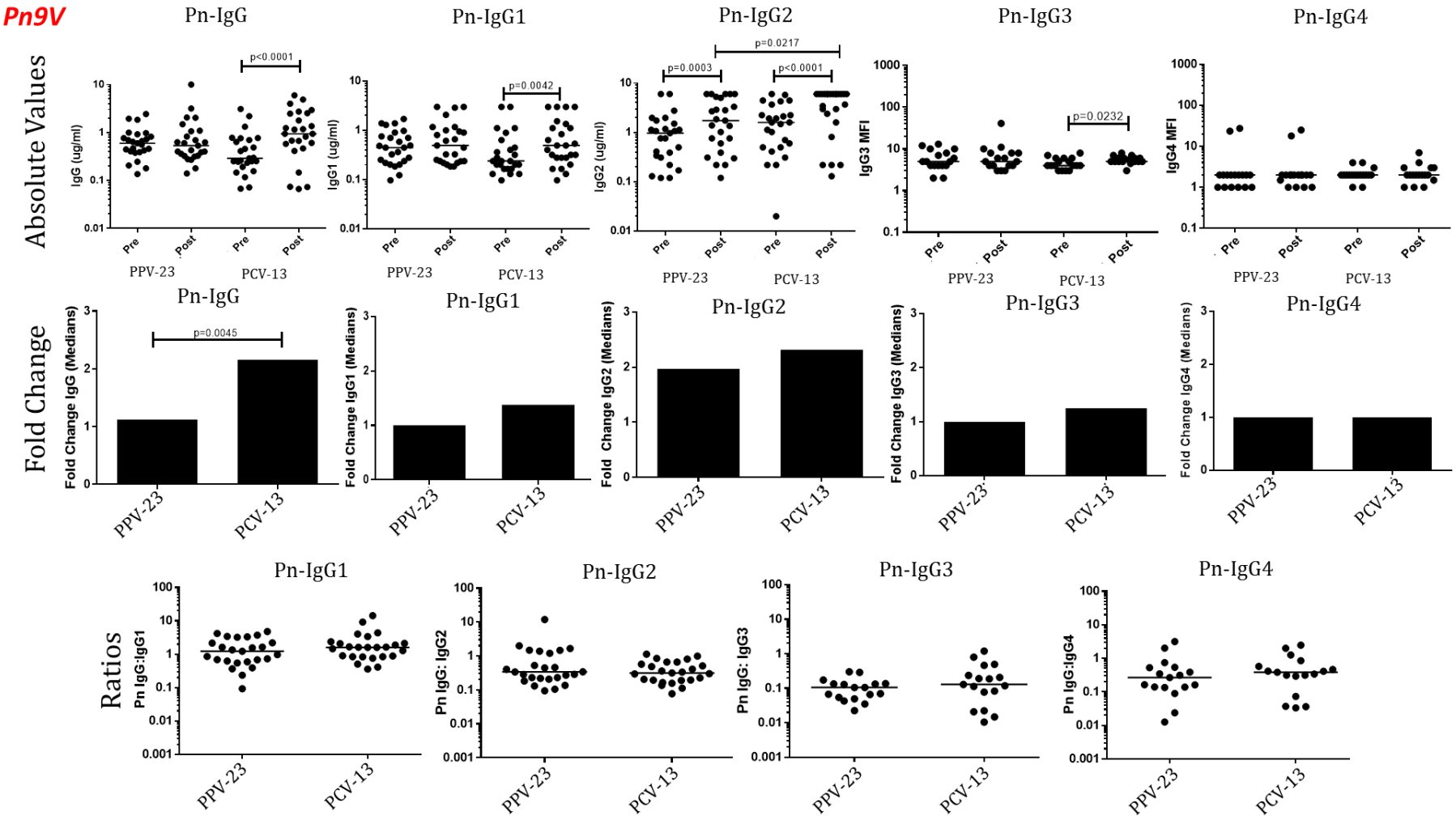


Figure 5.15. Pn-specific IgG, IgG1-4 responses to pneumococcal vaccination- Serotype 9V. *Each point represents one study participant. Solid horizontal lines indicate medians. Absolute values are represented as µg/mL. Fold changes are represented as medians. Ratios are represented as medians. P-values are significant at p<0.05.*

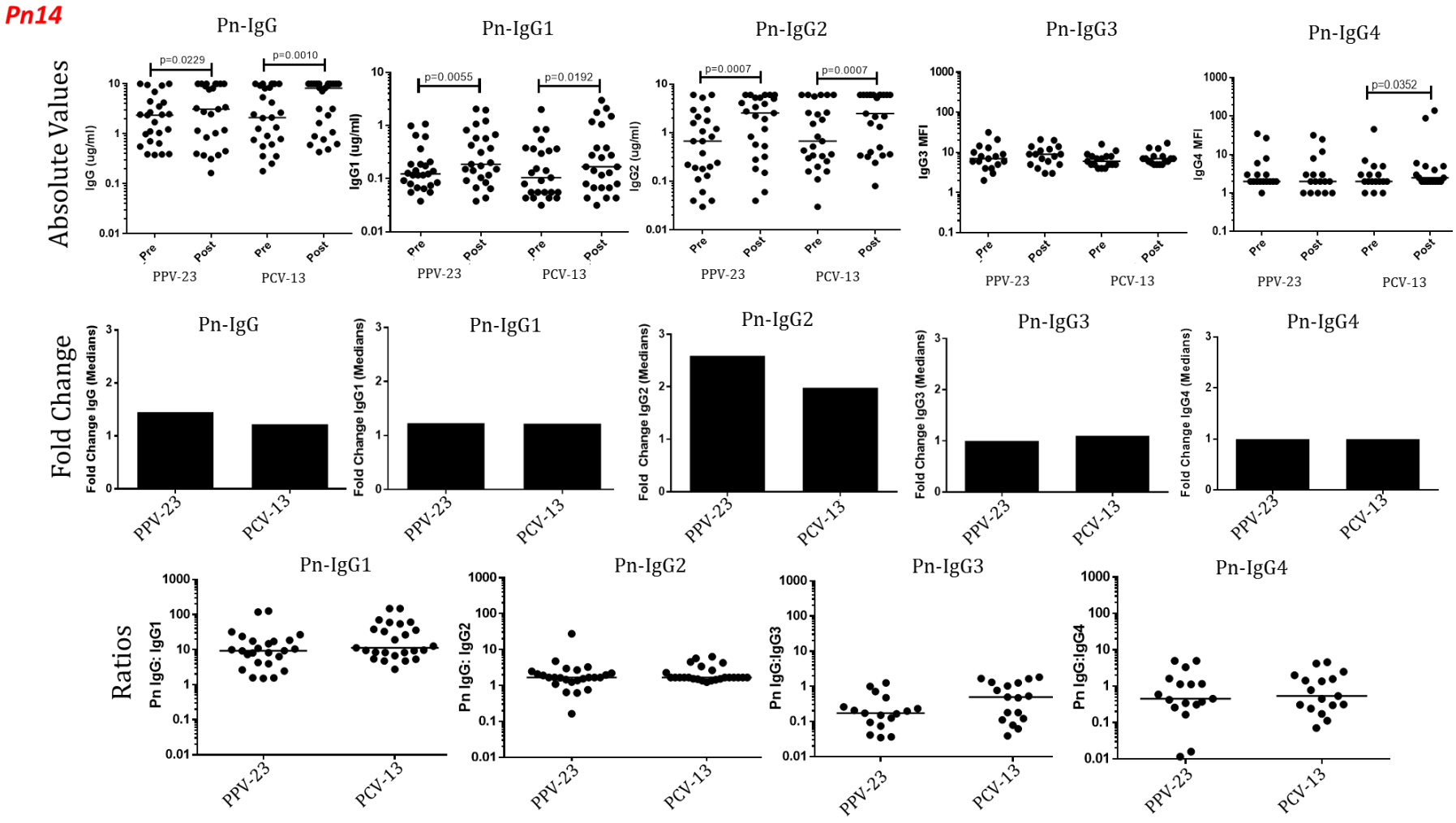


Figure 5.16. Pn-specific IgG, IgG1-4 responses to pneumococcal vaccination- Serotype 14. *Each point represents one study participant. Solid horizontal lines indicate medians. Absolute values are represented as µg/mL. Fold changes are represented as medians. Ratios are represented as medians. P-values are significant at p<0.05.*

Pn18C

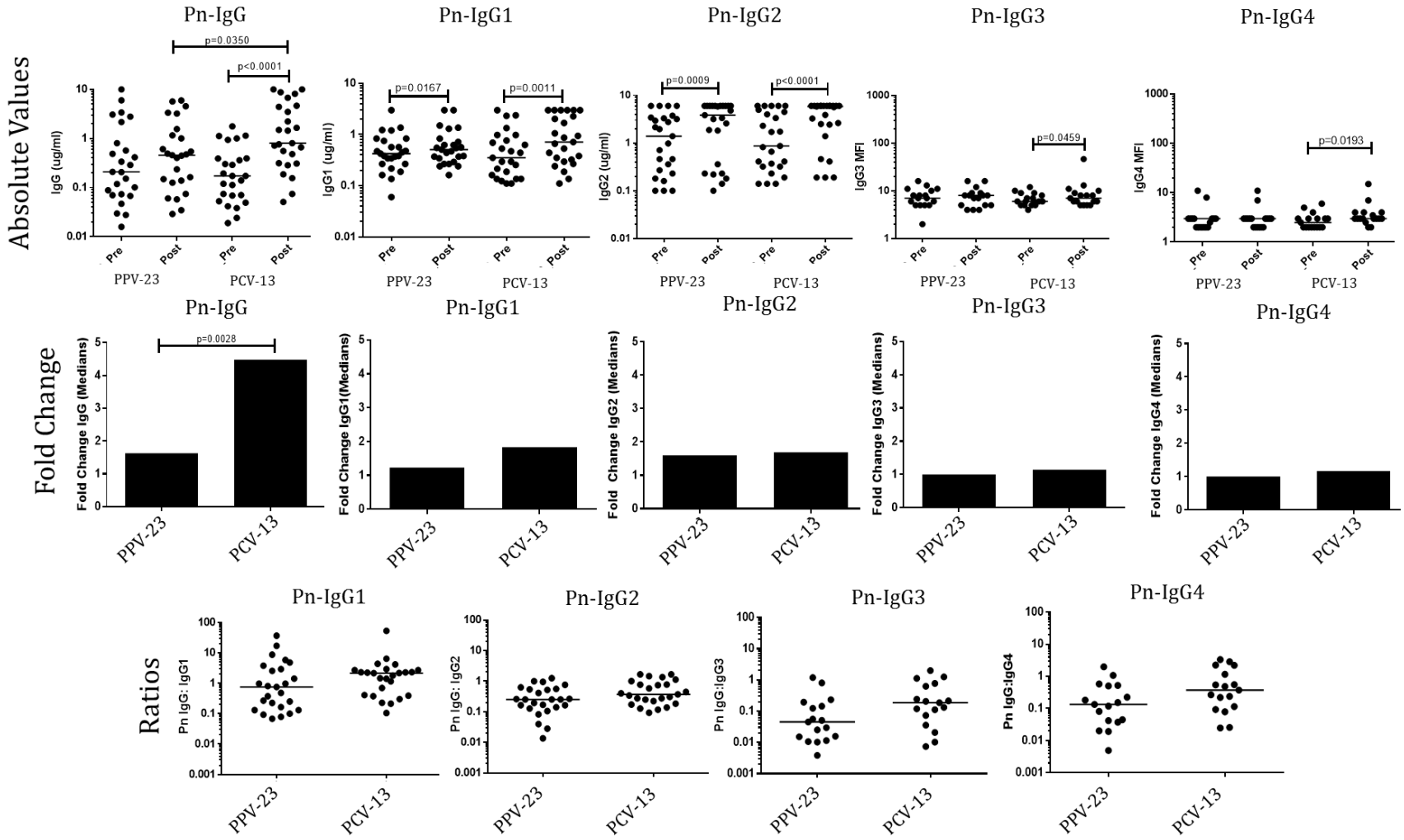


Figure 5.17. Pn-specific IgG, IgG1-4 responses to pneumococcal vaccination- Serotype 18C. *Each point represents one study participant. Solid horizontal lines indicate medians. Absolute values are represented as µg/mL. Fold changes are represented as medians. Ratios are represented as medians. P-values are significant at p<0.05.*

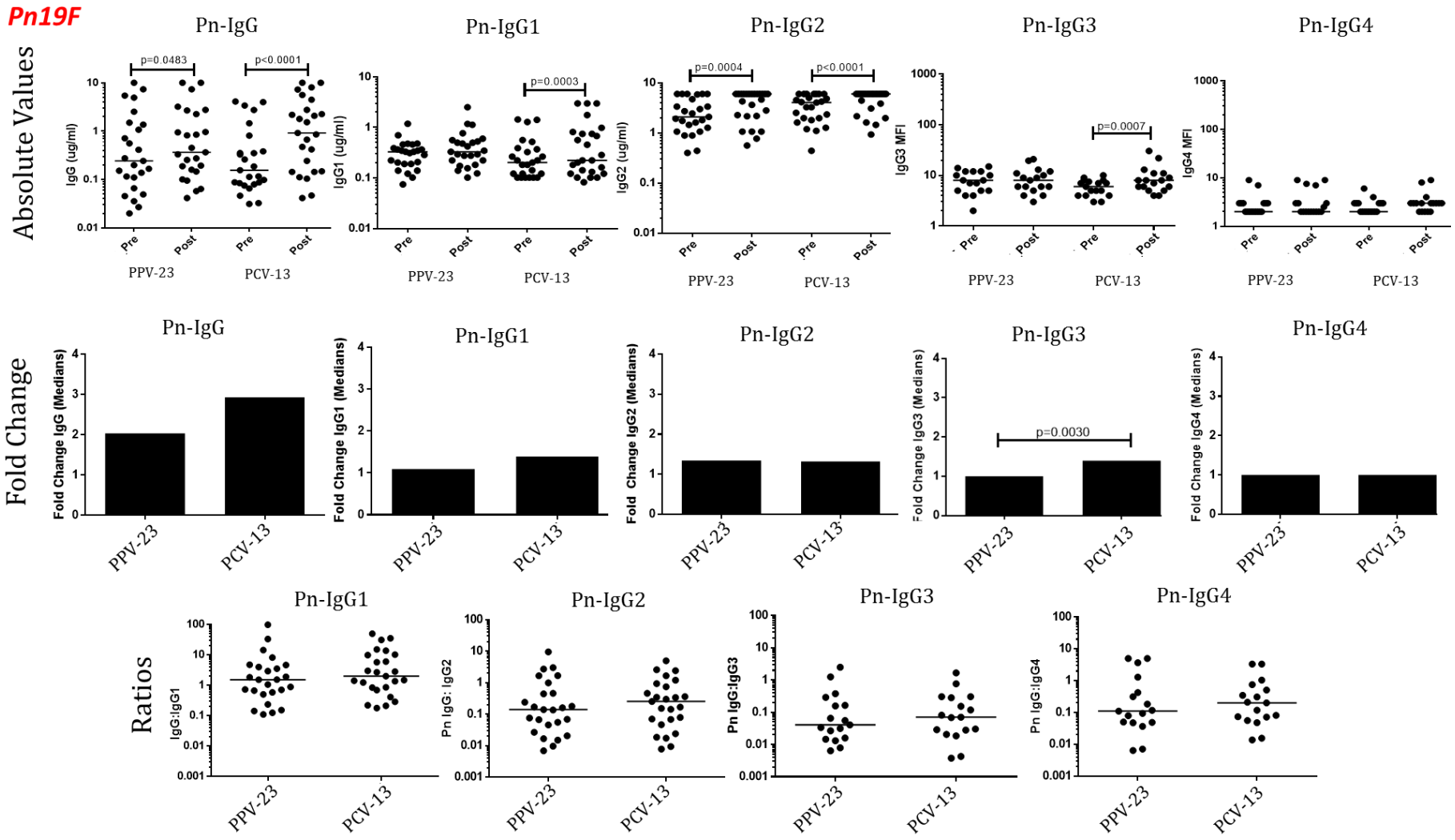


Figure 5.18. Pn-specific IgG, IgG1-4 responses to pneumococcal vaccination- Serotype 19F. *Each point represents one study participant. Solid horizontal lines indicate medians. Absolute values are represented as µg/mL. Fold changes are represented as medians. Ratios are represented as medians. P-values are significant at p<0.05.*

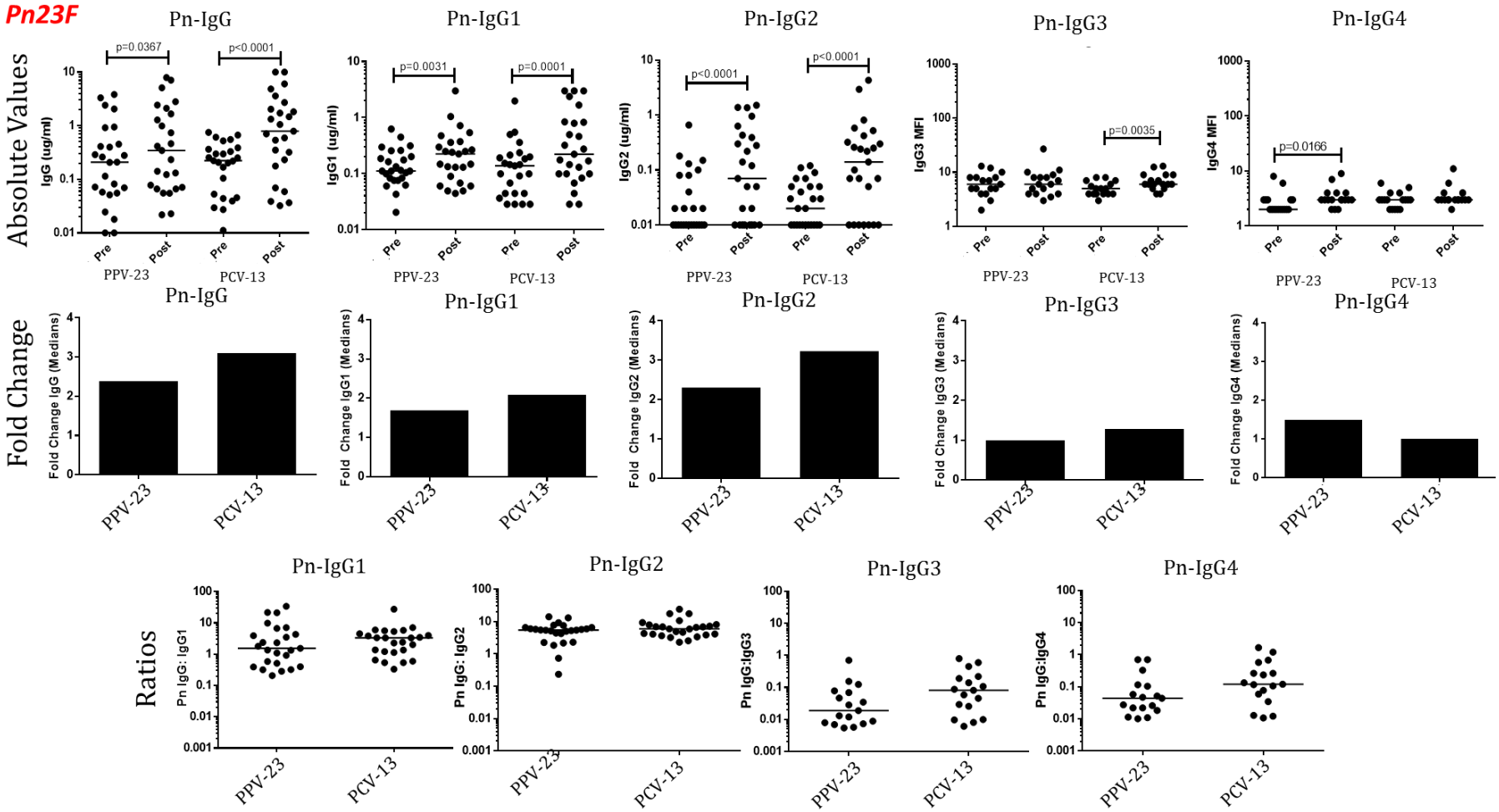


Figure 5.19. Pn-specific IgG, IgG1-4 responses to pneumococcal vaccination- Serotype 23F. *Each point represents one study participant. Solid horizontal lines indicate medians. Absolute values are represented as µg/mL. Fold changes are represented as medians. Ratios are represented as medians. P-values are significant at p<0.05.*

Table 5.4. Pn-specific IgG values pre-and post-pneumococcal vaccination – absolute IgG values (µg/mL) and fold changes.

