# Humoral immunity in patients with chronic kidney disease and their response to pneumococcal immunization.

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## Dedication

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Table of Contents

## List of Tables

**Chapter 2**



**Chapter 3**



**Chapter 4A**



**Chapter 4B**

## List of Figures

**Chapter 2**



**Chapter 3**



**Chapter 4A**

**Chapter 4B**

**List of Abbreviations**

AP-1: Activator protein 1

APC: Allophycocyanin

ASC: antibody secreting cells

ANOVA: analysis of variance

BAFF: B cell activating factor

BAFFR: B cell activating factor receptor

BCR: B cell receptor

BMI: body mass index

BTK: Bruton's tyrosine kinase

CAP: community acquired pneumonia

CBC: complete blood count

CCR7: C-C chemokine receptor type 7

CD: cluster of differentiation

CKD: chronic kidney disease

CI: confidence intervals

CIRN: Canadian immunization research network

CS memory: class switched memory B cell

COPD: chronic obstructive pulmonary disease

$CO_2$: carbon dioxide

CpG-ODN: CpG-Oligonucleotide

CVID: common variable immunodeficiency

$CRM_{197}$: nontoxic variant of diphtheria toxin isolated from Corynebacterium diphtheriae strain

CXCR5: C-X-C chemokine receptor type 5

C7 (β197) grown in a casamino acids and yeast extract-based medium

C1q: complement component 1q

°C: degree Celsius

DL1: delta-like 1

DN: Double Negative

ELISA: enzyme-linked immunosorbent assay

ELISPOT: enzyme-linked immunospot assay

FBS: fetal bovine serum

FL: Flow cytometry fluorescence channel

FITC: Fluorescein isothiocyanate

FN: First Nations

FN-CKD: First Nations adults with chronic kidney disease

FNNO: First Nations adults from northwestern Ontario

FNSO: First Nations adults from southern Ontario

GFR: glomerular filtration rate

GMC: geometric mean concentration

GMT: geometric mean titre

HbOC: Hib oligomers conjugated to the mutant diphtheria toxin $CRM_{197}$

*H. influenzae/* Hi: *Haemophilus influenzae*

HC: healthy control

HIV: human immunodeficiency virus

HSCT: hematopoietic stem cell transplantation

HRP: horseradish peroxidase

Ig: immunoglobulin

IL: interleukin

IPD: invasive pneumococcal disease

*K. pneumoniae*: *Klebsiella pneumoniae*

L: liter

LOS: lipooligosaccharide

LPS: lipopolysaccharide

MAML1: mastermind-like 1 protein

MHC II: major histocompatibility complex class II

mHSA: methylated human serum albumin

µg: microgram

mL: milliliter

NFAT: nuclear factor of activated T cells

NFN-CKD: non-First Nations adult with chronic kidney disease

NFNC: non-Indigenous elderly non-frail adults from across Canada

NFNK: non-First Nations adults from Kenora

NFNT: non-First Nations adults from Thunder Bay

NF-kB: nuclear factor kB

NML: National microbiology laboratory

NTHi: nontypeable *Haemophilus influenzae*

OCAP: Ownership, Control, Access, and Possession

OPA: opsonophagocytic assay

*P. aeruginosa: Pseudomonas aeruginosa*

PBS: phosphate buffered saline

PBMC: peripheral blood mononuclear cells

PCV (PCV7, PCV10, PCV13): pneumococcal protein-polysaccharide conjugate vaccine

PCV13: 13-valent pneumococcal protein-polysaccharide conjugate vaccine

PE: Phycoerythrin

PerCP-Cy™5.5: peridinin chlorophyll protein coupled to the cyanine dye Cy™5.5

Pneumococcus/ *S. pneumoniae: Streptococcus pneumoniae*

PPV23: 23-valent pneumococcal polysaccharide vaccine

PRP: polysaccharide polyribosylribotol phosphate

PRP-D: polysaccharide polyribosylribotol phosphate covalently linked to diphtheria toxoid

PRP-OMP: polysaccharide polyribosylribotol phosphate covalently linked to *Neisseria meningitidis* group B outer membrane protein