Absolute Values Pn-IgG	Pre-Vaccine			Post-Vaccine			Pre-Post	Pre-Post	Fold Change		
Serotype	***PPV-23 (N=25)***	***PCV-13 (N=25)***	***p-Value***	***PPV-23 (N=25)***	***PCV-13 (N=25)***	***p-Value***	***PPV-23 (N=25) p-Value***	***PCV-13 (N=25) p-Value***	***PPV-23 (N=25)***	***PCV-13 (N=25)***	***p-Value***
Pn1	0.08 (0.03-0.13)	0.09 (0.05-0.19)	0.3858	0.18 (0.10-0.41)	0.93 (0.27-5.21)	***0.001***	***0.0074***	***<0.0001***	3.14 (1.12-6.30)	7.00 (2.46-31.11)	***0.0108***
Pn3	0.13 (0.07-0.19)	0.06 (0.05-0.16)	***0.0445***	0.11 (0.08-0.32)	0.19 (0.07-0.41)	0.6034	0.1817	***<0.0001***	1.44 (0.65-2.04)	2.22 (1.32-3.89)	***0.0052***
Pn4	0.07 (0.04-0.16)	0.14 (0.07-0.20)	0.1077	0.11 (0.05-0.26)	0.33 (0.16-1.81)	***0.0009***	***0.0255***	***<0.0001***	1.54 (1.08-2.55)	2.41 (1.36-9.51)	0.0686
Pn5	0.14 (0.11-0.31)	0.21 (0.08-0.46)	0.5768	0.32 (0.11-1.17)	0.70 (0.24-2.22)	***0.0518***	***0.0088***	***<0.0001***	2.34 (0.99-4.16)	2.45 (1.41-7.99)	0.1132
Pn6B	0.24 (0.12-0.65)	0.23 (0.07-00.72)	0.4297	0.29 (0.13-2.73)	0.61 (0.15-5.78)	0.2545	***0.0315***	***<0.0001***	2.03 (0.76-4.41)	3.58 (1.70-9.15)	0.0642
Pn7F	0.90 (0.53-1.26)	0.45 (0.29-0.45)	***0.045***	0.98 (0.48-1.78)	0.83 (0.41-1.99)	0.7878	0.1014	***0.0025***	1.37 (0.69-1.68)	1.33 (0.97-2.78)	0.5735
Pn9V	0.60 (0.40-0.87)	0.29 (0.21-0.73)	0.0568	0.53 (0.33-1.08)	0.94 (0.52-2.50)	***0.0869***	0.5249	***<0.0001***	1.12 (0.56-1.70)	2.16 (1.17-5.08)	***0.0045***
Pn14	2.36 (0.68-4.34)	2.13 (0.69-8.86)	0.7433	3.12 (0.65-10.00)	8.22 (0.98-10.00)	0.245	***0.0229***	***0.001***	1.45 (0.98-2.81)	1.22 (1.00-2.14)	0.8814
Pn18C	0.21 (0.07-1.49)	0.17 (0.06-0.39)	0.3548	0.46 (0.13-1.37)	0.80 (0.31-0.80)	***0.035***	0.1645	***<0.0001***	1.63 (0.69-5.50)	4.48 (2.12-20.52)	***0.0028***
Pn19F	0.24 (0.09-1.44)	0.16 (0.08-0.60)	0.4643	0.37 (0.15-0.37)	0.92 (0.15-3.62)	0.3937	***0.0483***	***<0.0001***	2.04 (0.84-6.16)	2.93 (1.37-7.60)	0.3938
Pn23F	0.21 (0.06-0.69)	0.22 (0.05-0.38)	0.769	0.35 (0.07-1.89)	0.79 (0.20-2.38)	0.2528	***0.0367***	***<0.0001***	2.39 (1.01-5.48)	3.11 (1.27-12.36)	0.1623

Data are reported as medians and interquartile ranges, with p-values from Mann-Whitney tests (unpaired) and Wilcoxon matched-pairs tests. Bold p-values are significant at p<0.05.

Table 5.5. Pn-specific IgG1 values pre-and post-pneumococcal vaccination – absolute IgG values (µg/mL) and fold changes.

Absolute Values Pn-IgG1	Pre-Vaccine			Post-Vaccine			Pre-Post	Pre-Post	Fold Change		
Serotype	*PPV-23 (N=25)*	*PCV-13 (N=25)*	*p-Value*	*PPV-23 (N=25)*	*PCV-13 (N=25)*	*p-Value*	*PPV-23 (N=25) p-Value*	*PCV-13 (N=25) p-Value*	*PPV-23 (N=25)*	*PCV-13 (N=25)*	*p-Value*
Pn1	0.04 (0.02-0.07)	0.08 (0.03-0.15)	0.0587	0.17 (0.07-0.42)	0.34 (0.15-0.97)	0.0594	***0.0001***	***<0.0001***	2.99 (1.38-6.92)	3.09 (1.54-7.32)	0.8475
Pn3	0.38 (0.24-0.60)	0.29 (0.15-0.38)	0.1425	0.45 (0.37-0.97)	0.39 (0.24-0.93)	0.2526	***0.0098***	***0.0002***	1.25 (0.93-1.74)	1.72 (1.12-2.37)	0.1774
Pn4	0.27 (0.18-0.50)	0.28 (0.12-0.86)	0.8891	0.69 (0.38-1.18)	2.12 (0.34-3.00)	0.1485	***0.0004***	***<0.0001***	2.04 (1.34-3.94)	2.91 (1.35-5.19)	0.3278
Pn5	0.29 (0.18-0.39)	0.16 (0.09-0.25)	***0.0237***	0.29 (0.18-0.69)	0.22 (0.11-0.51)	0.1396	***0.0229***	***0.006***	1.54 (0.84-1.95)	1.21 (1.00-1.59)	0.9501
Pn6B	0.21 (0.12-0.38)	0.13 (0.07-0.47)	0.3828	0.39 (0.13-0.87)	0.23 (0.12-0.95)	0.4551	***0.0004***	***0.0007***	1.52 (1.07-3.68)	1.53 (1.00-2.24)	0.5601
Pn7F	0.15 (0.10-0.36)	0.09 (0.06-0.36)	0.2771	0.26 (0.15-0.67)	0.18 (0.10-0.74)	0.5096	***0.0067***	***0.0001***	1.45 (1.12-3.75)	1.48 (1.21-2.82)	0.7473
Pn9V	0.45 (0.23-0.83)	0.24 (0.17-0.54)	0.0847	0.49 (0.24-1.09)	0.49 (0.28-1.23)	0.9885	0.0717	***0.0042***	1.00 (0.86-1.93)	1.38 (1.00-2.83)	0.2200
Pn14	0.13 (0.09-0.20)	0.10 (0.06-0.36)	0.5027	0.19 (0.10-0.63)	0.17 (0.07-0.71)	0.5667	***0.0055***	***0.0192***	1.23(0.89-2.92)	1.22 (1.00-2.13)	0.9962
Pn18C	0.42 (0.27-0.79)	0.35 (0.15-0.87)	0.5537	0.51 (0.32-0.78)	0.71 (0.31-2.64)	0.2766	***0.0167***	***0.0011***	1.23 (1.00-1.39)	1.83 (1.00-3.94)	0.1314
Pn19F	0.33 (0.19-0.43)	0.20 (0.11-0.38)	0.2101	0.33 (0.20-0.55)	0.22 (0.13-0.78)	0.6406	0.0605	***0.0003***	1.09 (0.90-1.49)	1.39 (1.00-2.10)	0.0904
Pn23F	0.11 (0.08-0.23)	0.14 (0.04-0.21)	0.6269	0.22 (0.08-0.37)	0.22 (0.10-0.83)	0.4322	***0.0031***	***0.0001***	1.69 (0.91-2.48)	2.09 (1.00-5.04)	0.1870

Data are reported as medians and interquartile ranges, with p-values from Mann-Whitney tests (unpaired) and Wilcoxon matched-pairs tests. Bold p-values are significant at p<0.05.

Table 5.6. Pn-specific IgG2 values pre-and post-pneumococcal vaccination – absolute IgG values (µg/mL) and fold changes.

Absolute Values Pn-IgG2	Pre-Vaccine			Post-Vaccine			Pre-Post	Pre-Post	Fold Change		
Serotype	*PPV-23 (N=25)*	*PCV-13 (N=25)*	*p-Value*	*PPV-23 (N=25)*	*PCV-13 (N=25)*	*p-Value*	*PPV-23 (N=25) p-Value*	*PCV-13 (N=25) p-Value*	*PPV-23 (N=25)*	*PCV-13 (N=25)*	*p-Value*
Pn1	0.03 (0.01-0.09)	0.10 (0.02-0.30)	0.0958	0.23 (0.11-0.68)	1.66 (0.21-6.00)	0.0213	***<0.0001***	***<0.0001***	5.79 (2.59-16.18)	9.38 (3.77-26.24)	0.3937
Pn3	0.03 (0.02-0.06)	0.03 (0.02-0.07)	0.8957	0.08 (0.02-0.16)	0.09 (0.04-0.27)	0.3768	0.0614	***<0.0001***	1.70 (0.52-4.01)	2.39 (1.05-5.17)	0.2606
Pn4	0.02 (0.01-0.06)	0.05 (0.03-0.09)	0.0654	0.08 (0.02-0.08)	0.20 (0.07-0.82)	0.014	***0.0003***	***<0.0001***	2.06 (1.23-4.02)	3.68 (1.48-7.35)	0.2776
Pn5	0.51 (0.36-2.99)	0.60 (0.36-2.48)	0.9885	1.81 (0.63-6.00)	1.92 (0.78-6.00)	0.864	***<0.0001***	***0.0001***	1.82 (1.02-4.67)	1.97 (1.27-3.40)	0.9424
Pn6B	0.05 (0.02-0.31)	0.10 (0.02-0.56)	0.4025	0.34 (0.05-1.95)	0.53 (0.07-5.14)	0.4422	***<0.0001***	***<0.0001***	3.25 (1.00-7.80)	3.25 (1.87-9.46)	0.6472
Pn7F	0.67 (0.13-1.45)	0.67 (0.31-1.78)	0.5032	1.85 (0.46-4.45)	1.39 (0.61-3.40)	0.8888	***<0.0001***	***0.0002***	2.47 (1.50-5.70)	2.12 (1.07-4.43)	0.2629
Pn9V	0.97 (0.33-1.63)	1.60 (0.50-2.97)	0.1246	1.74 (0.50-5.17)	6.00 (1.99-6.00)	0.0217	***0.0003***	***<0.0001***	1.97 (1.00-3.63)	2.32 (1.02-4.49)	0.5859
Pn14	0.67 (0.16-1.95)	0.67 (0.28-4.08)	0.2601	2.54 (0.43-5.25)	2.47 (0.38-6.00)	0.3439	***0.0007***	***0.0007***	2.59 (1.10-7.33)	1.98 (1.00-3.32)	0.3316
Pn18C	1.39 (0.25-3.36)	0.87 (0.32-4.72)	0.7759	3.83 (0.30-6.00)	5.83 (1.92-6.00)	0.4527	***0.0009***	***<0.0001***	1.60 (1.00-2.69)	1.69 (1.00-5.14)	0.4782
Pn19F	2.09 (1.17-5.31)	4.04 (1.89-6.00)	0.1345	6.00 (2.17-6.00)	6.00 (3.47-6.00)	0.2367	***0.0004***	***<0.0001***	1.34 (1.01-2.12)	1.32 (1.00-1.77)	0.5510
Pn23F	0.01 (0.01-0.08)	0.02 (0.01-0.05)	0.85	0.07 (0.01-0.41)	0.14 (0.01-0.37)	0.6926	***<0.0001***	***<0.0001***	2.31 (1.00-8.14)	3.23 (1.03-23.40)	0.2638

Data are reported as medians and interquartile ranges, with p-values from Mann-Whitney tests (unpaired) and Wilcoxon matched-pairs tests. Bold p-values are significant at p<0.05.

Table 5.7. Pn-specific IgG3 values pre-and post-pneumococcal vaccination – absolute IgG values (µg/mL) and fold changes.

Absolute Values Pn-IgG3	Pre-Vaccine			Post-Vaccine			Pre-Post	Pre-Post	Fold Change		
Serotype	*PPV-23 (N=25)*	*PCV-13 (N=25)*	*p-Value*	*PPV-23 (N=25)*	*PCV-13 (N=25)*	*p-Value*	*PPV-23 (N=25) p-Value*	*PCV-13 (N=25) p-Value*	*PPV-23 (N=25)*	*PCV-13 (N=25)*	*p-Value*
Pn1	5.0 (3.00-15.50)	6.00 (4.75-9.00)	0.6633	9.00 (6.00-18.75)	14.00 (10.00-27.50)	0.1720	***0.0011***	***<0.0001***	1.33 (1.25-2.23)	1.80 (1.33-3.75)	0.1675
Pn3	7.00 (5.25-12.50)	5.00 (4.50-7.50)	0.0842	10.00 (5.00-16.00)	7.00 (5.00-9.00)	0.2678	0.3166	0.1147	1.06 (0.84-2.03)	1.25 (0.90-1.75)	0.8443
Pn4	5.00 (3.00-10.00)	7.00 (4.50-14.00)	0.1548	6.00 (4.50-12.50)	13.00 (7.75-26.50)	***0.0230***	***0.0204***	***0.0024***	1.25 (1.00-1.75)	1.60 (1.00-2.12)	0.3342
Pn5	5.00 (4.50-9.50)	5.00 (4.00-6.00)	0.4723	5.00 (4.00-10.00)	5.00 (4.50-6.00)	0.8821	0.8879	0.5049	1.00 (0.80-1.27)	1.00 (0.93-1.25)	0.4645
Pn6B	5.00 (3.50-8.50)	4.00 (4.00-6.50)	0.5966	6.00 (4.00-11.00)	7.00 (5.00-7.50)	0.9249	***0.0317***	***0.0002***	1.25 (0.95-1.61)	1.25 (1.07-1.50)	0.5782
Pn7F	4.00 (4.00-9.00)	5.00 (3.50-6.00)	0.5971	5.00 (4.00-10.00)	6.00 (4.50-8.50)	0.7003	0.1176	***0.0038***	1.05 (0.89-1.42)	1.20 (1.00-1.50)	0.3692
Pn9V	5.00 (4.00-9.00)	4.00 (3.50-6.00)	0.1994	5.00 (4.00-8.00)	5.00 (5.00-6.00)	0.9101	0.5605	***0.0232***	1.00 (0.93-1.38)	1.25 (1.00-1.50)	0.1648
Pn14	7.00 (4.50-11.50)	6.00 (5.00-8.00)	0.4983	9.00 (5.00-13.00)	7.00 (5.25-8.00)	0.3961	0.8398	0.1583	1.00 (0.84-1.16)	1.10 (0.94-1.23)	0.2608
Pn18C	7.00 (5.00-10.50)	6.00 (5.00-8.50)	0.4222	8.00 (5.00-9.00)	7.00 (5.50-9.50)	0.8040	0.8223	***0.0459***	1.00 (0.80-1.26)	1.14 (1.00-1.50)	0.0793
Pn19F	8.00 (5.00-12.00)	6.00 (4.00-7.75)	0.0911	8.00 (5.50-11.50)	8.00 (5.50-11.00)	0.9659	0.7112	***0.0007***	1.00 (0.85-1.16)	1.40 (1.20-1.69)	***0.0030***
Pn23F	6.00 (4.50-8.00)	5.00 (4.00-6.50)	0.1511	6.00 (4.00-8.50)	6.00 (5.50-9.00)	0.6366	0.5713	***0.0035***	1.00 (0.84-1.29)	1.29(1.00-1.57)	0.0759

Data are reported as medians and interquartile ranges, with p-values from Mann-Whitney tests (unpaired) and Wilcoxon matched-pairs tests. Bold p-values are significant at p<0.05.

Table 5.8. Pn-specific IgG4 values pre-and post-pneumococcal vaccination – absolute IgG values (µg/mL) and fold changes.

Absolute Values Pn-IgG4	Pre-Vaccine			Post-Vaccine			Pre-Post	Pre-Post	Fold Change		
Serotype	*PPV-23 (N=25)*	*PCV-13 (N=25)*	*p-Value*	*PPV-23 (N=25)*	*PCV-13 (N=25)*	*p-Value*	*PPV-23 (N=25) p-Value*	*PCV-13 (N=25) p-Value*	*PPV-23 (N=25)*	*PCV-13 (N=25)*	*p-Value*
Pn1	1.00 (1.00-1.50)	1.00 (1.00-1.00)	0.1969	1.00 (1.00-5.00)	2.00 (1.00-8.50)	0.3337	***0.0156***	***0.0258***	1.00 (1.00-2.25)	2.00 (0.75-4.98)	0.5247
Pn3	1.00 (1.00-1.00)	1.00 (1.00-2.00)	0.557	1.00 (1.00-3.00)	1.00 (1.00-2.00)	0.3801	0.1172	>0.9999	1.00 (1.00-2.00)	1.00 (0.58-1.50)	0.1021
Pn4	1.00 (1.00-2.00)	1.00 (1.00-2.00)	0.3217	1.00 (1.00-3.50)	2.00 (1.00-3.00)	0.2634	0.2656	0.0781	1.00 (1.00-1.67)	1.00 (1.00-2.25)	0.4397
Pn5	1.00 (1.00-1.50)	1.00 (1.00-2.00)	0.2565	1.00 (1.00-2.00)	1.00 (1.00-2.00)	0.9824	0.75	0.2734	1.00 (1.00-1.00)	1.00 (0.50-1.00)	0.1547
Pn6B	1.00 (1.00-2.00)	1.00 (1.00-2.00)	0.9961	1.00 (1.00-5.50)	1.00 (1.00-2.00)	0.4442	0.1172	0.585	1.00 (1.00-1.75)	1.00 (0.83-1.75)	0.4186
Pn7F	2.00 (1.25-2.00)	2.00 (1.50-3.00)	0.6405	1.00 (1.00-2.00)	2.00 (1.50-2.00)	0.9637	0.8906	0.2813	1.00 (0.83-1.00)	1.00 (0.83-1.00)	0.9894
Pn9V	2.00 (1.00-2.00)	2.00 (2.00-2.00)	0.2524	2.00 (1.25-2.00)	2.00 (1.75-3.00)	0.4478	0.8906	0.8606	1.00 (0.83-1.75)	1.00 (0.58-1.75)	0.9408
Pn14	2.00 (2.00-4.50)	2.00 (2.00-4.00)	0.6441	2.00 (1.00-5.50)	2.50 (2.00-5.00)	0.2483	0.7041	***0.0352***	1.00 (0.58-1.09)	1.00 (1.00-2.00)	***0.0159***
Pn18C	3.00 (2.00-3.00)	2.50 (2.00-3.00)	0.8005	3.00 (2.00-3.00)	3.00 (3.00-4.00)	0.1051	0.875	***0.0193***	1.00 (1.00-1.00)	1.17 (1.00-1.50)	0.1544
Pn19F	2.00 (2.00-3.00)	2.00 (2.00-3.00)	0.9147	2.00 (2.00-5.00)	3.00 (2.00-3.00)	0.3561	0.2109	0.1992	1.00 (1.00-1.39)	1.00 (1.00-1.50)	0.6112
Pn23F	2.00 (2.00-3.00)	3.00 (2.00-3.50)	0.1364	3.00 (3.00-4.00)	3.00 (3.00-4.00)	0.6793	***0.0166***	0.1851	1.50 (1.00-2.00)	1.00 (1.00-1.67)	0.5339

Data are reported as medians and interquartile ranges, with p-values from Mann-Whitney tests (unpaired) and Wilcoxon matched-pairs tests. Bold p-values are significant at p<0.05.

Table 5.9. Pn-specific IgG:IgG subclass values post-pneumococcal vaccination

Pn IgG:IgG1 ratio	Post-Vaccine		
Serotype	*PPV-23 (N=25)*	*PCV-13 (N=25)*	*p-Value*
Pn1	1.47 (0.74-2.26)	2.32 (1.23-5.92)	***0.0170***
Pn3	0.24 (0.13-0.35)	0.43 (0.22-0.82)	***0.0028***
Pn4	0.17 (0.12-0.27)	0.55 (0.20-1.03)	***0.0011***
Pn5	0.95 (0.38-1.33)	2.56 (1.34-5.37)	***0.0014***
Pn6B	1.32 (0.65-2.17)	2.94 (0.78-5.32)	0.0614
Pn7F	3.09 (1.44-6.83)	2.99 (1.45-5.29)	0.9693
Pn9V	1.23 (0.61-2.73)	1.61 (0.85-2.26)	0.3067
Pn14	9.33 (4.12-18.04)	11.22 (7.50-46.07)	0.1068
Pn18C	0.76 (0.15-3.37)	2.17(0.40-2.72)	0.1823
Pn19F	1.51 (0.54-4.29)	1.99 (0.77-9.92)	0.3159
Pn23F	1.55 (0.46-5.51)	3.33 (1.18-1.95)	0.3727

Pn IgG:IgG2 ratio	Post-Vaccine		
Serotype	*PPV-23 (N=25)*	*PCV-13 (N=25)*	*p-Value*
Pn1	0.85 (0.46-1.18)	0.90 (0.75-1.67)	0.1885
Pn3	1.91 (0.91-2.51)	1.76 (1.13-2.84)	0.773
Pn4	1.37 (1.14-2.41)	2.18 (1.66-3.05)	0.0518
Pn5	0.21 (0.12-0.33)	0.38 (0.22-0.65)	***0.0059***
Pn6B	1.71 (0.97-4.20)	1.67 (1.31-2.27)	0.9234
Pn7F	0.61 (0.35-1.19)	0.72 (0.43-1.04)	0.8626
Pn9V	0.34 (0.21-1.30)	0.32 (0.19-0.62)	0.5004
Pn14	1.67 (1.32-2.34)	1.67 (1.54-2.46)	0.5454
Pn18C	0.25 (0.13-0.55)	0.37 (0.20-0.89)	0.0643
Pn19F	0.14 (0.04-0.73)	0.26 (0.06-0.84)	0.5802
Pn23F	5.47 (3.30-6.61)	6.00 (4.01-8.05)	0.2799

Pn IgG:IgG3 ratio	Post-Vaccine		
Serotype	*PPV-23 (N=17)*	*PCV-13 (N=17)*	*p-Value*
Pn1	0.02 (0.01-0.03)	0.03 (0.01-0.22)	0.2181
Pn3	0.02 (0.01-0.02)	0.01 (0.01-0.03)	0.6098
Pn4	0.01 (0.01-0.02)	0.02 (0.01-0.06)	0.0529
Pn5	0.04 (0.02-0.10)	0.11 (0.04-0.30)	***0.041***
Pn6B	0.07 (0.03-0.40)	0.05 (0.02-0.15)	0.8651
Pn7F	0.15 (0.08-0.25)	0.09 (0.05-0.28)	0.394
Pn9V	0.11 (0.05-0.14)	0.13 (0.05-0.35)	0.3223
Pn14	0.17 (0.08-0.37)	0.49 (0.12-1.28)	0.0933
Pn18C	0.05 (0.01-0.17)	0.19 (0.05-0.70)	0.0675
Pn19F	0.04 (0.02-0.23)	0.07 (0.02-0.29)	0.7339
Pn23F	0.02 (0.01-0.07)	0.08 (0.02-0.21)	0.0623

Pn IgG:IgG4 ratio	Post-Vaccine		
Serotype	*PPV-23 (N=17)*	*PCV-13 (N=17)*	*p-Value*
Pn1	0.11 (0.4-0.19)	0.21 (0.04-0.75)	0.3801
Pn3	0.09 (0.05-0.16)	0.06 (0.03-0.21)	0.2719
Pn4	0.04 (0.02-0.12)	0.12 (0.07-0.22)	***0.0216***
Pn5	0.22 (0.05-0.43)	0.42 (0.12-1.44)	0.1329
Pn6B	0.29 (0.10-1.03)	0.37 (0.13-1.54)	0.8919
Pn7F	0.56 (0.14-1.06)	0.48 (0.17-0.97)	0.8651
Pn9V	0.27 (0.14-0.54)	0.39 (0.18-0.71)	0.4536
Pn14	0.45 (0.29-1.39)	0.54 (0.27-1.79)	0.9382
Pn18C	0.13(0.04-0.51)	0.38 (0.10-1.68)	0.0730
Pn19F	0.11 (0.05-0.86)	0.20 (0.06-0.63)	0.7277
Pn23F	0.04 (0.02-0.11)	0.12 (0.05-0.42)	0.0575

Data are reported as medians and interquartile ranges, with values from Mann-Whitney tests (unpaired). Bold p-values are significant at p<0.05.

5.3.4. Pneumococcal serotypes versus novel less complex 'whole' pneumococcal assays

Pneumococcal serotype assays offer information about the breadth of the vaccine response. The downside to pneumococcal assays is that they are expensive and take a considerable amount of laboratory staff time to complete in terms of routine clinical testing. There is also the additional complexity of reporting and interpreting multi-parameter results for clinicians.

Whole PCP IgG assays are available for ELISAs, but not for MIAs. Therefore, we decided to conjugate both PPV-23 and PCV-13 to Luminex beads in order to compare patient antibody concentrations to the existing Pn serotype assays in two novel whole vaccine bead assays.

5.3.4.1. Whole vaccine bead assays compared to 8/12 Pn serotypes as gold standard (WHO)

When comparing the whole PPV-23 and PCV-13 beads with the Pn serotype data, ROC curve analysis for the prediction of patients who reach threshold for the Pn-serotype specific IgG assay (WHO) returned an area under the curve of 0.68 ($p<0.0001$) against the whole PPV-23 bead (Figure 5.20) and 0.87 ($P<0.0001$) against the whole PCV-13 bead (Figure 5.21).

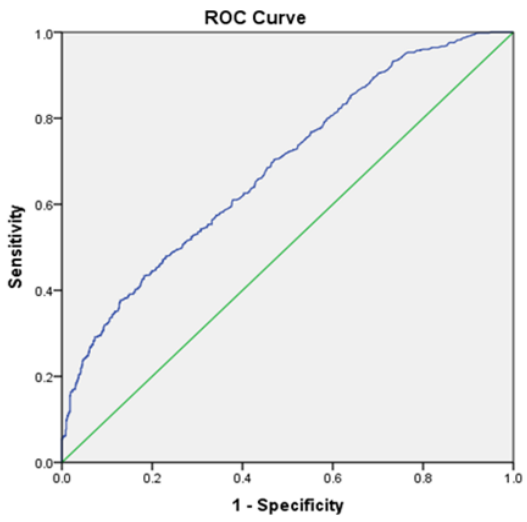


Figure 5.20. ROC curve for PPV-23 whole vaccine beads as predictive of 8/12 Pn serotype (WHO) response.

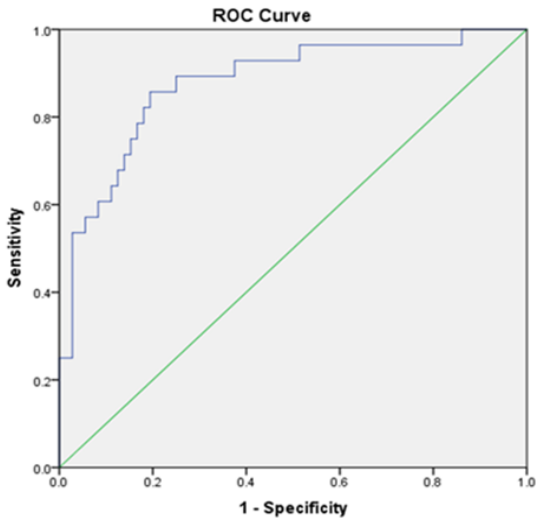


Figure 5.21. ROC curve for PCV-13 whole vaccine bead as predictive of 8/12 Pn serotype (WHO) response.

For the PPV-23 whole vaccine bead, if a measured patient sample achieved a response of 309.5 MFI, this was considered indicative of a predictive response to 8/12 Pn serotypes with a sensitivity of 43% and specificity of 82%. For the PCV-13 whole vaccine bead, if a measured patient sample achieved a response of 1307.5 MFI, this was considered indicative

of a predictive response to 8/12 Pn serotypes with a sensitivity of 85.7% and specificity of 80.6%.

We then decided to investigate if there were any differences post-vaccination according to type of pneumococcal vaccine administered. We found that the PCV-13 whole vaccine bead is better at predicting protection to 8/12 Pn serotypes post-PPV-23 and PCV-13 vaccination. The area under the curve for the PPV- 23 whole vaccine bead was 0.619 and 0.825 for the PCV-13 whole vaccine bead post-PPV-23 vaccination (Figure 5.22). The area under the curve for the PPV-23 whole vaccine bead was 0.731 and 0.870 for the PCV-13 whole vaccine bead post-PCV-13 vaccination. (Figure 5.23).

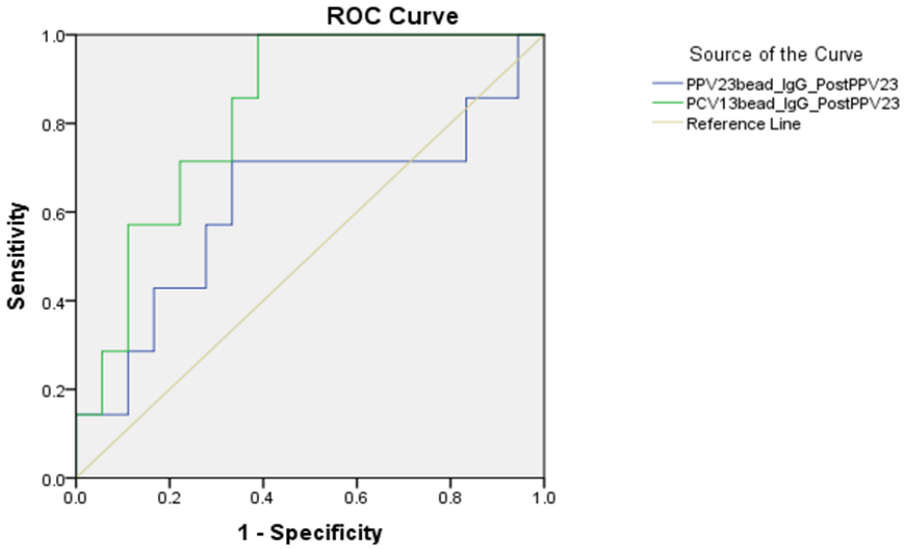


Figure 5.22. ROC curves for PPV-23 and PCV-13 whole vaccine beads predicting protection against 8/12 Pn serotypes post- PPV-23 vaccination.

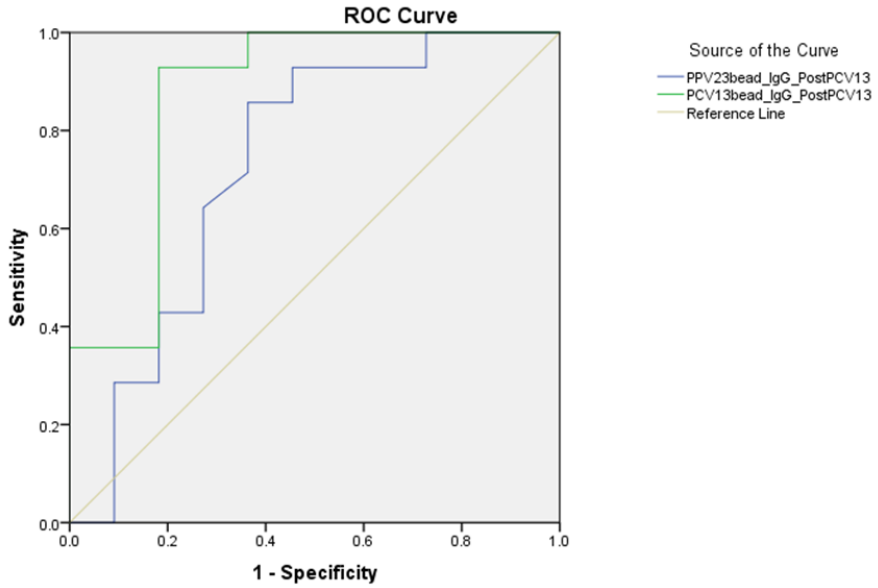


Figure 5.23. ROC curves for PPV-23 and PCV-13 whole vaccine beads predicting protection against 8/12 Pn serotypes post-PCV-13 vaccination.

5.3.5. Comparison of novel whole vaccine bead assays with the Binding-Site PCP-IgG assay

For Pn-specific IgG serotype assays, protection is deemed significant when two-thirds of measured serotypes reach the WHO threshold (179). The Binding Site anti-PCP IgG Assay is an ELISA using a polyclonal secondary IgG antibody against 23 different serotypes found in PPV-23. When the Pn serotype data was compared with the whole PCP-IgG (Binding Site) assay, 36.4% of patients that were deemed protected by the PCP-IgG assay, had a significant number of serotypes that failed to reach threshold (Figure 5.24).

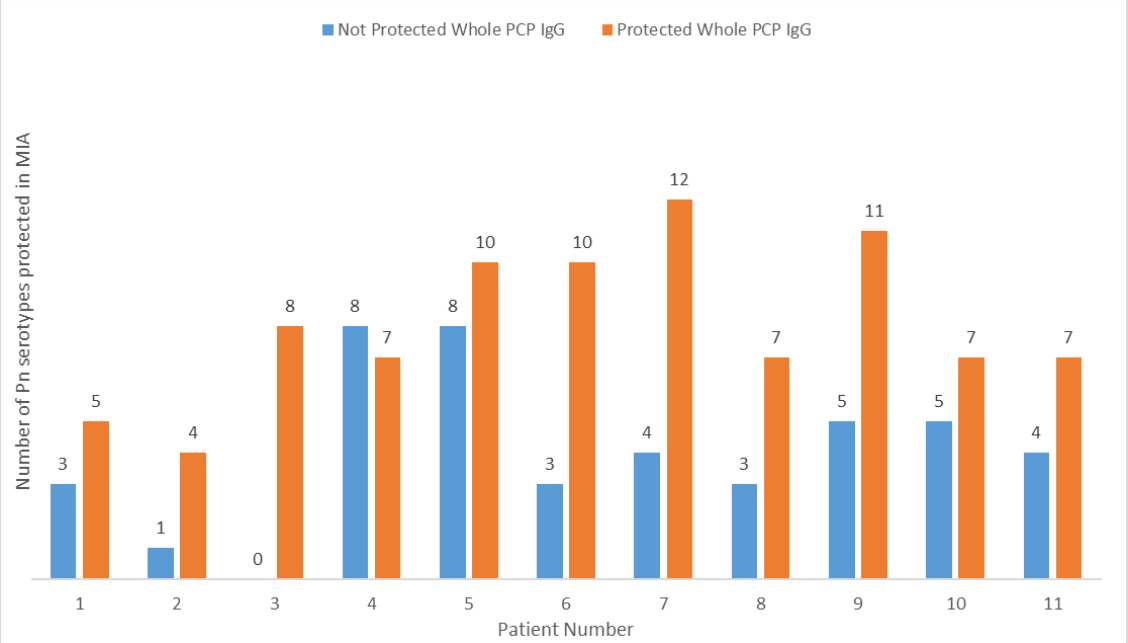


Figure 5.24. Number of Pn serotypes protected in MIA compared with protection in the whole PCP-IgG Binding Site Assay.

5.3.6. Comparison of the Pn-IgG2 MIA with the Binding-Site PCP-IgG2 assay

For the PCP-IgG2 (Binding Site) assay (210), there were no post-vaccine differences between the PPV-23 and PCV-13 cohorts similar to the Pn-IgG2 MIA data (Figure 5.25). There were no differences between the total anti-PCP IgG2 produced post-vaccination; however, patients that received a first dose of PPV-23 initially produced a greater amount of IgG2 than patients that received a first dose of PCV-13 (Figure 5.26).

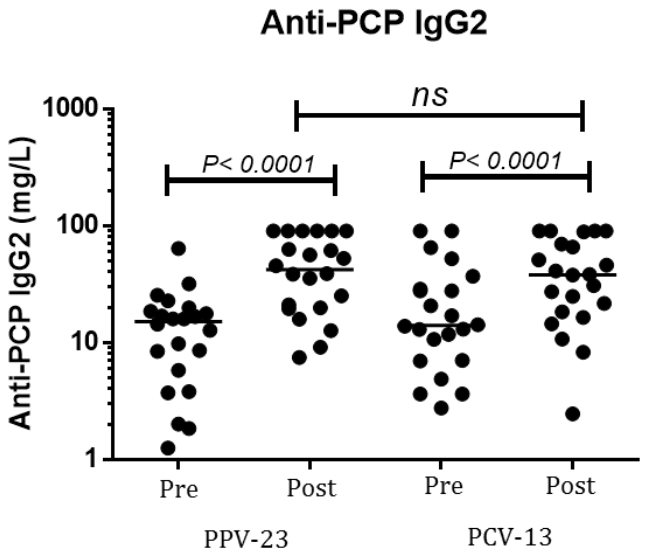


Figure 5.25. Anti-PCP IgG2 titres (Binding Site) for a single dose of PPV-23 or PCV-13 vaccination. *For post-vaccine values, Mann-Whitney tests (non-parametric, two-tailed) P<0.05 is significant. For pre-post vaccine values, Wilcoxon matched-pairs test (non-parametric, two-tailed), P<0.05 is significant.*

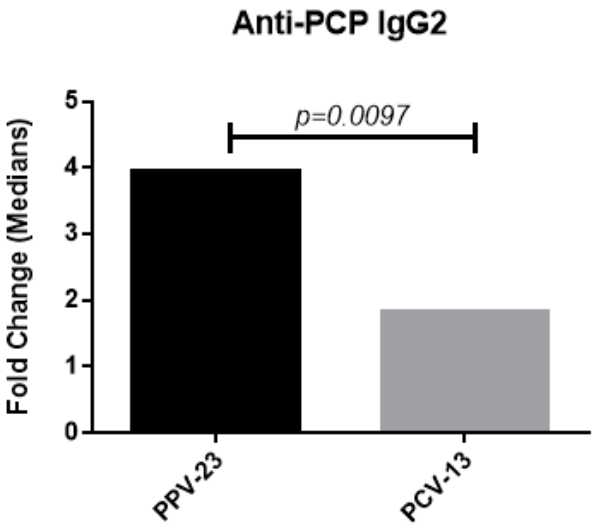


Figure 5.26. Fold changes for anti-PCP IgG2 titres (Binding Site) for a single dose of PPV-23 or PCV-13 vaccination. *Mann-Whitney tests (non-parametric, two-tailed) P<0.05 is significant.*

Furthermore, we investigated fold changes for our Pn-IgG2 assay and compared it with the Binding Site anti-PCP IgG2 assay (Figure 5.27). We found that the Pn-specific IgG2 response in the MIA varied widely (1.34-9.38 median fold change) (Table 5.6) between Pn serotypes and vaccine cohorts. These differences cannot be seen in the whole PCP-IgG2 assay (anti-PCP IgG2 (PPV-23 (pre-vaccine (15.09 mg/L (5.27-18.83 IQR)) and post-Vaccine (42.02 mg/L (19.72-90.00 IQR)); median fold change 3.95 (1.96-5.75 IQR))) and PCV-13 (pre-vaccine (13.99 mg/L (6.98-30.68 IQR) and post-vaccine (37.88 mg/L (17.81-74.20 IQR); median fold change 1.82 (1.16-3.35 IQR))). Pn1-IgG2 produced the greatest fold change in both vaccine cohorts (PPV-23 (5.79 median) and PCV-13 (9.38 median)). A greater than 4-fold difference was produced for both in the MIA assay, which was potentially masked in the anti-PCP IgG2 Binding Site assay.

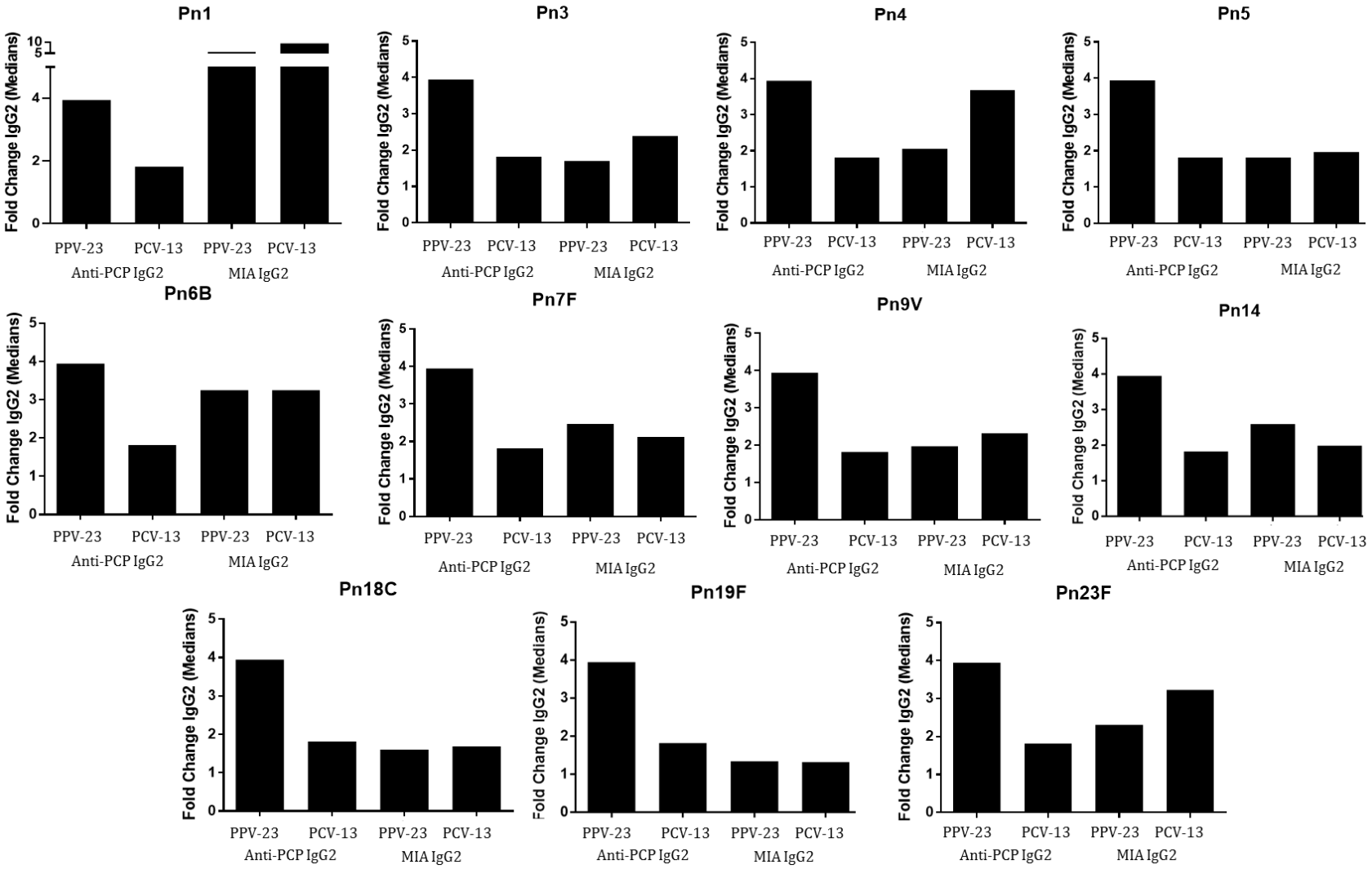


Figure 5.27. Comparison of fold changes for anti-PCP IgG2 (Binding Site) and MIA Pn-IgG2 assays for a single dose of PPV-23 or PCV-13 vaccination. *Mann-Whitney tests (non-parametric, two-tailed) P<0.05 is significant.*

5.4. Discussion

This study has demonstrated differences in IgG subclass responses to PPV-23 and PCV-13 vaccines. Furthermore, it challenges previous thinking that a plain polysaccharide response is predominantly IgG2 and that a conjugate vaccine response is predominantly IgG1. This is a really interesting notion as previously, studies have shown that the response to pneumococcal polysaccharides and other carbohydrate epitopes are notoriously, IgG2-directed (236,237). Our results from the PPV-23 vaccine cohort suggest that there is still a strong IgG1 response in addition to an IgG2 response. Von Gunten et. al. previously reported similar results suggesting that the IgG response is composed of a wide array of anti-carbohydrate antibodies that are not only restricted to the IgG2 subclass response (238). Furthermore, it has been suggested that $CD4^+$ T-cells can provide help in generating carbohydrate-specific antibodies following glycoconjugate vaccination (239). Avci et al. established that the carbohydrate portion of glyconjugate vaccines can bind MHC-Class II to produce cytokines including IL-4 and IL-2, which are involved in the maturation of B-cells and the subsequent generation of carbohydrate-specific IgG antibodies (240).