PRP-T: polysaccharide polyribosylribotol phosphate covalently linked to tetanus toxoid

PVDF: Hydrophobic Polyvinylidene Fluoride

RBP-Jk: Recombination signal binding protein for immunoglobulin-Jk

ROS: reactive oxygen species

RPMI: Roswell Park Memorial Institute Medium

SAC: *Staphylococcus aureus* Cowan strain protein A

SBA: serum bactericidal assay

SLMHC: Sioux Lookout Meno Ya Win Health Center

SOS: Serious Outcomes Surveillance

ST: serotype

TCPS2: Tri-Council Policy Statement: Ethical Conduct for Research Involving Humans

TIV: trivalent inactivated influenza vaccine

T1 / T2 B cell: transitional 1/ transitional 2 B cell

TLR4: Toll-like receptor 4

TBRHSC: Thunder Bay Regional Health Sciences Centre

TIBDN: Toronto Invasive Bacterial Diseases Network

**Abstract**

In northwestern Ontario the Indigenous First Nations (FN) population have an increased prevalence of chronic kidney disease (CKD). There is an increased rate of infectious disease caused by *Streptococcus pneumoniae* (pneumococcus) and *Haemophilus influenzae* in the FN population. The 23-valent pneumococcal polysaccharide vaccine (PPV23) and 13-valent pneumococcal protein-polysaccharide conjugate vaccine (PCV13) are used to prevent pneumococcal disease in Canada. Since the implementation of *H. influenzae* type b (Hib) vaccine programs, invasive *H. influenzae* type a (Hia) disease has replaced invasive Hib disease in the FN population. However, there is no vaccine to prevent disease caused by non-Hib strains.

To maintain immunological memory in adults, stimulation of pre-existing memory is achieved through immunization. To determine if patients with CKD have the ability to respond to conjugate vaccines specific for Hia or pneumococcus, naturally acquired humoral immunity against these pathogens must be evaluated. Immunization with PPV23 is recommended for adults with CKD, however PPV23 is suboptimal at inducing antibody responses in adults with CKD compared to healthy adults. PCV13 is recommended for certain immunocompromised adults, but only two studies evaluated the immunogenicity of PCV13 in adults with CKD with conflicting results. To determine if PCV13 is immunogenic in patients with CKD and the effect of previous PPV23 immunization on subsequent immunization with PCV13, quantification of B-cell subpopulations, pneumococcal specific B cells and their relationship with antibody responses to PCV13 must be evaluated.

The results suggest that the increased prevalence of invasive disease caused by pneumococcus and Hia in the FN population is not due to decreased numbers of B cells or concentrations of naturally acquired antibodies. The increased prevalence of diseases such as

CKD causing immune dysfunction in a higher proportion of the FN population increases their risk for invasive disease. Results suggest that a new Hia-conjugate vaccine may be immunogenic in adults with CKD, as it will potentially re-activate Hia specific immunological memory. Our findings suggest that previous immunization with PPV23 has a negative effect on the humoral immune response that results in long-term changes in B-cell subpopulations and decreases antibody responses to subsequent immunization with PCV13 in patients with CKD.

## Co-authorship statement

This dissertation consists of six chapters, two of the chapters have been published and the remaining chapters (except chapters 1 and 5) are part of larger studies that are in preparation for publication but are continuing to collect data. For the published studies (chapters 2 and 4A) the roles of each contributing author will be outlined, and for the unpublished studies (chapters 3 and 4B) the roles of individuals that contributed significantly to these chapters will be outlined and will be referred to as authors. Chapters 2 – 4B each have an acknowledgment section which describes additional contributions.

**Chapter 1**: Literature Review

Author: Gabrielle Nicole Gaultier

**Chapter 2**: Natural immunity against *Haemophilus influenzae* type a and B-cell subpopulations in adult patients with severe chronic kidney disease.

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Authors contributions: MU and WM conceived the study; MU supervised the study; GNG recruited participants and collected samples, optimized and performed experiments, analyzed the data and drafted the manuscript; MU revised the manuscript draft. All authors reviewed the results and text and approved the final version of the manuscript.