Investigating IgG subclass antibody responses is important to understanding the immune response to Pn vaccination because Pn-specific antibodies can bind pathogens with a high degree of specificity and form immune complexes, which activate mechanisms downstream of antigen binding that lead to subsequent removal from the immune system (241).

Additionally, IgG1 and IgG3 antibodies are robust classical route complement activators, whereas IgG2 and IgG4 do not bind C1q as effectively (242–244). This could be investigated further by comparing Pn-specific IgG subclass serum antibody concentrations (post fractioning of the IgG subclasses from patient serum) to opsonophagocytic killing concentrations to their specific Pn serotypes in a future study. Furthermore, it has been postulated that the long hinge region of the IgG3 subclass allows for a more exposed C1q binding site, thus allowing for more effective activation of complement in the immune response (245,246).

It is important to highlight that the success of glyconjugate vaccines is dependent on the specific polysaccharide structure and the carrier protein that it is attached to i.e. pneumococcal polysaccharides conjugated to CRM_{197} in PCV-13. Previous studies have suggested that antigen processing of CRM_{197} is altered depending on the specific Pn serotype and its structure. Furthermore, the binding of the Pn polysaccharide to CRM_{197} can

alter the exposed epitopes produced if the proteolysis or MHC-binding of CRM_{197} is compromised (247,248).

This study has also highlighted the variety of assays available for the investigation of Pn-IgG and IgG subclass responses.

Additionally, the serotype-specific assays demonstrate that responses to individual serotypes are very different for both PPV-23 and PCV-13 vaccines and that this granularity cannot be visualised in combined serotype assays.

Chapter 6: Impact of HIV infection on B-cell populations following conjugate pneumococcal vaccination

6.1 Introduction

6.1.1 Aims and Objectives

The aim of this chapter is to examine the effect of pneumococcal conjugate (PCV-13) vaccination on B-cell populations in the context of HIV-infection.

1) This will be investigated by examining B- and T-cell populations, total immunoglobulins (IgG, IgA, and IgM), and Pn-specific IgG antibodies pre-and post-vaccination (day 7 and 30).

6.1.2. Dysregulated humoral immunity in HIV infection

HIV infects $CD4^+$T-cells, primarily (249). However, with induction of $CD8^+$T-cells that neutralise HIV, $CD4^+$T-cell counts can be preserved with the usage of ARVs. Conversely, without ARV treatment, HIV can lead to the infection and depletion of $CD4^+$T-cells, which results in subsequent opportunistic infections, immunodeficiency, AIDS, and ultimately, death (250).

HIV infection is also known to have an impact on other lymphocytes, particularly, B-cells (251,252). Hypergammaglobulinaemia caused by aberrant polyclonal B-cell activation, autoimmunity, and B-cell malignancy are very well characterised in HIV-infection (111,253,254).

HIV has been known to interact with B –cells. The gp120 (glycoprotein) on the HIV virus can bind to variable region three (VH3) immunoglobulin genes that are expressed on specific B-cell populations (approximately half of VH-expressing cells in peripheral B-cell populations) (255–259). VH3 antibodies are essential for immune defence against bacterial and viral pathogens, most notably, *S. pneumoniae* (260–264). The binding of gp120 to VH3-expressing B-cells leads to B-cell clonal expansion and resulting hypergammaglobulinaemia in HIV-infected patients (265). Furthermore, these cell populations are proven to be depleted in later stages of HIV-infection (266). The subsequent depletion of these VH3-expressing cells may

contribute to dysregulated B-cell maturation, which may lead to decreased memory B-cell populations in HIV-infected individuals (267).

Additionally, HIV can interact with B-cells through the complement receptor, CD21, which is expressed on the majority of B-cells, and complement proteins bound to opsonised HIV viruses. These events can lead to subsequent infection of $CD4^+$T-cells and altered B-cell responses and ultimately, humoral dysregulation (268–270).

Hyperactivation is also common in HIV infection and increased antibody secretion can be due to hyperactivated naïve B-cells, as well as clonally expanded CD21 low B-cell populations including activated, mature activated, and exhausted B-cells (see Table 7.1) (106,271,272). Furthermore, CD21 low B-cell populations have been known to produce autoreactive antibodies, which has been evidenced in HIV-infection (273,274). Autoreactive antibodies are defined as antibodies that are produced in response to self-antigens (275).

Furthermore, circulating peripheral B-cells seen in HIV-infection are prone to abberant proliferation and hyperactivation (276). Furthermore, HIV infection predisposes B-cells to terminal differentiation, which in patients with high viral loads, can lead to increased cell death/apoptosis (277).

Chronic inflammation and hyperactivation can lead to B-cell exhaustion (CD27 lowCD21 low subpopulations) (117,273). $CD27^+$CD21 low B-cells are prominent in HIV infection and are a result of chronic activation and differentiation into plasmablasts (113). $CD10^+$CD21 low (immature transitional) B-cells are also a key feature of HIV infection, which results due to increased T-cell reduction in viraemic individuals (118).

Exhausted B-cells phenotypically express high levels of inhibitory receptors such as PD1 (programmed cell death 1) and CTLA4 (cytotoxic T-lymphocyte antigen 4), which normally are associated with HIV-specific $CD4^+$ and $CD8^+$ T-cells (278–280). Furthermore, exhausted B-cell subpopulations show reduced levels of expressed homing receptors (CXC-chemokine receptor 4 and 5), CXCR4 and CXCR5, which are essential for the migration of B-cells to germinal centres where T-cell dependent B-cell interactions occur, which is important for the pneumococcal glycoconjugate vaccination response (55,281). Other features of B-cell exhaustion in HIV infection include reduced proliferation and effector functions of naïve and memory B-cells (273,282,283).

6.1.3. Peripheral B-cell populations and HIV infection

Circulating peripheral B-cell populations vary widely amongst HIV-infected and HIV-negative individuals (284). Table 6.1 describes the B-cell populations investigated in this thesis and a comparison of normal healthy control data versus HIV-infected individuals.

Table 6.1. $CD19^+$ B-cell subpopulations in normal healthy adults and HIV-infected individuals

B-cell subpopulation name	*Significance of population (References)*	*Normal Healthy Adult Control (Reference Ranges)*	*HIV-related changes compared to normal healthy adult controls (References)*
Naïve B-cells (IgM^+ IgD^+CD27^-)	B- cells that have not encountered specific antigen and therefore, have not responded to it (285).	42.6-82.3% (284,286)	N/A; some studies suggest that these populations are increased/decreased (287,288,122,289)
Marginal Zone (MZ) B-cells (IgM^+ IgD^+CD27^+)	A population of B-cells found in the marginal zones of the spleen that respond rapidly to pathogenic encapsulated bacteria (283)	7.2-32.5% (284,286,291)	Decreased(292,293)
Switched Memory B-cells (IgM^- IgD^-CD27^+)	$CD27^+$ B-cells that do not express IgD and have class-switched following somatic hypermutation in germinal centres post exposure to antigens. These cells express IgG, IgA, or IgE (287).	6.5-31.4% (284,286,291)	Decreased(105,108,124,122,292,295)
Activated B-cells (CD38 low CD21 low)	Rare population of B-cells in the blood of healthy controls, but an expansion of >10% is associated with autoimmune disease (286,296)	0.9-7.6% (284,286,291)	Increased (273,113)
Mature Activated B-cells ($CD27^+$ CD21 low)	Mature B-cells in HIV-infected individuals that show increased activation and terminal differentiation (277)	N/A; not relevant in normal healthy control adults.	Increased (277)
Exhausted B-cells ($CD27^-$ CD21 low)	Tissue-like memory B-cells found in HIV-infected patients. They express high levels of inhibitory receptors, have shorter replication histories, and lower immunoglobulin diversity (162,273)	N/A; not relevant in normal healthy control adults.	Increased (162,273)
Plasmablasts B-cells ($CD38^{+++}IgM^-$)	Short-lived, proliferating, antibody-secreting B-cells that either die after a few days or undergo terminal differentiation into plasma cells, where they either migrate to the bone marrow (long-lived) or to the lymphoid organs (short-lived) to continue antibody secretion (297).	0.4-3.6% (286,291)	Increased (277,113,298,299)
Transitional B-cells ($CD38^{hi}IgM^{hi}$)	B-cells that are an intermediate between immature bone-marrow derived B cells and fully mature naïve B –cells in the peripheral blood (300,301)	0.6-3.5% (286,291)	Increased (118,293,302–304)

6.1.4 Impact of CD4+T-cells, Viral Load (VL), and Antiretroviral therapy (ARV) on B-cells in HIV infection

HIV viral load can impact peripheral B-cell populations early on in HIV-infection, which may have detrimental effects on B-cell reconstitution in later stages of HIV infection (305).

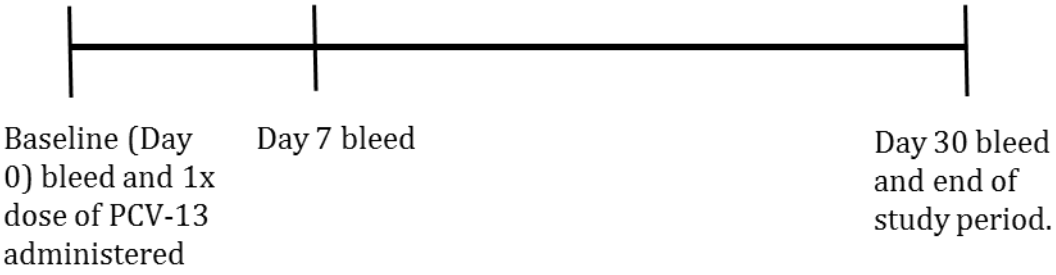
The introduction of ARVs has allowed for the reversal of aberrant antibody production and the restoration of antibody-producing VH3 B-cells (306,307). Furthermore, naïve B-cell populations may not be as severely affected by HIV-infection compared to memory B-cell populations (288,308). One study suggests that even after years of ARV treatment, dysregulation in the $CD27^+$ memory B-cell compartment persists even with undetectable viral loads (295).

6.2. Materials and Methods

6.2.1. Study Design

This study investigated the effects of pneumococcal conjugate vaccination (PCV-13) on B-cell populations in HIV-infected adults by examining B- and T-cell populations, total IgG, IgA, and IgM immunoglobulins, and Pn-specific IgG antibodies (Pn serotypes 1, 3, 4, 5, 6B, 7F, 9V, 14, 18C, 19A, 19F, and 23F).

Pneumococcal vaccine naïve HIV-infected adults were administered one PCV-13 vaccine and monitored over the course of one month with 3 time-points (Day 0 (pre-vaccine), Day 7 and 30 (post-vaccination)) at which lymphocyte (B and T-cell populations) and serum samples were analysed (Figure 6.1). HIV-infected adults ($\geq$18 years old) were recruited from the UHB HIV-department at the QE Hospital in Edgbaston, Birmingham. Patients were formally consented and a baseline (Day 0) blood sample was taken and one PCV-13 vaccine was administered intramuscularly.



- 20 mls of blood taken at Baseline, Day 7 and 30 post-vaccination into lithium-heparin tubes (lymphocyte preparation and immunophenotyping) and coagulated blood tubes (serum antibody quantification).

Figure 6.1. Schematic of PCV-13 Study Design

6.2.2. Ethical Approval

Ethical approval was obtained from the West Midlands-Edgbaston Research Ethics Committee (REC reference number 16/WM/0313; B-cell Immune Responses to Pneumococcal Vaccination in HIV-infected patients). Informed written consent was obtained from all eligible study participants.

6.2.3. Vaccination Schedule

All eligible study participants were administered a single dose of Prevenar-13™ (PCV-13) (Pfizer, UK) by intramuscular injection into a chosen deltoid muscle.

6.2.4. Patient Recruitment

20 HIV-infected adults were recruited from the UHB HIV- Department at the QE Hospital, Birmingham (Patients are still being recruited for this study at the time of writing this thesis so an interim analysis was performed on 10 HIV-infected adults who have completed the study). Patients were eligible for inclusion in the study if they were 18 years and older, had confirmed HIV-infection, were pneumococcal vaccine naïve (Pneumovax™ (Merck Inc, UK) or Prevenar-13™), and were able to give informed consent. Patients were excluded and deemed ineligible for the study if they were previously vaccinated with pneumococcal vaccines, had history of a pre-existing allergy to any component of PCV-13 or diphtheria toxoid vaccines, and/or were not able to give informed consent.

6.2.5. Patient Demographics

The demographics of the study participants are described in Table 6.2.

Table 6.2. HIV-infected study participant demographical data

	HIV-infected Study Participants
N (total number of study participants)	10
Age (Median + Range-years)	42.5 (20.0-64.0)
Female	2
Male	8
Viral Load at Baseline (pre-vaccination) (Median + Range-copies/mL)	1484 (<40-55,210)
Viral Load at Day 30 (post-vaccination)	160 (<40-581)
ARV status at baseline (Yes/No)	Yes

6.2.5. Data Collection

ARV status was taken at baseline after collecting data from clinical records. HIV viral loads (VL) were also assessed at baseline and day 30 post-vaccination, as well as collected from clinical records. The HIV VL was calculated using an HIV-1 RNA assay (Abbott RealTime HIV-21; Abbott Molecular, Inc., USA). The lower limit of detection for the HIV VL assay is 40 copies/mL.

6.2.6. Preparation of samples

6.2.6.1. Lymphocyte sample preparation for flow cytometry staining

Blood for lymphocyte immunophenotyping (15 mL) was collected in lithium heparin vacutainer plastic tubes at each time point. Lymphocytes were isolated by Ficoll density centrifugation using Leucosep™ centrifuge tubes (Grenier Bio-One, Stonehouse, UK) and frozen in 10% DMSO and heat inactivated fetal bovine serum (Sigma, Dorset, UK) for batch processing.

6.2.6.1.1. B-cell subpopulations

Lymphocytes were gated based on forward (FSC) and side scatter (SSC). The P1 gate (as seen in Figure 6.2) is adjusted wide enough to include most of the lymphocytes. B-cells are selected by staining with CD19 and CD45. The remaining gated B-cell lymphocyte populations are further gated on IgD, CD27, CD21, CD38, and IgM (as described in Table 6.1/Figure 6.2). Transitional B-cells and plasmablast gating is based on B-cell phenotypes from the EUROclass trial in CVID (Common Variable Immunodeficiency) patients and healthy adults (286). Exhausted and mature activated B-cells were gated initially with CD19 and CD45 and then on CD21 and CD27 (Figure 6.3). The template was set up to acquire 5,000 $CD19^{+}$ population events in the P1 lymphocyte gate (Figure 6.2/6.3). All lymphocyte

samples were acquired on the FACSCanto II flow cytometer (BD Biosciences, Oxford, UK). Daily calibration and monitoring of the instrument was performed using CS and T beads (Cytometer Setup and Tracking beads) (BD Biosciences, Oxford, UK). Rainbow calibration particles (BD Biosciences, Oxford, UK) were used to standardise EuroFlow instrument settings (309). BD CompBeads (BD Biosciences, Oxford, UK) were used to apply compensation. Compensation was performed using fluorescence minus one (FMO) techniques in order to identify and gate cells of interest due to the multiple fluorochrome staining in the investigated panels (310).

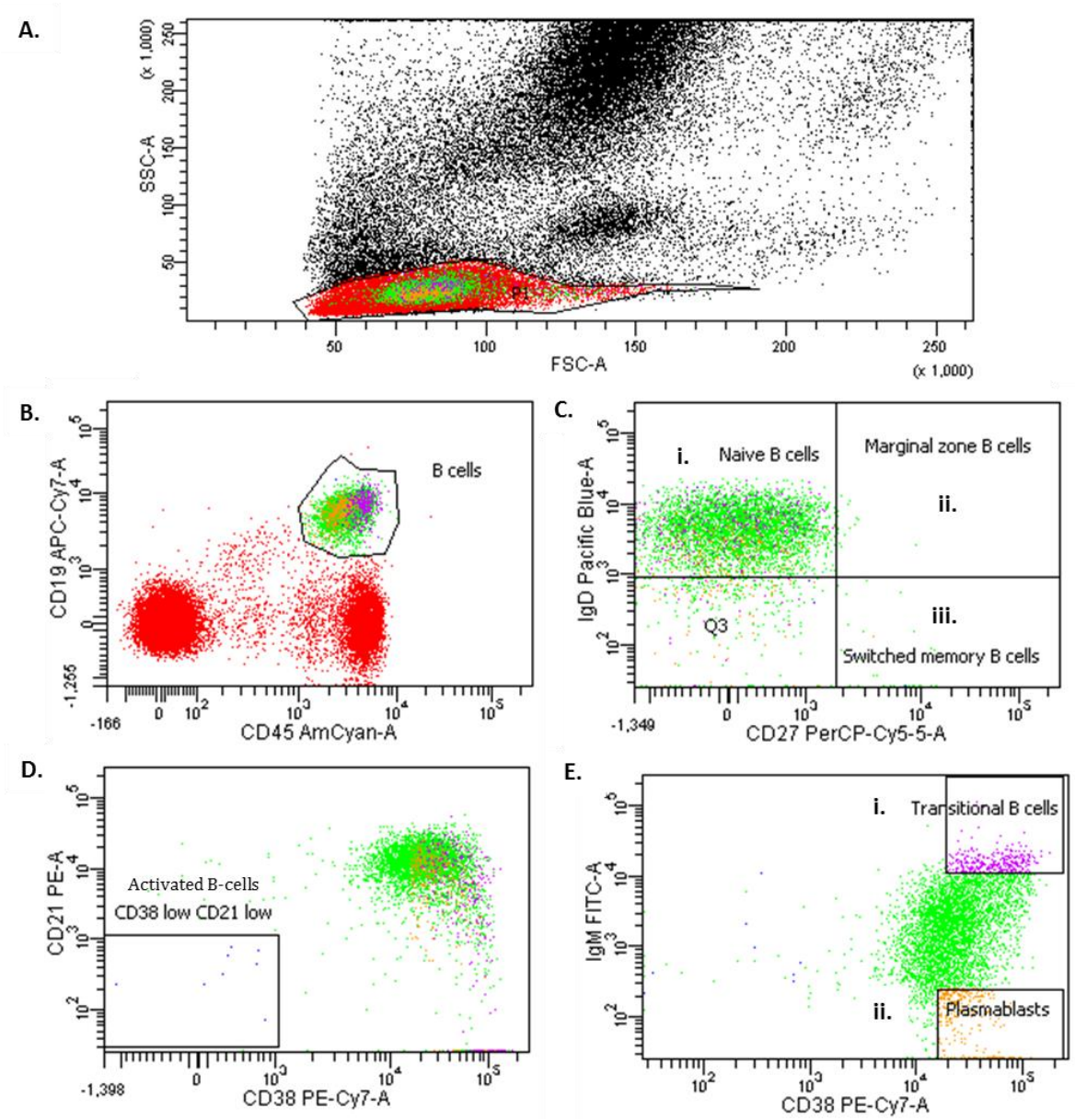


Figure 6.2. Example gating strategy for B-cell phenotyping in the HIV PCV-13 study to identify CD19⁺ subpopulations. *Gating is shown on a study participant sample. A. Gate is set on the P1 lymphocyte population. B. Gate from P1 set on CD19⁺ and CD45⁺ B-cells C. Gated on the CD19⁺ population, i. Naïve B-cells (IgD⁺ CD27⁻) ii. Marginal zone B-cells (IgD⁺ CD27⁺) iii. Switched memory B-cells (IgD– CD27⁺) D. Gated on the CD19⁺ population, Activated B-cells (CD38 low CD21 low) E. Gated on the CD19⁺ population, i. Transitional B-cells (CD38 high, IgM high) ii. Plasmablasts (CD38 low IgM low).*