**Chapter 3**: Antibodies specific for *Streptococcus pneumoniae* serotypes 6B and 14 in Canadian First Nations and Non-First Nations adults.

Authors: Gabrielle N. Gaultier, Eli B. Nix, Joelle Thorgrimson, William McCready, Douglas Boreham, Marina Ulanova

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**Chapter 4B**: Association of pneumococcal antibody levels with B-cell subpopulations in the peripheral blood of patients with CKD.

Authors: Gabrielle Nicole Gaultier, Angele Desbiens-Forget, Brenda Huska, William McCready, Marina Ulanova

Authors contributions: MU and WM conceived the study; MU supervised the study; GNG and AD recruited participants and collected samples; GNG performed all B cell experiments; AD, and BH performed ELISA experiments; GNG analyzed the data and drafted the manuscript; MU revised the manuscript draft.

## Chapter 1A: Literature Review

### Introduction

Chronic kidney disease (CKD) is a serious disease that is characterized by high mortality rates. Cardiovascular disease and infection are the top two causes of mortality in this group. In northwestern Ontario the Indigenous population (First Nations peoples) have an increased incidence of CKD (1). First Nations peoples in northwestern Ontario also have an increased rate of infectious disease, including disease caused by *Streptococcus pneumoniae* and *Haemophilus influenzae* (2). In this review the issues associated with CKD resulting in immune dysfunction, focus on humoral immune responses and the importance of immunological memory will be discussed.

There are currently two types of pneumococcal vaccines available in Canada (PPV23: 23-valent pneumococcal polysaccharide (PPV23) and PCV13: 13-valent pneumococcal protein polysaccharide vaccine) to prevent disease caused by *S. pneumoniae* in infants, immunocompromised adults and the elderly. However, studies have shown that the capsular polysaccharide vaccines are less immunogenic in some immunocompromised adults compared to protein-polysaccharide conjugate vaccines (3, 4). This chapter will describe the differences between the types of pneumococcal vaccines, as well as the vaccine recommendations for adults at increased risk for invasive pneumococcal disease (IPD). Current data available on the humoral immune responses of patients with CKD in response to pneumococcal immunization will investigate the differences in immunogenicity between pneumococcal vaccines.

As of present, there is no vaccine available to prevent disease caused by non-*H. influenzae* type b strains. Since the implementation of publicly funded *H. influenzae* type b (Hib) vaccine programs, invasive disease caused by *H. influenzae* type a (Hia) has replaced the

invasive Hib disease in the Indigenous population. To understand the need for the development of a Hia vaccine, current epidemiological data on the prevalence of invasive Hia disease as well as naturally acquired humoral immunity against Hia in the northwestern Ontario First Nations population will be investigated.

**Chronic kidney disease**

CKD is a common condition and is considered a major public health issue with 3,467,822 (95% uncertainty interval (UI): 3,213,111 – 3,766,495) Canadians being affected by this disease as of 2017. CKD affects approximately 9.1% (95% UI: 8.5 – 9.8) of the global population (5). Mortality rates are high amongst this population, more than double the rate of people without CKD at 103.0 per 1000 patient-years when adjusted for age, sex, and ethnicity (6). CKD is defined as the presence of kidney damage for more than 3 months, and the 5 stages of CKD are based on the glomerular filtration rate (GFR). A rate of less than 60 mL/ min/ 1.73 $m^2$ is considered abnormal, and greater than this is considered abnormal if accompanied by abnormal urine sediment, abnormal imaging or biopsy results. Stages 1 to 3 range from kidney damage with normal/ increased GFR (stage 1, $\geq$ 90 mL/ min/ 1.73 $m^2$) or mildly decreased GFR (stage 2, 60-89 mL/ min/ 1.73 $m^2$) to moderately decreased GFR (stage 3, 30-59 mL/ min/ 1.73 $m^2$). Severe or end-stage CKD includes stages 4 (15-29 mL/ min/ 1.73 $m^2$) and 5 (< 15 mL/ min/ 1.73 $m^2$) (7).