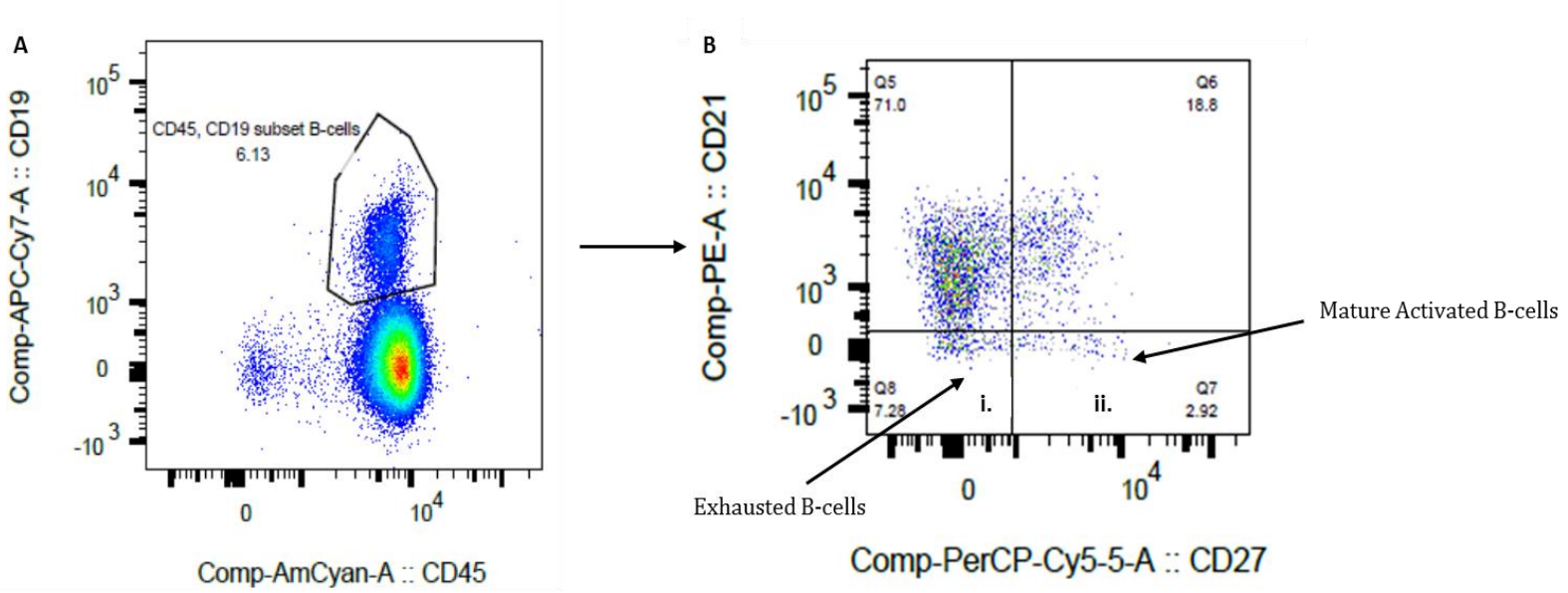


Figure 6.3. Example gating for Exhausted and Mature Activated B-cell phenotyping in the HIV PCV-13 study. *Gating is shown on a study participant sample. A. Gate from P1 (Figure 7.2) set on CD19⁺ and CD45⁺ B-cells B. Gated on the CD19⁺ population, i. Exhausted B-cells (CD27– CD21 low) ii. Mature Activated B-cells (CD27⁺ CD21 low).*

6.2.6.1.2. T-cell subpopulations

Lymphocytes were gated based on forward (FSC) and side scatter (SSC). The P1 gate (as seen in Figure 6.4) is adjusted wide enough to include most of the lymphocytes. T-cells are selected by staining with CD3 and CD45. The remaining gated T-cell lymphocyte populations are further gated on CD4 and CD8 in order to determine CD4:CD8 ratios. Gating is based on T-cell phenotypes from the ALPS (Autoimmune Lymphoproliferative Syndrome) classification panel in the Clinical Immunology Service (311). The template was set up to acquire 20,000 $CD3^+$ population events in the P1 lymphocyte gate (Figure 6.4). All lymphocyte samples were acquired on the FACSCanto II flow cytometer (BD Biosciences, Oxford, UK). Compensations were applied as described in section 6.2.6.1.1..

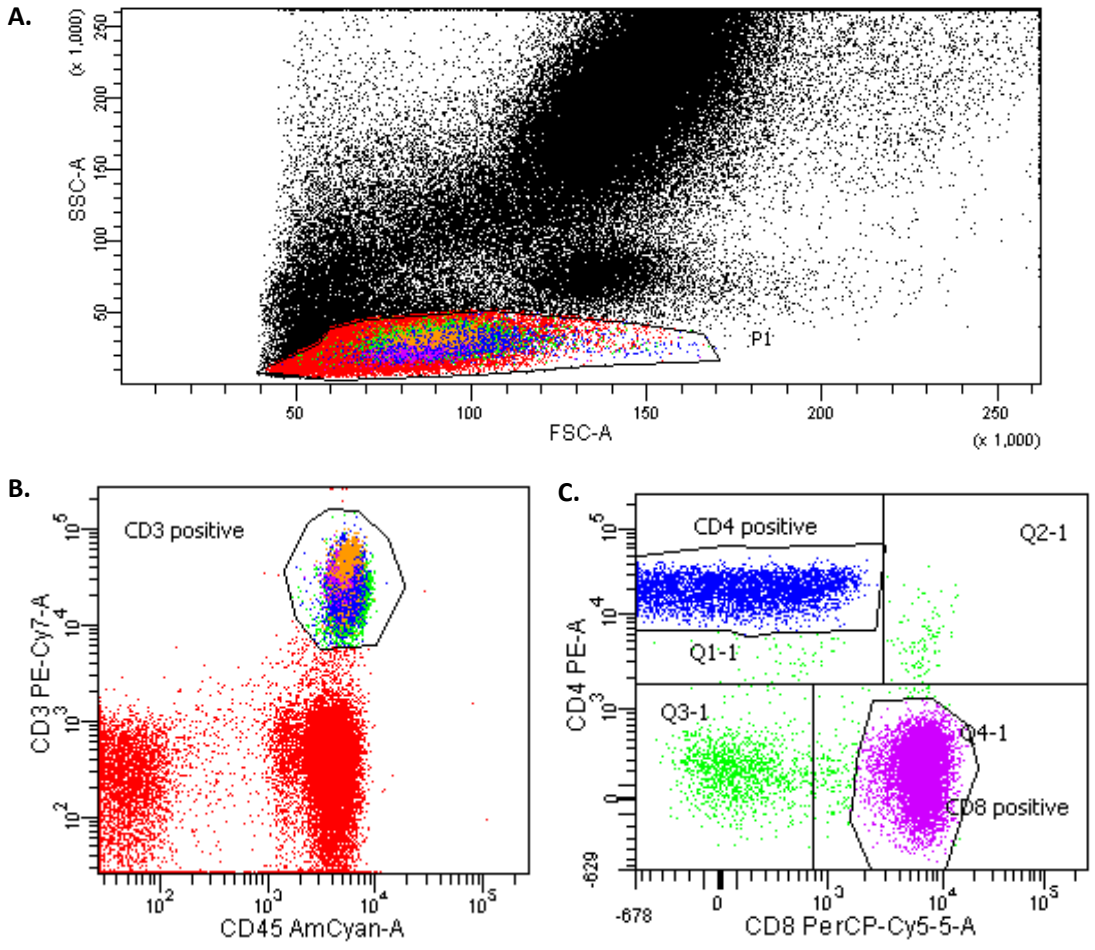


Figure 6.4. Example gating strategy for T-cell phenotyping in the HIV PCV-13 study to identify $CD3^+$ subpopulations. *A. Gate is set on the P1 lymphocyte population. B. Gate from P1 set on $CD3^+$ and $CD45^+$ T-cells C. Gated on $CD4^+$ and $CD8^+$ populations.*

6.2.6.1.3. Flow cytometry staining

Study participant cells were resuscitated from frozen by placing a frozen cryovial (5-10 x 10^6 cells/mL) into a 37°C water bath and gently swirling until a few ice crystals remained. Cryovial contents were transferred into a pre-labelled Falcon with 10 mL warmed complete RPMI (10% HI FCS and 1% penicillin/streptomycin). The falcon was then topped up to 50 mL with complete RPMI and spun at 300g for 10 minutes. The supernatant was then decanted and cells were resuspended in their residual volume. 2 mL of warmed complete RPMI was then added to each falcon in addition to 1 µl of Benzonase ® Nuclease (25 U/µl) (Merck Millipore, Hertfordshire, UK) for every 1 x 10^6 cells frozen down for 1 hour at 37°C, 5% CO_2 in order to reduce cell clumping and increase cell viability post-resuscitation (312). The falcon was then topped up to 50 mL with complete RPMI and spun at 300g for 10 minutes. The supernatant was then decanted and cells were resuspended in 10 mL of warmed complete RPMI and cells were counted using an automated Horiba Cell Counter (ABX Micro ES60, Horiba Ltd., Northampton, UK). The falcon was then topped up to 50 mL with complete RPMI and spun at 300g for 10 minutes. The supernatant was then decanted and cells were resuspended at the desired concentration (approximately 3 x 10^5 cells per FACS tube) for use in the flow cytometry experiment.

The resuspended cells were then incubated with a mastermix of extracellular staining antibodies (described in Table 6.3; all antibodies obtained from BD Biosciences, Oxford, UK). 2 µl of each of the antibodies in Table 6.3 were added for each patient tube. The tubes were then incubated in the dark at 4°C for 15 minutes. Immediately after incubation, 500 µl of BD FACS lysis and fixative fluid (BD Biosciences, Oxford, UK) was added to each patient tube and incubated in the dark at 4°C for 15 minutes. Then, 2 mLs of BD FACS CellWASH buffer (BD Biosciences, Oxford, UK) was added to each patient tube and then spun at 1000 RCF for 7 minutes. Post-centrifugation, the supernatant was poured off of each patient tube and then resuspended with 200 µl of BD FACS CellWASH buffer (BD Biosciences, Oxford, UK) in order to immediately acquire on the FACSCanto II. Patient tubes were kept in the dark at 4°C until they were acquired.

Table 6.3. Antibody mastermixes for B and T-cell lymphocyte staining

Patient Sample Tube	*FITC*	*PE*	*PerCP*	*PE Cy7*	*APC H7*	*V450*	*V500*
Tube 1 (B-cells)	IgM	CD21	CD27	CD38	CD19	IgD	CD45
Tube 2 (T-cells)	-	CD4	CD8	-	CD3	-	-

6.2.6.1.4. Flow cytometry analysis

Analysis was performed using a FACSCanto II and FACSDiva™ (BD Biosciences, Oxford, UK) software. Lymphocytes were gated on FSC and SSC. B and T- lymphocytes were gated as described in 6.2.6.1.1 and 6.2.6.1.2, respectively. Compensation was performed automatically using FACSDiva™ software and compensation FMO controls previously in the Clinical Immunology Service. Additionally, analysis of the B and T-cell lymphocyte data was performed using FlowJo software, version 10.4.1. (Treestar, Inc., Oregon, USA). $CD19^+$ B-cells and $CD3^+$ T-cell percentages were derived from the total lymphocyte count. Gating is described in section 6.2.6.1.1 and 6.2.6.1.2. Data was analysed by absolute counts and percentages. Additionally, $CD19^+$ B-cells and $CD3^+$ T-cell subpopulations were expressed as percentages of the total $CD19^+$ and $CD3^+$ counts.

6.2.6.2. Serum sample preparation for Total IgG, IgA, and IgM, and Pn-specific IgG antibody quantification

Blood for serum studies (5 mL) was taken into vacutainer plastic tubes with a clotting activator at each time point. Serum was then separated from whole blood by centrifugation at 3000 rpm for 5 min, aliquoted, and stored at -80°C for future studies. Blood was collected and serum was isolated/frozen down at -80°C within 24 hours of collection from venepuncture in the Clinical Immunology Service, University of Birmingham, Birmingham.

6.2.6.2.1. Total IgG, IgA, and IgM serum antibody quantification

Total IgG, IgA, and IgM antibody concentrations were quantified using nephelometry techniques, according to instructions described by the manufacturer (Roche Diagnostics GmbH, Mannheim, Germany). Goat anti-human IgG antibodies react with antigen in the patient samples to form an antigen/antibody complex. Following antibody/antigen agglutination, the antigen/antibody complex is measured turbidimetrically using a COBAS c 311/501 analyser in the Clinical Immunology Service, University of Birmingham, Edgbaston, UK. Normal ranges for healthy adults are as follows: total IgG (6.0-16.0 g/L), IgA (0.8-4.0 g/L), and IgM (0.5-2.0 g/L) (313).

6.2.6.2.2. Pn-specific IgG antibody quantification

Pn-specific IgG concentrations were measured as described in section 2.5.2.3.

6.2.7. Statistical Analysis

Continuous data was presented as medians and interquartile ranges unless stated otherwise. Non-parametrical statistical tests were used throughout. Differences between time-points were analysed by Wilcoxon signed rank tests. Univariate correlations were performed using a Spearman's Rank method. Mann-Whitney U tests were performed to detect differences

between $CD19^+$ B-cell populations and serum antibody vaccine responses at pre- and post-vaccination. Statistical analysis was performed using GraphPad Prism software (GraphPad Software, Inc., version 6). P-values of <0.05 were considered significant throughout. This was an observational pilot study; therefore, no formal power calculation was performed. The primary analysis for this study was to investigate the proportion of HIV-infected patients that produced a memory B-cell response to a single dose of Prevenar-13. The secondary outcomes were to investigate how HIV infection impacts the B-cell phenotype and correlates with quantifiable Pn-specific IgG and total serum antibody concentrations.

6.3. Results

6.3.1. Lymphocyte phenotyping

6.3.1.1. Evaluation of B-cell populations at baseline and post-vaccination responses with PCV-13

$CD19^+$ B-cell populations were investigated in this section with descriptions and associated changes in the context of HIV-1 infection described in Table 6.1.

6.3.1.1.1. Naïve B-cells

The percentages of naïve B-cell populations in HIV-infected adults were similar compared to normal healthy controls across all post-vaccination time points (Figure 6.5/Table 6.5) (reference ranges described in Table 6.1).

6.3.1.1.2. Marginal Zone B-cells

A novel finding was that there were very low marginal zone (MZ) populations (<10%) compared to normal healthy controls across all post-vaccination time points (Figure 6.5/Table 6.5) (reference ranges described in Table 6.1).

6.3.1.1.3. Switched Memory B-cells

Additionally, switched memory B-cells were relatively similar to normal healthy controls across all post-vaccination time points; however, there was a marked reduction from baseline to day 30 post-vaccination (p=0.0039) and day 7 to day 30 post-vaccination (p=0.0039)(Figure 6.5/Table 6.5) (reference ranges described in Table 6.1).

6.3.1.1.4. Activated B-cells

Activated B-cells were expanded compared to normal healthy controls (>10%) and there was a marked increase from day 7 to 30 post-vaccination (p=0.0195). However, mature activated B-cells were reduced from day 7 to 30 (p=0.0039) (Figure 6.5/Table 6.5).

6.3.1.1.5. Exhausted B-cells

Exhausted B-cell populations were expanded in this HIV-infected cohort; however, there were no significant differences between pre- and post-vaccination time points. It is important to note that mature activated and exhausted B-cell populations are unique to HIV-infection; therefore, it was not possible to make comparisons with normal healthy control reference ranges (Figure 6.5/Table 6.5).

6.3.1.1.6. Plasmablasts

Plasmablasts were expanded in the HIV-infected cohort compared to normal healthy control reference ranges (>5%); however, there was a reduction from day 7-30 post vaccination ($p=0.0098$) (Figure 6.5/Table 6.5) (reference ranges described in Table 6.1).

6.3.1.1.7. Transitional B-cells

The proportion of transitional B-cells were reduced compared to normal healthy control reference ranges (<0.5%) and there was a significant reduction from baseline to day 30 post-vaccination ($p=0.0488$) (Figure 6.5/Table 6.5) (reference ranges described in Table 6.1).

6.3.1.2. Evaluation of T-cell populations post-vaccination with PCV-13

The percentages of $CD3^+$, $CD4^+$, and $CD8^+$ T-cells were evaluated in this HIV-infected cohort across all time points pre- and post-vaccination. There were no significant differences found between pre- and post-vaccination time points (Figure 6.6 and Table 6.6).

$CD4^+$ T-cell percentages were relatively normal across all pre-and post-vaccination time points. At baseline, the percentage of $CD4^+$ T-cells (median 35.1% (IQR 24.0-43.1%) were normal and they did not change post-vaccination at day 7 (median 32.5%(IQR 24.4-42.4%)) and day 30 (median 35.1%(IQR 27.0-44.6%)).

$CD8^+$ T-cell percentages were expanded above normal reference ranges (Table 6.4) across all pre and post-vaccination time points. At baseline, the percentage of $CD8^+$ T-cells were higher than normal (median 53.4% (IQR 41.1-66.1%)) and this did not change at day 7 (median 51.5% (IQR 42.5-63.6%)) and day 30 (median 51.0% (IQR 41.3-61.4%)) post-vaccination.

The CD4:CD8 ratios per study participant was below 1 at baseline (median 0.65 (IQR 0.37-1.05)), day 7 (median 0.63 (IQR 0.38-1.12)), and day 30 (median 0.74 (IQR 0.45-1.12)) post-vaccination (Figure 6.6 and Table 6.7). Inverse CD4:CD8 ratios are considered as markers of immunosenescence and predictors of mortality (314,315). Two studies investigating influenza and hepatitis B vaccination have associated inverse CD4:CD8 ratios and an impaired ability to respond to vaccination (316,317).

Table 6.4. Normal T-cell reference ranges for healthy adults (18-65 years old)

T-cell lymphocyte population	*Normal Healthy Adult Reference Range*
CD3+	49.1-83.6 (318)
CD3+CD4+	28.2-62.8 (318)
CD3+CD8+	10.2-40.1 (318)
CD4:CD8 ratio	≥1 (314)

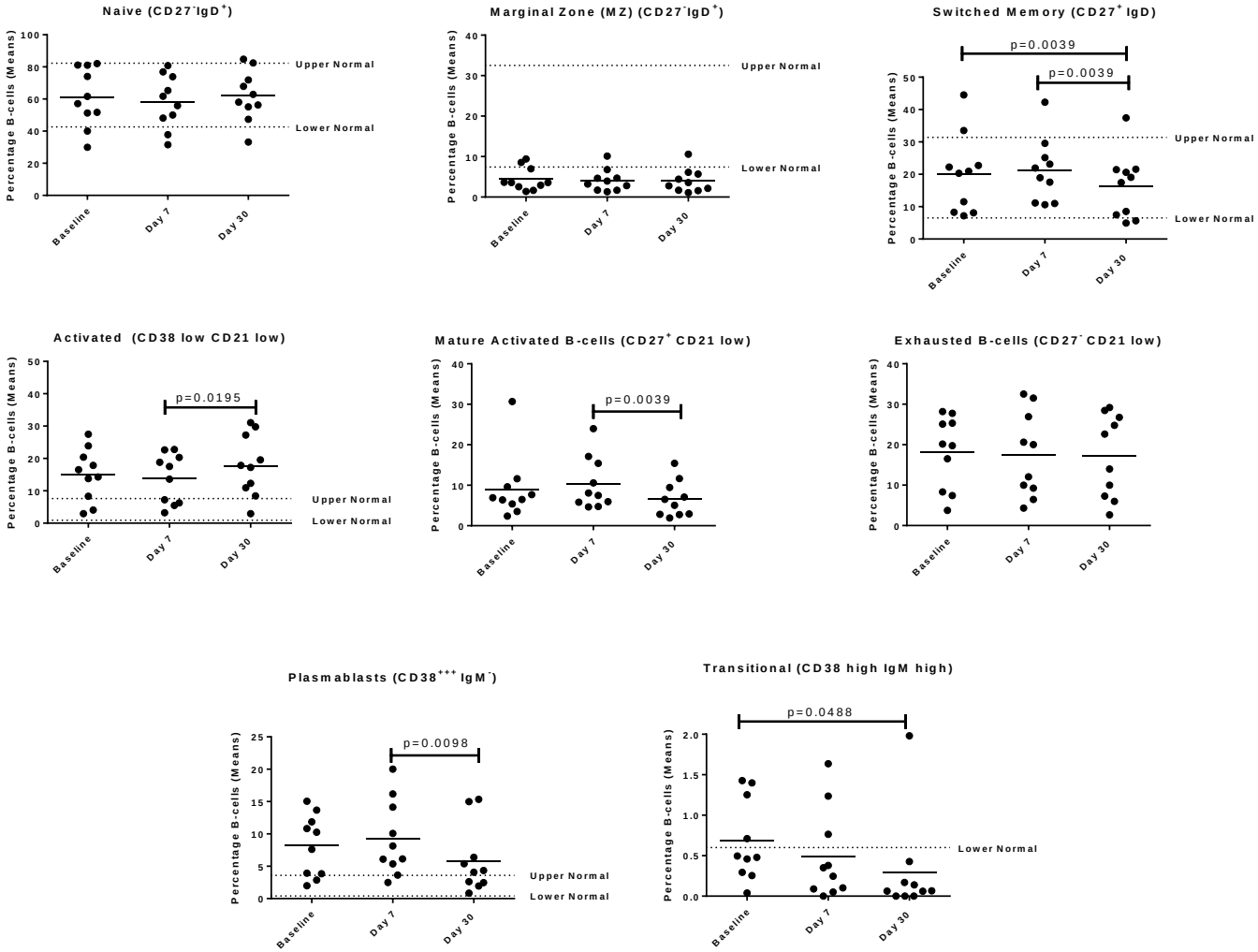


Figure 6.5. Changes in percentages of CD19⁺ B-cell subpopulations in HIV-infected adults pre- and post-vaccination with PCV-13. *Data are reported as percentages of total CD19+ populations, medians. and interquartile ranges with p-values from Wilcoxon-matched pairs signed rank tests. Bold p-values are significant at p<0.05.*

Table 6.5. Percentages of CD19⁺ B-cell subpopulations in HIV-infected adults pre- and post-vaccination with PCV-13.

	Timepoint			*P-values*		
CD19⁺ B-cell population	***Baseline (N=10)***	***Day 7 (N=10)***	***Day 30 (N=10)***	***Baseline vs Day 7 post-vaccination***	***Day 7 vs Day 30 post-vaccination***	***Baseline vs Day 30 post-vaccination***
Naïve	59.4 (48.5-81.0)	58.8 (45.5-74.6)	60.5 (53.2-74.5)	0.4922	0.084	0.375
Marginal Zone	3.58 (2.32-7.38)	3.59 (1.71-5.20)	3.18 (1.65-5.78)	0.625	0.4922	0.4922
Switched Memory	20.6 (8.22-25.4)	20.4 (11.1-26.3)	18.3 (7.02-21.5)	0.625	***0.0039***	***0.0039***
Activated	15.4 (7.25-21.3)	15.6 (6.07-20.9)	17.5 (10.3-27.9)	0.5566	***0.0195***	0.1934
Plasmablasts	8.93 (3.61-12.3)	7.13 (4.94-14.7)	4.21 (2.34-8.53)	0.4316	***0.0098***	0.1934
Transitional	0.49 (0.28-1.29)	0.30 (0.08-0.88)	0.06 (0.0-0.23)	0.2324	0.25	***0.0488***
Mature Activated	6.69 (4.91-10.12)	7.79 (5.55-15.9)	5.77 (2.80-10.0)	0.375	***0.0039***	0.1934
Exhausted	20.0 (8.08-25.9)	16.0 (8.55-28.1)	18.3 (6.96-27.2)	0.5566	0.9219	0.9219

Data are reported as medians and interquartile ranges with p-values from Wilcoxon-matched pairs signed rank tests
Bold p-values are significant at p<0.05

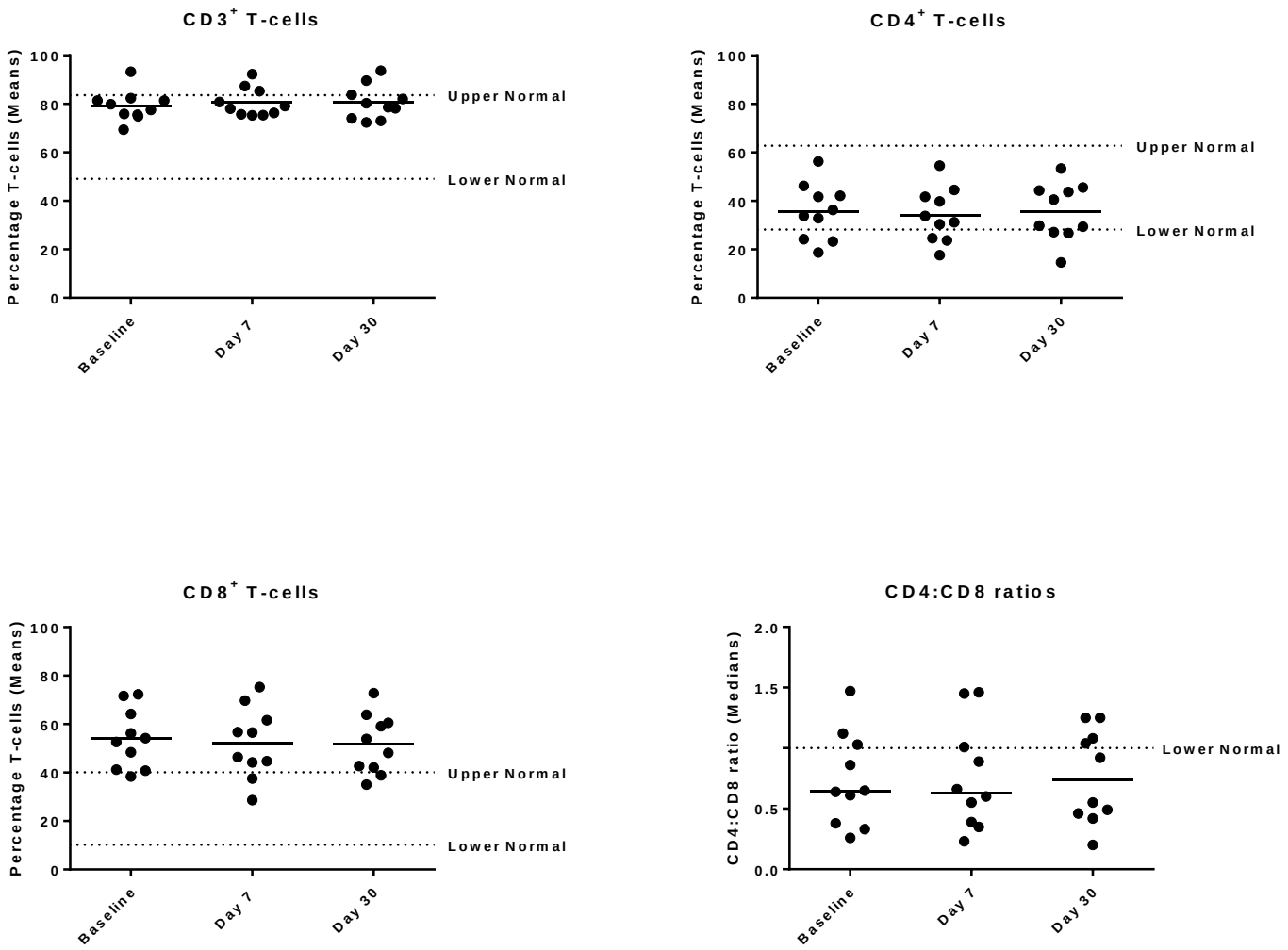


Figure 6.6. Changes in percentages of CD3+ T-cell subpopulations in HIV-infected adults pre- and post-vaccination with PCV-13. *Data are reported as percentages of total CD3+ populations, medians. and interquartile ranges with p-values from Wilcoxon-matched pairs signed rank tests. Bold p-values are significant at p<0.05.*

Table 6.6. Percentages of $CD3^+$ T-cell subpopulations in HIV-infected adults pre- and post-vaccination with PCV-13.

	Timepoint			P-values		
$CD3^+$ T-cell population	Baseline (N=10)	Day 7 (N=10)	Day 30 (N=10)	Baseline vs Day 7 post-vaccination	Day 7 vs Day 30 post-vaccination	Baseline vs Day 30 post-vaccination
CD3	78.7 (75.4-81.7)	78.6 (75.6-85.8)	79.5 (73.8-85.3)	0.3613	0.9805	0.3477
CD4	35.1 (24.0-43.1)	32.5 (24.4-42.4)	35.1 (27.0-44.6)	0.1309	0.5313	0.9219
CD8	53.4 (41.1-66.1)	51.5 (42.5-63.6)	51.0 (41.3-61.4)	0.375	0.8457	0.2754

Data are reported as medians and interquartile ranges with p-values from Wilcoxon-matched pairs signed rank tests
Bold p-values are significant at p<0.05

Table 6.7 CD4:CD8 ratios in HIV-infected adults pre- and post-vaccination with PCV-13

	Timepoint			P-values		
	Baseline (N=10)	Day 7 (N=10)	Day 30 (N=10)	Baseline vs Day 7 post-vaccination	Day 7 vs Day 30 post-vaccination	Baseline vs Day 30 post-vaccination
CD4:CD8 ratio	0.65 (0.37-1.05)	0.63 (0.38-1.12)	0.74 (0.45-1.12)	0.8652	>0.9999	0.623

Data are reported as medians and interquartile ranges with p-values from Wilcoxon-matched pairs signed rank tests
Bold p-values are significant at p<0.05

6.3.2. Pn-specific IgG response to a single dose of PCV-13

Patient serum samples obtained at baseline, day 7 and day 30 post-vaccination were analysed for Pn-specific IgG antibodies (Pn1, 3, 4, 5, 6B, 7F, 9V, 14, 18C, 19A, 19F, and 23F) (Figures 6.7-6.10/Table 6.8-6.9).

At baseline, 10% of patients reached the ≥8 out of 12 Pn serotypes WHO (≥0.35 µg/mL) threshold. By day 7 post-vaccination, 20% had an increase in protective antibody concentrations and by day 30, 70 % had an increased response to ≥8 out of 12 Pn serotypes (Figure 6.7).

The median antibody concentrations for the HIV-infected study participants increased from baseline to day 7 for Pn 4, 6B, 9V, and 23F serotypes (Figures 6.7-6.10/Table 6.8-6.9). The median antibody concentrations for the HIV-infected study participants increased from day 7-day 30 post-vaccination for Pn 1, 3, 19A, 19F, and 23F serotypes (Figures 6.7-6.10/Table 6.8-6.9).From baseline to day 30 post-vaccination, the median antibody concentrations increased for Pn 1, 3,4,6B, 18C, 19A, 19F, and 23F serotypes (Figures 6.7-6.10/Table 6.8-6.9).

There were greater fold changes from baseline to day 30 post-vaccination compared with baseline-day 7 post-vaccination for Pn 1, 6B, 7F, 9V, 14, 18C, 19A, 19F, and 23F serotypes (Figures 7.7-7.10/Table 7.8-7.9); No significant increases in fold change were produced for Pn 4 and 5. Additionally, there were greater fold changes produced for day 7-30 compared with baseline-day 7 post-vaccination for Pn3 and 19F (Figures 6.7-6.10/Table 6.8-6.9). Also, there were greater fold changes produced from baseline-day 30 post-vaccination compared with day 7-day 30 post-vaccination for Pn4 and 23F (Figures 6.7-6.10/Table 6.8-6.9).

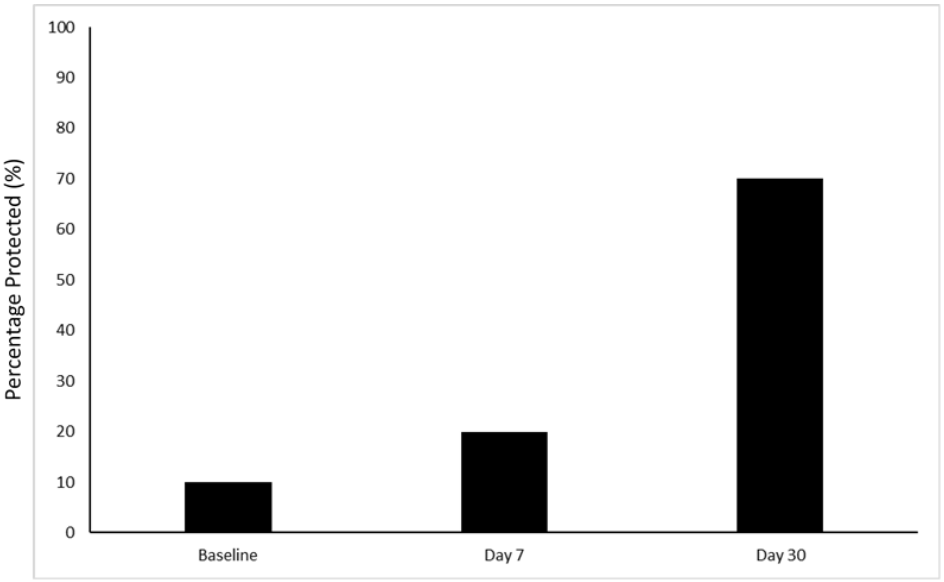


Figure 6.7. Percentage of HIV-infected study participants that reach WHO threshold (0.35 µg/mL) and ≥ 8 out of 12 Pn serotypes protected pre and post-vaccination (Day 7 and 30) with PCV-13.

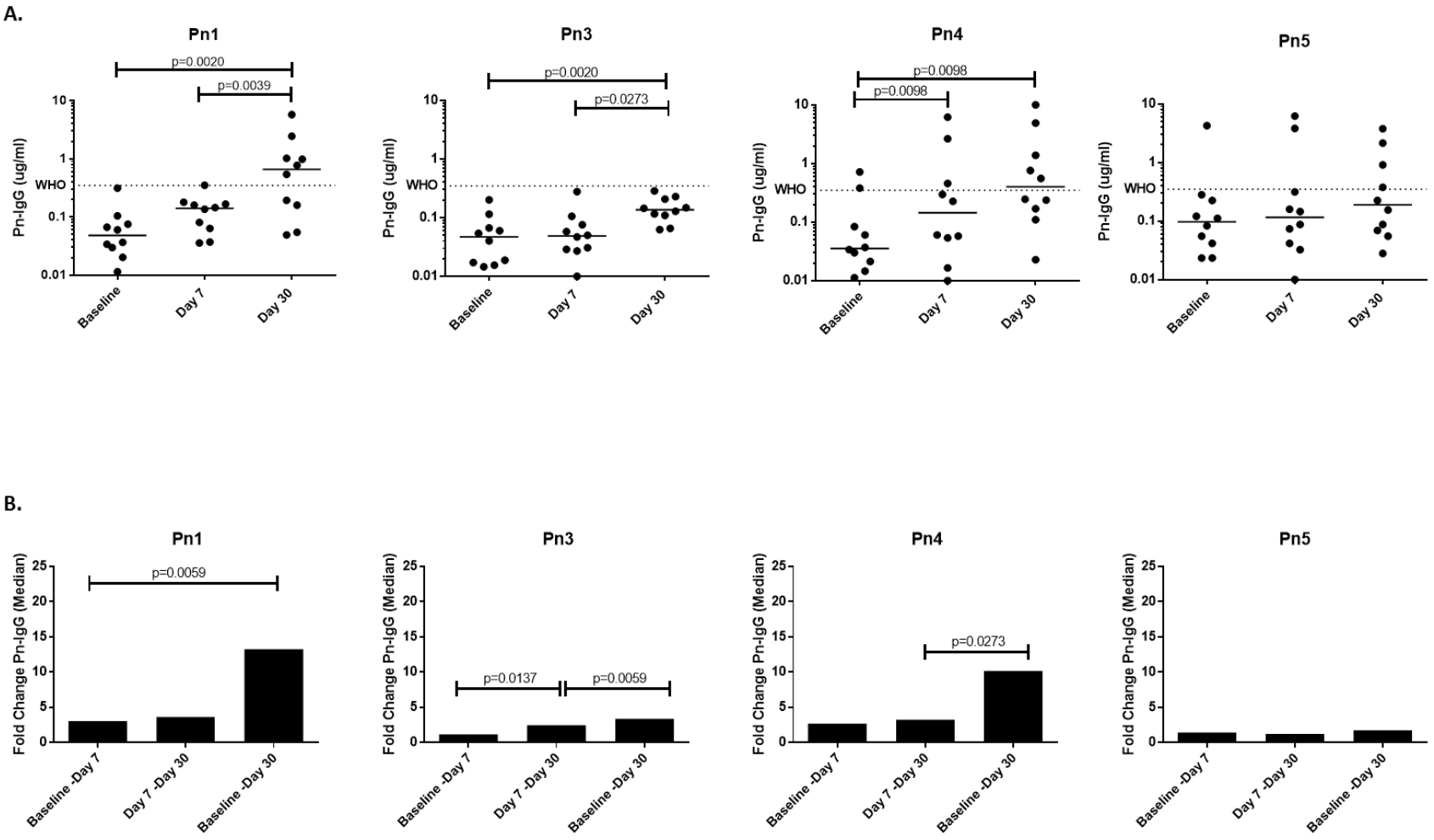


Figure 6.8 Pn-specific IgG (Pn 1, 3, 4 , and 5) vaccine responses (absolute values and fold changes) to a single dose of PCV-13. A. *Pn-specific IgG values are reported as µg/mL. WHO Pn threshold antibody titres (0.35 µg/mL) are denoted by dotted lines B. Fold Changes Pn-specific IgG .Data are reported as medians with p-values from Wilcoxon-matched pairs signed rank tests. Bold p-values are significant at p<0.05.*

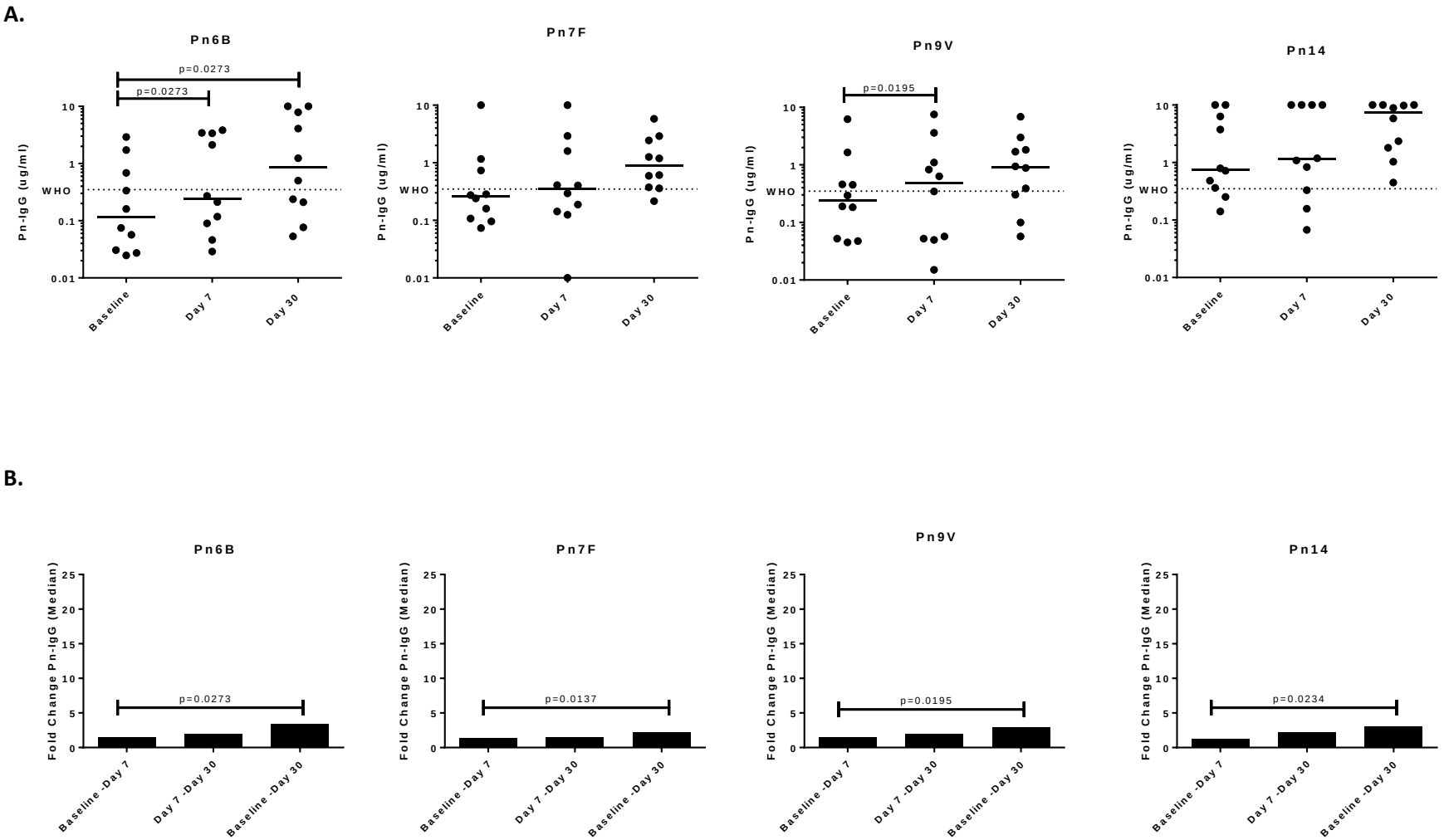


Figure 6.9. Pn-specific IgG (Pn 6B. 7F, 9V, and 14) vaccine responses (absolute values and fold changes) to a single dose of PCV-13. A. *Pn-specific IgG values are reported as µg/mL. WHO Pn threshold antibody titres (0.35 µg/mL) are denoted by dotted lines B. Fold Changes Pn-specific IgG .Data are reported as medians with p-values from Wilcoxon-matched pairs signed rank tests. Bold p-values are significant at p<0.05.*