Once a patient has reached a GFR of less than 15 mL/ min/ 1.73 $m^2$ they require renal replacement therapy which can be achieved through dialysis or kidney transplantation (8). Hemodialysis was the first intervention that was able to partially replace lost kidney function and is used to prevent or decrease the progression of CKD by decreasing further damage caused by

waste materials (9, 10). This process involves blood diffusing through a semipermeable membrane by means of an electrochemical concentration gradient to remove excess waste products such as urea. (11). A solution called dialysate, containing sodium, potassium, calcium, magnesium, chloride, bicarbonate and glucose or dextrose is required for hemodialysis as it aids in the removal of low molecular weight molecules such as urea and restores the extracellular pH and concentration of electrolytes (12).

**Uremic syndrome and oxidative stress**

Uremic syndrome (uremia) is the retention of compounds normally excreted in the urine by the kidneys. Uremia causes significant metabolic issues and is a serious condition which affects the whole body. The increased amounts of these compounds interfere with biological functions and result in toxicity. These retained compounds can become altered through oxidation causing them to become pro-inflammatory (13). Oxidative stress contributes to the progression of CKD by promoting apoptosis and inflammation. Oxidative stress is also a consequence of other conditions that are commonly associated with CKD such as diabetes, hypertension, and atherosclerosis that also contribute to kidney damage. The hemodialysis process has been found to promote the production of reactive oxygen species (ROS), as well as inflammation. In patients undergoing hemodialysis, the amounts of ROS in plasma are increased and antioxidant levels are decreased. Exposure to the dialysis membrane has also been associated with increased complement factors, platelets and polymorphonuclear white blood cells (14).

**CKD and dysfunction of the immune system**

Increased inflammation is a major issue that results in the development and progression of cardiovascular disease, which is the number one cause of mortality in patients with CKD followed by infection (15, 16). Patients with CKD are at an increased risk for infection because they are immunocompromised. Patients with CKD are unique among other high-risk groups because they have multiple factors predisposing them to infection. Aging of the immune system is referred to as immunosenescence, and is a decline in the immune system resulting in increased susceptibility to disease, increased inflammation and poorer response to immunization (17). Severe CKD related changes in the immune system that have resulted in decreased immune function comparable to healthy elderly adults is known as premature immunological aging. In patients with CKD, this is characterized by uremia, increased oxidative stress and increased inflammation (18).

The principle clinical feature of uremia in regard to the immune system is low response to vaccination and increased sensitivity to infectious diseases (19). Chronic kidney disease and uremia affects multiple cells and functions of the immune system including: decreased bactericidal ability of neutrophils, decreased granulocyte and monocyte/ macrophage phagocytic function, defective antigen presenting capacity of antigen presenting cells, reduced numbers of B cells and decreased antibody producing capacity of plasma cells, increased T- and B cell apoptosis, decreased thymic T-cell output, and impaired activation of T-cell response (20) (21).

Among immune abnormalities, patients with CKD have noticeable alterations in B cells and B-cell functions. It is also thought that epigenetic changes caused by CKD decrease production of lymphoid cells at the hematopoietic stem cell level (22) resulting in patients with CKD having decreased absolute numbers of B cells and T cells (23). Pahl *et al.* 2010 found that patients with CKD had decreased numbers of B cells as well as decreased naïve and memory B

cells compared to healthy controls. They concluded that B cell lymphopenia in patients with CKD was due to uremia related decrease in transitional B cell BAFF receptor expression, causing a decrease their survival (24). Patients with CKD also have an increased incidence of underlying diseases which also contribute to their susceptibility to infections such as diabetes mellitus, hypertension, cardiovascular disease, COPD, cancer, dyslipidemia and peripheral vascular disease (25, 26). We must also consider that these patients have an increased exposure to infectious agents due to disruptions in the skin from needles and venous catheters, as well as an increased frequency of exposure to the hospital environment and proximity to other patients (27).