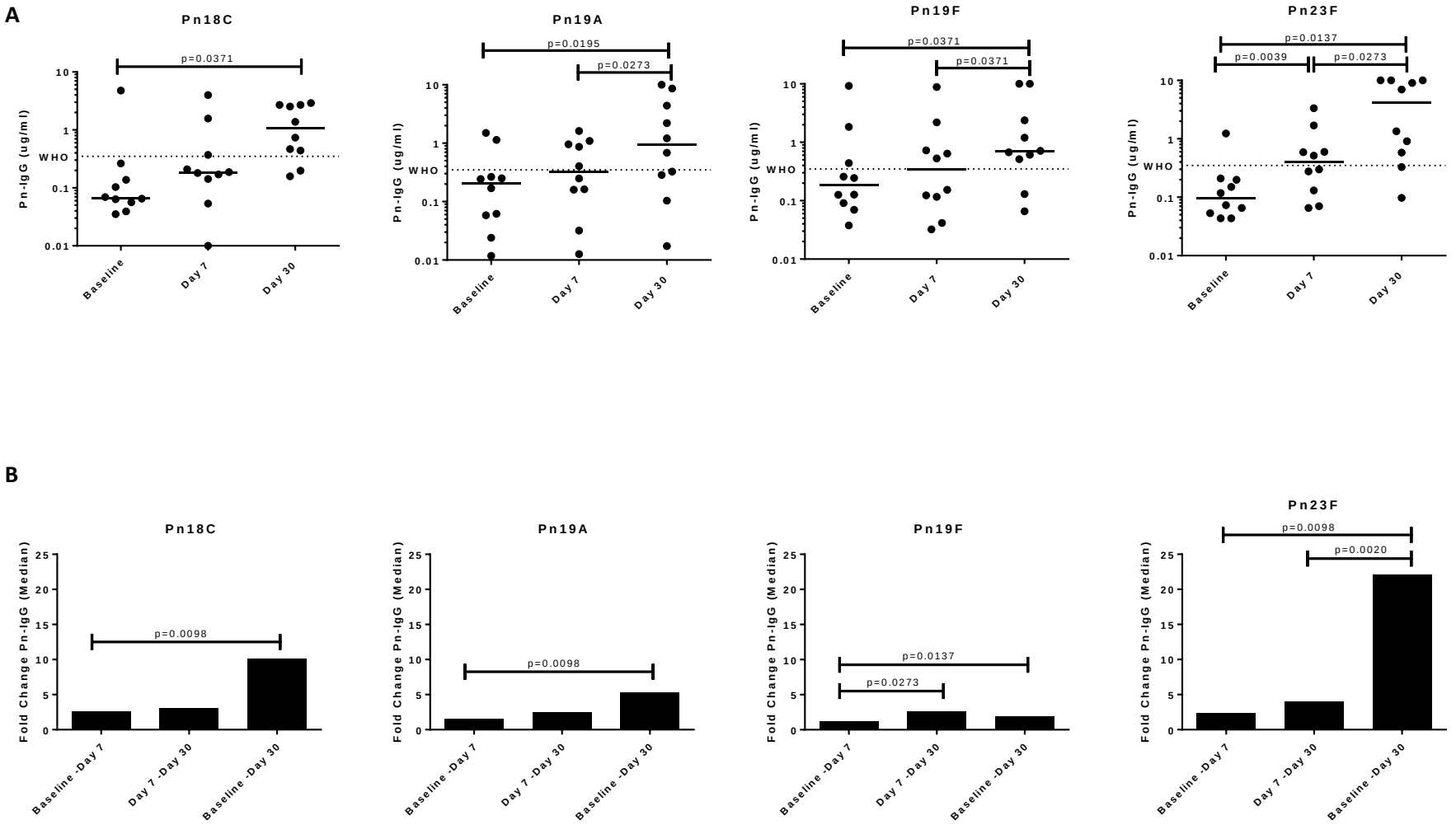


Figure 6.10. Pn-specific IgG (Pn 18C, 19A, 19F, and 23F) vaccine responses (absolute values and fold changes) to a single dose of PCV-13. A. *Pn-specific IgG values are reported as µg/mL. WHO Pn threshold antibody titres (0.35 µg/mL) are denoted by dotted lines B. Fold Changes Pn-specific IgG .Data are reported as medians with p-values from Wilcoxon-matched pairs signed rank tests. Bold p-values are significant at p<0.05.*

Table 6.8. Pn-specific IgG vaccine responses (absolute values) to a single dose of PCV-13.

Pn-specific IgG	*Timepoint*			*P-values*		
	Baseline (N=10)	*Day 7 (N=10)*	*Day 30 (N=10)*	*Baseline vs Day 7 post-vaccination*	*Day 7 vs Day 30 post-vaccination*	*Baseline vs Day 30 post-vaccination*
Pn1	0.05 (0.03-0.08)	0.14 (0.06-0.17)	0.66 (0.13-1.38)	0.0645	***0.0039***	***0.002***
Pn3	0.05 (0.02-0.08)	0.05 (0.03-0.08)	0.14 (0.10-0.21)	0.1934	***0.0273***	***0.002***
Pn4	0.04 (0.02-0.16)	0.14 (0.04-1.01)	0.40 (0.15-2.27)	***0.0098***	0.1602	***0.0098***
Pn5	0.10 (0.04-0.24)	0.12 (0.04-1.19)	0.19 (0.07-1.22)	0.084	0.2324	0.0645
Pn6B	0.12 (0.03-0.94)	0.24 (0.08-3.39)	0.87 (0.17-8.42)	***0.0273***	0.084	***0.0273***
Pn7F	0.26 (0.10-0.84)	0.35 (0.14-1.93)	0.90 (0.37-2.56)	0.0547	0.1055	0.084
Pn9V	0.24 (0.05-0.75)	0.49 (0.05-1.73)	0.91 (0.25-2.11)	***0.0195***	0.1602	0.0645
Pn14	0.76 (0.34-7.26)	1.14 (0.29-10.00)	7.36 (1.61-10.00)	0.0781	0.1563	0.0547
Pn18C	0.07 (0.05-0.17)	0.18 (0.12-0.67)	1.06 (0.38-2.70)	0.1055	0.0645	***0.0371***
Pn19A	0.21 (0.05-8.48)	0.33 (0.13-0.99)	0.95 (0.24-5.46)	0.1602	***0.0273***	***0.0195***
Pn19F	0.19 (0.09-0.79)	0.34 (0.10-1.09)	0.69 (0.42-4.28)	0.3223	***0.0371***	***0.0371***
Pn23F	0.10 (0.05-0.20)	0.41 (0.12-0.87)	4.15 (0.52-10.00)	***0.0039***	***0.0273***	***0.0137***

Data are reported as medians and interquartile ranges with p-values from Wilcoxon-matched pairs signed rank tests
Bold p-values are significant at p<0.05

Table 6.9. Pn-specific IgG vaccine responses (fold changes) to a single dose of PCV-13.

Pn-specific IgG Fold Change	*Timepoint*			*P-values*		
	Baseline vs Day 7 post-vaccination	*Day 7 vs Day 30 post-vaccination*	*Baseline vs Day 30 post-vaccination*	*Baseline-Day 7 vs Day 7-Day 30*	*Day 7-Day 30 vs Baseline-Day 30*	*Baseline-Day 7 vs Baseline-Day 30 post-vaccination*
Pn1	3.06 (1.24-4.84)	3.63 (1.33-8.67)	13.27 (2.39-31.1)	0.6953	0.084	***0.0059***
Pn3	1.15 (0.90-1.66)	3.36 (1.76-4.83)	2.45 (1.62-5.75)	***0.0137***	0.2754	***0.0059***
Pn4	2.71 (1.40-4.00)	3.24 (1.23-5.84)	10.16 (2.74-20.6)	0.9219	***0.0273***	0.1055
Pn5	1.42 (1.03-1.77)	1.27 (0.96-2.75)	1.75 (1.24-3.91)	0.7695	0.1602	0.084
Pn6B	1.54 (1.19-5.90)	3.45 (2.37-22.7)	1.93 (1.09-2.52)	0.625	0.084	***0.0273***
Pn7F	1.43 (1.03-2.22)	1.52 (1.20-4.73)	2.25 (1.90-5.90)	0.6953	0.1289	***0.0137***
Pn9V	1.51 (1.08-2.16)	2.02 (1.04-3.87)	2.90 (1.55-5.04)	0.375	0.084	***0.0195***
Pn14	1.22 (1.00-2.36)	2.18 (1.00-4.38)	3.07 (1.43-7.15)	0.2031	0.1953	***0.0234***
Pn18C	2.64 (1.23-3.72)	3.12 (1.45-7.02)	10.10 (3.16-20.7)	0.4922	0.1055	***0.0098***
Pn19A	1.54 (1.04-4.55)	2.52 (1.30-6.39)	5.32 (1.41-21.1)	0.4316	0.1055	***0.0098***
Pn19F	1.20 (0.97-1.58)	2.65 (1.11-8.04)	1.92 (1.07-8.19)	***0.0273***	0.1309	***0.0137***
Pn23F	2.31 (1.30-8.67)	4.00 (1.77-19.0)	22.1 (3.95-150.2)	0.375	***0.002***	***0.0098***

Data are reported as medians and interquartile ranges with p-values from Wilcoxon-matched pairs signed rank tests
Bold p-values are significant at p<0.05

6.3.3. Total immunoglobulin (IgG, IgA, and IgM) responses to a single dose of PCV-13

Patient serum samples obtained at baseline, day 7 and day 30 post-vaccination were analysed for total IgG, IgA, and IgM. Vaccine responsiveness was assessed to a single dose of PCV-13 in total IgG, IgA, and IgM (Figure 6.11/Table 6.10-6.11). HIV-infected study patients had relatively normal immunoglobulin concentrations compared with normal healthy adult reference ranges (described in section 6.2.6.2.1).

There were no changes or significant differences detected between any vaccination time points for total IgG and IgM concentrations. However, there was a greater fold change from baseline to day 30 post-vaccination in contrast to baseline to day 7 post-vaccination for total IgA (Figure 6.11/Table 6.10-6.11).

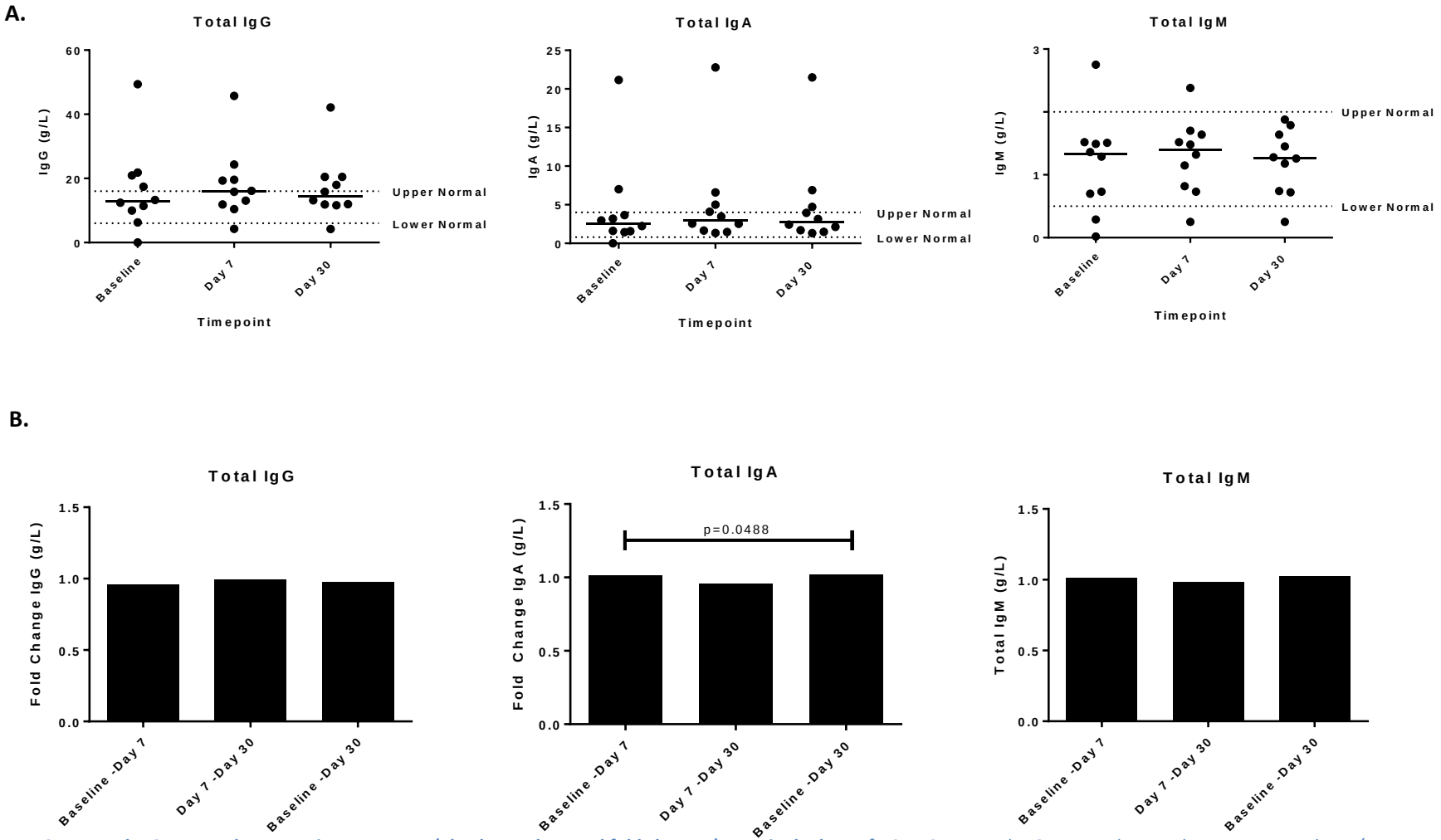


Figure 6.11. Total IgG, IgA, and IgM vaccine responses (absolute values and fold changes) to a single dose of PCV-13. A. *Total IgG, IgA, and IgM values are reported as g/L. Upper and lower normal reference values are depicted as dotted lines as described in section 7.2.6.2.1. B. Fold Changes for total IgG, A, and M. Data are reported as medians with p-values from Wilcoxon-matched pairs signed rank tests. Bold p-values are significant at p<0.05.*

Table 6.10. Total IgG, IgA, and IgM vaccine responses (absolute values) to a single dose of PCV-13

Absolute Values (g/L)	*Timepoint*			*P-values*		
Total Ig	*Baseline (N=10)*	*Day 7 (N=10)*	*Day 30 (N=10)*	*Baseline vs Day 7 post-vaccination*	*Day 7 vs Day 30 post-vaccination*	*Baseline vs Day 30 post-vaccination*
IgG	12.85 (9.05-21.1)	16.0 (11.5-20.8)	14.51 (11.8-20.5)	0.748	0.3594	0.625
IgA	2.6 (1.55-4.49)	3.01 (1.61-5.40)	2.79 (1.65-5.26)	0.4922	0.1055	0.7695
IgM	1.33 (0.60-1.51)	1.40 (0.80-1.66)	1.27 (0.74-1.68)	0.6953	0.4258	0.7695

Data are reported as medians and interquartile ranges with p-values from Wilcoxon-matched pairs signed rank tests
Bold p-values are significant at p<0.05

Table 6.11. Total IgG, IgA, and IgM vaccine response (fold changes) to a single dose of PCV-13

Fold Change	*Timepoint*			*P-values*		
Total Ig	*Baseline (N=10)*	*Day 7 (N=10)*	*Day 30 (N=10)*	*Baseline vs Day 7 post-vaccination*	*Day 7 vs Day 30 post-vaccination*	*Baseline vs Day 30 post-vaccination*
IgG	0.96 (0.91-1.11)	0.99 (0.84-1.06)	0.98 (0.83-1.07)	>0.9999	0.6953	0.4961
IgA	1.02 (0.92-1.17)	0.96 (0.93-1.02)	1.02 (0.86-1.12)	0.1602	0.5566	***0.0488***
IgM	1.01 (0.89-1.12)	0.98 (0.86-1.04)	1.02 (0.86-1.13)	0.4922	0.625	0.3594

Data are reported as medians and interquartile ranges with p-values from Wilcoxon-matched pairs signed rank tests
Bold p-values are significant at p<0.05

6.3.4 Associations between CD19⁺ B-cell populations and serum antibody responses to pneumococcal conjugate vaccination (PCV-13)

6.3.4.1 Do baseline B-cell populations predict WHO threshold responses to PCV-13?

There were associations found between baseline CD19⁺ B-cell subpopulations and Pn-specific IgG pre-and post- vaccine (day 7 and 30) antibody responses (Table 6.12). Marginal zone B-cell populations can predict Pn-specific IgG WHO threshold responses at day 7 (r=0.68, p=0.0342, strong correlation) and 30 post-vaccination (r=0.48, p=0.1593, moderate correlation). Switched memory B-cells can also predict WHO threshold responses at day 7 post-vaccination (r=0.48, p=0.1608, moderate correlation).

Switched memory (day 30), activated (day 7&30), mature activated (day 7&30), transitional (day 7 &30), and exhausted (day 30) B-cells (at baseline) were all found to predict Pn-specific WHO threshold responses; however, these associations ranged from very weak to moderate correlations.

However, naïve B-cells (day 7&30), plasmablasts (day 7&30), transitional (day 7&30), and exhausted (day 7) B-cells do not correlate with Pn-specific IgG antibody concentrations post-vaccination (as described in Table 6.12).

Table 6.12. Baseline CD19⁺ B-cell populations can predict WHO threshold responses to PCV-13

CD19⁺ B-cell population at Baseline	*Number of Pn serotypes protected at Day 7 post-vaccination*	*Number of Pn serotypes protected at Day 30 post-vaccination*
Naïve	*r= -0.65, p=0.0458*	r= -0.24, p=0.4936
Marginal Zone	***r= 0.68, p=0.0342***	r=0.48, p=0.1593
Switched Memory	r=0.48, p=0.1608	r=0.07, p=0.8529
Activated	r=0.22, p=0.5396	r=0.39, p=0.2663
Plasmablasts	r=-0.49, p=0.1546	r=-0.34, p=0.3360
Transitional	r=-0.02, p=0.9661	r=-0.28, p=0.4266
Mature Activated	r=0.23, p=0.5292	r=0.06, p=0.8673
Exhausted	r=-0.06, p=0.8708	r=0.31, p=0.3852

Univariate correlations were performed between CD19⁺ B-cell populations and number of Pn serotypes that reach WHO threshold (0.35 µg/ml) protection at Day 7 and 30 post-vaccination using Spearman rank correlation tests. The Spearman correlation coefficient is denoted as r (r=0.00-0.19; very weak correlation; r=0.20-0.39; weak correlation; r=0.40-0.59; moderate correlation; r=0.60-0.79; strong correlation; r=0.80-1.0; very strong correlation.
Bold and italicized p-values are significant at p<0.05.

6.3.4.2 Do patients who are classed as good responders versus non-responders to PCV-13 vaccination have different CD19⁺ B-cell profiles?

Patients who reach WHO threshold to $\geq$ 8 out of 12 Pn serotypes are classed as good responders in this analysis. Those who do not reach threshold values for $\leq$ 8 out of 12 Pn serotypes are classed as non-responders with impaired/absent Pn-specific IgG antibody.

There were no significant differences found between CD19⁺ B-cell subpopulation profiles and Pn-specific IgG pre-and post- vaccine (day 7 and 30) responses (Figure 6.12/Table 6.13).

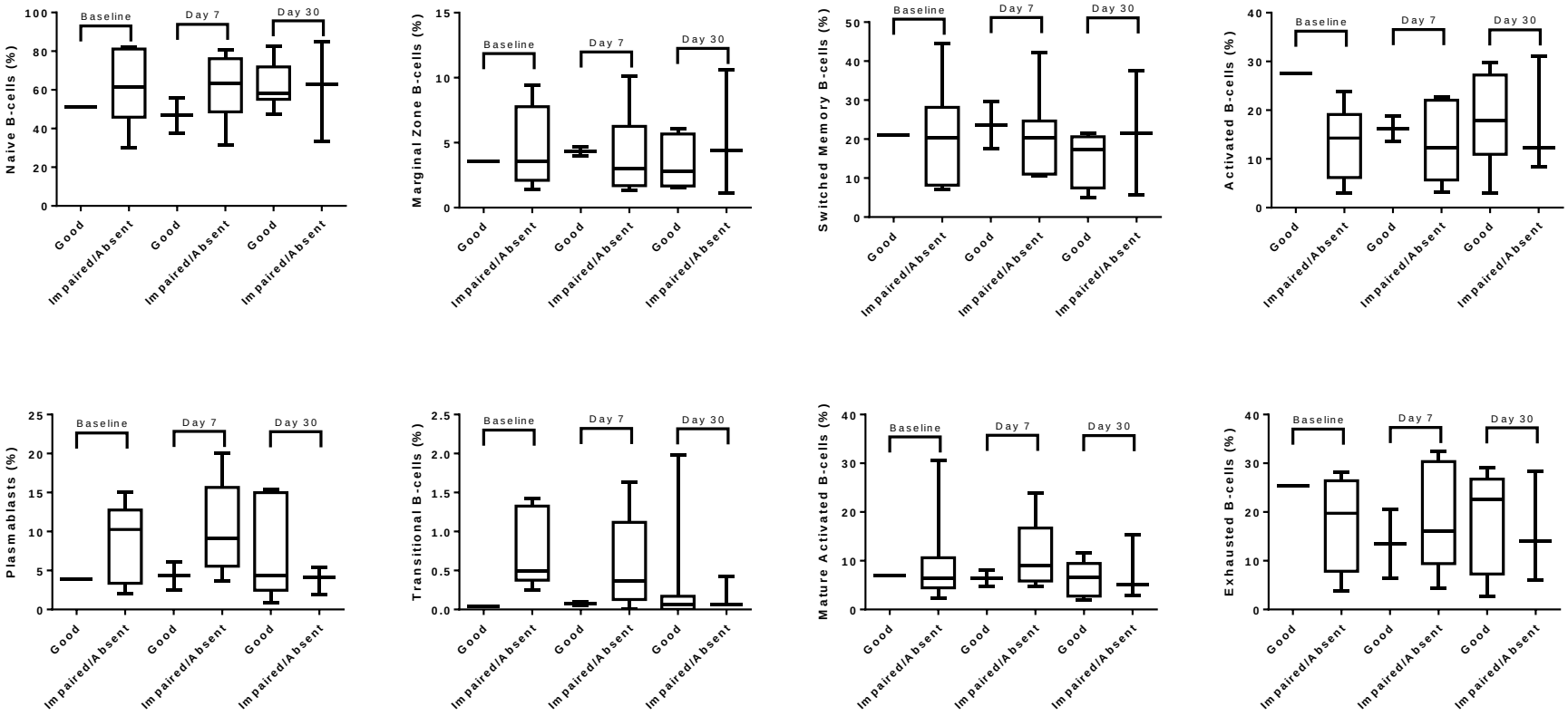


Figure 6.12. CD19⁺B-cell subpopulation percentages and relationships to good or impaired/absent responses to PCV-13 vaccination. *Good responses are classed as patients that reach WHO threshold (0.35 µg/mL) to ≥8 out of 12 Pn serotypes and impaired/absent responses are classed as patients that do not reach WHO threshold and have protection against <8 out of 12 Pn serotypes. The percentage of B-cell populations are depicted as box and whisker (minimum to maximum) plots. Mann-Whitney U non-parametrical tests were performed at all time points where p< 0.05 is considered significant.*

Table 6.13. Association between $CD19^+$ B-cell populations and vaccine responses at pre- and post-vaccination (day 7 and 30) (according to WHO threshold (0.35 µg/mL) and ≥ 8 out of 12 Pn serotypes protected)

	Timepoint (p-values)		
	Baseline (N=10)	***Day 7 (N=10)***	***Day 30 (N=10)***
$CD19^+$ B-cell population	***Good vs Impaired/Absent***	***Good vs Impaired/Absent***	***Good vs Impaired/Absent***
Naïve	N/A	0.4	>0.9999
Marginal Zone	N/A	0.5333	0.8333
Switched Memory	N/A	0.7111	0.5167
Activated	N/A	0.8889	>0.9999
Plasmablasts	N/A	0.2667	0.6667
Transitional	N/A	0.2667	0.6667
Mature Activated	N/A	0.4	0.8333
Exhausted	N/A	0.7111	>0.9999

Mann-Whitney U-tests were performed to assess relationships between $CD19^+$ B-cell populations and good versus impaired/absent vaccine responses (based on responses to WHO ≥ 8 out of 12 Pn serotypes) groups for each post-vaccination time point (day 7 and 30). Furthermore, baseline responses could not be assessed by Mann-Whitney U-tests as there was only one patient who mounted a good response to WHO ≥ 8 out of 12 Pn serotypes.
Bold p-values are significant at $p<0.05$.

6.3.4.3 Do baseline B-cell populations predict total immunoglobulin (IgG, IgA, and IgM) responses to PCV-13?

Very weak to strong correlations were found between baseline $CD19^+$ B-cell subpopulations and total IgG, IgA, and IgM (day 7 and 30) antibody responses (Table 6.14).

Transitional B-cells correlate strongly with total IgG antibody levels at day 7 post-vaccination (r=0.74, p=0.0174) and moderately at day 30 post-vaccination (r=0.54, p=0.1139). Exhausted B-cells were found to correlate moderately with total IgG at day 30 (r=0.41, p=0.2475) and total IgA at day 7 (r=0.47, p=0.1786). Marginal zone B-cells correlated moderately with total IgM at day 7 (r= 0.49, p=0.1548) and 30 (r=0.54, p=0.1139) post-vaccination. Switched memory B-cells also correlated moderately with total IgM at day 7 (r=0.47, p=0.1786) and 30 (r=0.43, p=0.2182).

Table 6.14. Baseline CD19⁺ B-cell populations can predict total immunoglobulin responses to PCV-13

CD19⁺ B-cell population at Baseline	*Total IgG (Day 7 post-vaccination)*	*Total IgG (Day 30 post-vaccination)*	*Total IgA (Day 7 post-vaccination)*	*Total IgA (Day 30 post-vaccination)*	*Total IgM (Day 7 post-vaccination)*	*Total IgM (Day 30 post-vaccination)*
Naïve	r=0.2, p=0.5837	r= 0.19, p=0.6073	r=-0.28, p=0.4271	r=-0.31, p=0.3869	r=-0.36, p=0.3129	r=-0.36, p=0.3129
Marginal Zone	r=-0.03, p=0.9460	r= -0.05, p=0.8916	r=0.04,p=0.9184	r=0.02, p=0.9730	r=0.49, p=0.1548	r=0.54, p=0.1139
Switched Memory	r=0.04, p=0.9184	r=0.05, p=0.8916	r=0.37, p=0.2957	r=0.39, p=0.2632	r=0.47, p=0.1786	r=0.43, p=0.2182
Activated	r=-0.13, p=0.7330	r=0.05, p=0.8916	r=0.39, p=0.2632	r=0.35, p=0.3304	r=-0.43, p=0.2182	r=-0.31, p=0.3869
Plasmablasts	r=0.15, p=0.6821	r=0.27, p=0.4483	r=0.08, p=0.8382	r=0.04, p=0.9184	r=0.31, p=0.3869	r=0.37, p=0.2957
Transitional	***r=0.74, p=0.0174***	r=0.54, p=0.1139	r= -0.26, p=0.4697	r=-0.30, p=0.4069	r=0.37, p=0.2957	r=0.09, p=0.8113
Mature Activated	r=0.02, p=0.9730	r=0.15, p=0.6821	r=0.30, p=0.4069	r=0.31, p=0.3869	r=0.35, p=0.3304	r=0.37, p=0.2957
Exhausted	r=0.19, p=0.6073	r=0.41, p=0.2475	r=0.47, p=0.1786	r=0.37, p=0.2957	r=-0.35, p=0.3304	r=-0.16, p=0.6567

Univariate correlations were performed between CD19⁺ B-cell populations and Total IgG and IgM serum titres using Spearman rank correlation tests. The Spearman correlation coefficient is denoted as r (r=0.00-0.19; very weak correlation; r=0.20-0.39; weak correlation; r=0.40-0.59; moderate correlation; r=0.60-0.79; strong correlation; r=0.80-1.0; very strong correlation. Bold and italicized p-values are significant at p<0.05.

6.4. Discussion

The impact of HIV-infection and changes to peripheral B-cell populations, total serum IgG, IgA, and IgM antibody concentrations, and Pn-specific IgG pre- and post-vaccination with PCV-13 at day 7 and 30 were investigated in this chapter.

Plasmablast populations were increased above normal ranges at baseline and day 7 post-vaccination with PCV-13; however, there was a reduction from day 7 to day 30 post-vaccination. Increased plasmablast populations are common in the context of HIV-infection (319,120). Interestingly, a significant reduction in the percentage of plasmablasts was seen at day 30 post-vaccination, which has not been established in prior pneumococcal conjugate vaccination studies in HIV-infection. This suggests that there is an attenuation in vaccine responses to conjugate vaccination regarding plasmablast populations. This may be due to the small group number and the fact that this is an interim analysis of a small pilot study.

It was chosen not to correlate CD4, VL, and ethnicity with vaccine responses as it was found that CD4 and VL are not robust predictors of pneumococcal vaccine responses (178,320).

Switched-memory B-cell populations were relatively normal compared to normal healthy adult reference ranges at baseline and post-vaccination time points; however, there were significant reductions seen from baseline to day 7 and 30 post-vaccination. The depletion of memory B-cell populations has been evidenced in previous studies, although not in the context of pneumococcal vaccination (124,122,289,292,293,295,321).

Marginal zone B-cell populations were very low compared to normal reference ranges as evidenced in the HIV-infected cohort across all vaccination time points. This is of concern as marginal zone B-cells play a major role in the immediate response and clearance of encapsulated bacteria, particularly pneumococcus (290) . Other studies (although not in the context of pneumococcal vaccination) have also supported my observation that $CD27^+$ B-cells (including marginal zone populations) are depleted in HIV-infection (295,322–324). This may be due to the fact that the splenic marginal zone may have atrophied following major disturbances to the germinal centres and marginal zone region even early on in HIV-infection despite being on ARV treatment (325). Interestingly, total IgM serum antibody concentrations following pre- and post-vaccination were similar to normal reference ranges with no increase in concentrations following PCV-13 vaccination. This is a novel

observation, which would need further exploration in future studies by investigating Pn-specific IgM concentrations.

All of the patients in this study had commenced ARV treatment, although due to the low number of patients in this interim analysis, correlations were not performed on the duration of ARV treatment and impact on changes to $CD19^+$ B-cell populations following pneumococcal conjugate vaccination. However, other studies (most notably, the International Network for Strategic Initiatives in Global HIV Trials (INSIGHT) Strategic Timing of AntiRetroviral Treatment (START)) have suggested that starting early ARV treatment (when patients have more than 500 $CD4^+$ T-cell counts per cubic millimetre) is beneficial than delaying to commence treatment once $CD4^+$ T-cell counts have reduced to 350 cells per cubic millimetre (274,324,326,121).

Furthermore, it was found in the HIV-infected patient cohort that there are increased activated B-cell populations pre- and post-vaccination, particularly at day 30 post-vaccination. There is also the presence of mature activated B-cells and exhausted B-cell populations, unique to HIV-infection as discussed previously in section 7.1.2. This reflects the notion that there are major disturbances to the peripheral B-cell repertoire in HIV-infection, which may reconstitute post-ARV treatment; however, as this is not a longitudinal study, it cannot be established this from this analysis.

Another interesting finding was the low numbers of immature transitional B-cells at pre-and post-vaccination time points, which also reduced further by day 30 post-vaccination suggesting attenuation post-vaccination with PCV-13. This is a novel finding as other studies have suggested that there is an increase in immature/transitional cells in HIV-infection (327,328).

Limitations of this work presented in this chapter include small group numbers as this is an interim analysis; however, these findings may be further elucidated following a final analysis including all of the patients that have completed the study. Another limitation of this work includes the gating of the transitional B-cell and plasmablast populations in section 6.2.6.1.1. These gates have been based on B-cell phenotypes from the EUROclass trial in CVID (286); however, both B-cell populations may benefit from using different gating strategies to further separate both populations. Furthermore, it is also planned to run Pn-specific B-cell ELISPOTs, which will help to identify the effects of HIV-infection and the memory B-cell response to pneumococcal conjugate vaccination (79,329).

It was also found that pre-existing immunity to pneumococcus is impaired at baseline prior to pneumococcal conjugate vaccination, with approximately 90% of the HIV-infected patients in this analysis classed as non-responders having < 8 out of 12 Pn serotypes that do not obtain WHO threshold values. However, by Day 30 post-vaccination, most patients (70%) had achieved WHO threshold protection ($\geq$ 8 out of 12 Pn serotypes).

However, total IgG, IgA, and IgM serum antibody concentrations (pre-and post-vaccination) are relatively normal compared to normal healthy adult control reference ranges. The total IgM serum antibody concentrations pre- and post-vaccination were also quite surprising considering that marginal zone B-cell populations (at all vaccination timepoints) and plasmablasts (at day 30 post-vaccination) were severely reduced in this HIV-infected cohort, which suggests attenuation of the immune response to pneumococcal conjugate vaccination. This would need to be further investigated by quantifying the Pn-specific IgM response at all pre- and post-vaccination time points. The total IgM antibody concentrations present at baseline, day 7, and day 30 post-vaccination may be due to natural, pre-existing antibody and potentially, IgM secreted by marginal zone cells (330–334).

The total IgA antibody levels appeared to decrease at day 7 and recovered by day 30 post-vaccination. This is a novel finding as one study suggested that IgA antibody levels peak at day 14 post-vaccination and then decline very rapidly (335). Another previous study found that monomeric IgA is important in the opsonophagocytic response to pneumococcal infections

Low baseline levels of Pn-specific IgG may be due to impairment and loss of memory B-cell subsets and has been documented in HIV-infection (124). This study by Titanji et al. has suggested that loss of these essential memory B-cell subsets can lead to impairment and maintenance of long-term serological memory responses (to prior infections, colonisation, and subsequent vaccination) during HIV-infection (124). Furthermore, chronic hyperactivation established in HIV-infection may lead to increased numbers of naïve B-cells, susceptible to apoptosis, which may affect the generation of circulating memory B-cell populations(288). However, increased numbers of naïve B-cells were not seen in this study, although I have evidenced that there is a reduction in switched memory B-cells post-vaccination from baseline and day 7 to day 30.

There were no associations found between circulating $CD19^{+}$B-cell responses and good versus impaired/absent responses to pneumococcal conjugate vaccination (PCV-13) at all

pre-and post-vaccination time points, but this should be considered carefully since these results reflect an interim analysis and may change once all of the patients have completed the study. Furthermore, there were not many strong correlations found between circulating $CD19^{+}$B-cells and total IgG and IgM responses except for a strong relationship found between transitional B-cells and total IgG serum antibody concentrations at day 7 post-vaccination and mature activated B-cell responses and total IgM serum antibody concentrations at day 30 post-vaccination.

In a previous study, human transitional B-cells were driven by CpG to terminal differentiate into plasmablasts producing Pn-specific IgM; however, my observation is a novel finding and will need to be further investigated in future studies (336).

The relationship between mature activated B-cell responses and total IgM serum antibody concentrations at day 30 post-vaccination could be attributed to hypergammaglobulinemia, which is well established in HIV infection, even though total IgM concentrations appear to be within normal reference ranges compared to healthy adults in my study (272). This would need to be examined by quantifying Pn-specific IgM concentrations at all vaccination time points in my study.

Several studies have associated reduced memory B-cell subsets and impaired vaccine responses in older individuals and in HIV-infected individuals (124,292,293,295,337,338). However, although I have evidenced that there are reduced plasmablasts, switched memory, marginal zone, and transitional B-cells post-vaccination, it does not appear to influence the quantitative Pn-specific IgG response to PCV-13 in HIV-infection. However, this will need to be justified by performing qualitative serum antibody testing in opsonophagocytic killing assays once all of the HIV-infected patients have completed my pilot study discussed in this chapter.

Chapter 7. Summary of findings

7.1 HIV-infection impacts the response to pneumococcal plain polysaccharide, Pneumovax-23® PPV-23, vaccination

In the AIR study, PPV-23 induced poor responses in HIV-infected individuals compared to HIV-negative controls. There was also lack of association between $CD4^+$ T-cell counts, viral load (VL), and antiretroviral treatment (ARVs) on pneumococcal vaccine responses, which challenges previous thinking that individuals with low $CD4^+$ T-cell counts, high viral loads, and delaying ARV treatment can benefit responses to pneumococcal vaccination. These findings suggested that HIV-infected patients were at risk of bacterial pneumonia and invasive pneumococcal disease even if they had high $CD4^+$ T-cell counts and were already on ARV treatment. From these findings, a single dose of PPV-23 appeared to be insufficient in the HIV-infected cohort. Thus, it was decided to investigate responses to pneumococcal conjugate vaccination with the intent of inducing a sustained protective Pn-specific antibody response in the HIV-infected cohort.

7.2 A single dose of Prevenar-13®, PCV-13, is more immunogenic than a single dose of Pneumovax-23®, PPV-23 in HIV-infected patients.

Low proportions of HIV-infected patients were found to have pre-existing Pn-specific IgG at baseline, thus there was a need to vaccinate against pneumococci. A single dose of PCV-13 was found to be more immunogenic than a single dose of PPV-23 in HIV-infected patients. A higher proportion of patients that received a single dose of PCV-13 achieved WHO threshold protection against $\geq$8 out of 12 pneumococcal serotypes included in the PCV-13 vaccine compared to those that received a single dose of PPV-23. Furthermore, opsonophagocytic (OPKA) titres did not correlate with Pn-specific IgG antibody concentrations indicating that the quantity does not relate to the quality of Pn-specific IgG, perhaps indicating humoral dysregulation in the context of HIV-infection. Further assessment of OPKA titres will need to be taken into consideration with regards to opsonic ability of Pn-specific IgG post-vaccination in future studies.

7.3 A higher proportion of HIV-infected patients achieve WHO threshold protection after two PCV-13 vaccines

A higher percentage of HIV-infected individuals achieved WHO threshold protection against pneumococcal serotypes post-vaccination with two doses of PCV-13. Patients that received a single dose of PPV-23 followed by a booster dose of PCV-13 achieved similar rates of protection to those who received a single dose of PCV-13. Additionally, patients that received a single dose of PPV-23 followed by two booster doses of PCV-13 had similar rates of protection compared to those who received two doses of PCV-13. The lower rates of threshold protection after receiving a single booster dose of PCV-13 following an initial dose of PPV-23 suggest hyporesponsiveness. However, this study demonstrates the effectiveness of boosting and that hyporesponsiveness could be overcome by boosting with PCV-13 in HIV-infected adults.

7.4 A single dose of PCV-13 provides a longer period of WHO threshold protection compared to a single dose of PPV-23

HIV-infected patients in the AIR study were followed for a period of 6 years following receipt of their initial pneumococcal vaccine dose. Patients who received an initial dose of PCV-13 demonstrated that they could retain WHO threshold protection in $\geq$ 8 out of 12 Pn serotypes for a significantly longer period (2 years) than those whose initial dose was PPV-23 (<1 year). This is an important finding as patients who are administered a dose of PPV-23 cannot be given another dose of PPV-23 or PCV-13 within five years of their initial dose due to hyporesponsiveness, thus leaving a significant proportion of patients vulnerable to pneumococcal infection and or invasive pneumococcal disease.

7.5 There are differences in IgG subclass responses to PPV-23 and PCV-13 vaccination

I have established novel MIA Pn-specific IgG subclass assays, including the assignment of Pn-specific IgG1 and IgG2 antibody concentrations to 007sp from 89SF, that allow for the investigation of pneumococcal vaccine responses. This study challenged the idea that a plain polysaccharide response is predominantly IgG2-directed and the conjugate vaccine, IgG1-directed; patients that received PPV-23 vaccination still produced a strong IgG1 response.

Furthermore, my study has highlighted that the Pn-specific IgG1 and IgG2 responses are equivalent at 4-weeks post- PCV-13 vaccination. Additionally, the Pn-specific IgG3 response was evidenced to be higher in the PCV-13 vaccine cohort compared to the PPV-23 vaccine

cohort. I have also established that there is a granularity in IgG subclass responses, which is essential to understanding the IgG response to different pneumococcal vaccines. This study shows that Pn-serotype specific assays demonstrate that responses to individual serotypes are widely variable and that this granularity cannot be visualised in combined or commercially available serotype assays.

7.6 HIV-infection impacts the immune response to PCV-13

7.6.1 Pn-specific IgG antibodies

Pn-specific IgG antibodies were found to be low at baseline in HIV-infected adults in this pilot study (B-cell responses to pneumococcal conjugate vaccination), which provides validation for pneumococcal vaccination in the context of HIV-infections. Pn-specific antibodies increase slightly at day 7 post-vaccination with a higher proportion of HIV-infected adults achieving threshold protection rates at day 30 post-vaccination. This analysis also shows the significant variations in Pn-serotype and individual responses.

7.6.2 Total Immunoglobulins (IgG, IgA, and IgM)

This analysis has shown that there is no evidence of hypergammaglobulinaemia in total immunoglobulin antibody concentrations in this HIV-infected cohort. Furthermore, the total antibody concentrations do not change with pneumococcal vaccination at day 7 or 30.

7.6.3 $CD19^{+}$ B-cell populations associated changes with pneumococcal vaccination

This analysis has found that there were low percentages of marginal zone (MZ) B-cells at baseline and following PCV-13 vaccination at day 7 and 30, which is a concern as MZ B-cells secrete IgM. Switched memory B-cells decreased post-vaccination from baseline to day 30 post-vaccination; however, there were no significant decreases in total or Pn-specific IgG at day 30 post-vaccination. There was an increased number of plasmablasts at baseline, day 7 and 30 post-vaccination, which could be due to chronic activation of the immune system in the context of HIV-infection. Additionally, activated, mature activated, and exhausted B-cells were present at baseline and post-vaccination, which confirms previous reports of these cell populations in HIV-infected adults. Lastly, transitional B-cells were activated at baseline and appeared to decrease by day 30 post-vaccination.

7.7 Summary

This study describes the immune response to pneumococcal vaccination in the context of HIV-infection. A variety of immunological methods were employed to assess the quantity and quality of Pn-specific antibodies produced in response to vaccination. Vaccine-naïve HIV-infected patients have low levels of pre-existing Pn-specific IgG antibody, thus vaccination is essential in the prevention of pneumococcal infection and IPD. It was established that PPV-23 induces poor responses in HIV-infected adults, which is not associated with CD4 counts, viral load, or ARV treatment. Additionally, the AIR study evidenced that PCV-13 induces a robust immune response in HIV-infected patients compared to PPV-23. Patients who receive an initial dose of PCV-13 can maintain threshold protective antibody concentrations for a longer duration than those who receive an initial dose of PPV-23. Furthermore, this study has evidenced that boosting is effective in HIV-infected adults and can potentially overcome hyporesponsiveness. Additionally, the Pn-specific antibody response is multi-faceted and IgG subclass responses are important in understanding the granularity of the immune response to PPV-23 and PCV-13 vaccination. There were perturbations to the $CD19^{+}$ B-cell compartment in HIV-infected patients at baseline because of humoral dysregulation, which was associated with responses to PCV-13 vaccination. Collectively, these findings should be taken into careful consideration when deciding optimal pneumococcal vaccination schedules in HIV-infected patients.

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