**CKD in the Canadian Indigenous population**

In North America, CKD disproportionally affects Indigenous populations. The rate of CKD amongst Indigenous people is two to four times higher than in non-Indigenous people (28). The burden of CKD among Indigenous peoples is mostly influenced by the high prevalence of obesity and type 2 diabetes mellitus, which results in an increased rate of diabetic nephropathy, and an increased prevalence of glomerulonephritis (29, 30). In northwestern Ontario, the population of First Nations Indigenous peoples has been found to have a higher proportion of adults with CKD compared to non-Indigenous adults. Specifically, CKD has a high prevalence in the First Nations population of northwestern Ontario. The rate of CKD in the First Nations population (7%) is double the rate of the general Canadian population. This high rate of CKD along with other conditions (e.g. diabetes) results in an increased rate of cardiovascular disease (1).

## Humoral Immunity

Adaptive immunity can be separated into humoral immunity and cell mediated immunity. Cell mediated immunity involves two types of T cells, CD4+ (helper) and CD8+ T cells (cytotoxic). Humoral immunity is mediated by B cells, which upon activation can proliferate and differentiate into memory B cells and plasma cells which produce antibodies. Response to immunization is assessed by quantifying the humoral immune response. The production of class switched antigen-specific antibodies and class switched memory B cells are indicators that immunological memory against a pathogen has been established. This next section will discuss the components of humoral immunity and B-cell responses to antigens.

## Cellular development and maturation of B cells

B cells originate from hemopoietic stem cells in bone marrow, beginning as common lymphoid progenitor cells. B cells develop from pro-B cell to pre-B cell to immature B cells through the rearrangement of light and heavy chain gene segments. The B cells leave the bone marrow once the surfacing of pre- B cell receptor μ chains results in the expression of IgM. These B cells migrate through the blood as transitional B cells to the secondary lymphoid organs such as the lymph nodes and spleen. Transitional B cells then develop into mature B cells that are called either follicular or marginal zone B cells after entering the secondary lymphoid organs (31). It is important to note that in the secondary lymphoid organs in humans, the follicles are not separated from the marginal zone by sinuses like in murine models which are often described in the literature (32). There are three lineages of B cells which have been identified in rodents B-1, B-2 and marginal zone B cells. However, it has been suggested that human B cells are more similar to B-2 cells, which give rise to both follicular and marginal zone B cells. Human

marginal zone B cells have the ability to re-circulate unlike murine models which remain in the spleen. Some human marginal zone B cells have undergone somatic mutations (IgM memory B cells, IgM+CD27+) unlike murine marginal zone B cells which are not mutated and are described as pre-activated naïve B cells that do not undergo genetic diversification. Human follicular B cells and rodent B-2 cells are the main subsets in humans and rodents. Similarly, both undergo somatic hypermutation and class switching (33).

The process of a B-2 cell becoming either a follicular or marginal zone B cell begins with transitional 1 (T1) B cells maturing into transitional 2 B cells (T2). This is achieved through tonic BCR signals, B cell activating factor (BAFF) survival signals and non-canonical nuclear factor-κB (NF-κB) signals. Weak BCR signals initiate the process of a T2 cell becoming a marginal zone B cell. Signals from delta-like 1 (DL1) and Notch2 through interactions with endothelial cells, causes the activation of mastermind-like 1 (MAML1) and RBP-Jκ. When BAFF interacts with the BAFF receptor (BAFFR), this activates the canonical NF-κB pathway. Additional signals from integrin signaling and a coupled Gα12 or Gα13 serpentine receptor help B cells to remain in the marginal zone. Follicular B cells develop when T2 B cells receive strong BCR signals, this activates Bruton's tyrosine kinase (BTK) which blocks Notch2 signaling and leads to the activation of the canonical NF-κB pathway. This results in commitment to the follicular B cell fate also known as follicular type 1 B cell. BAFFR activation is not required for dedication to this cell fate but is required for survival. There is another type of follicular B cell (follicular type 2) that derives from T2 B cells through tonic BCR signals, BAFF survival signals and non-canonical NF-κB signals. It has also been thought that these cells are a potential reservoir for marginal zone B cells (33).

**B cell receptors**

B cell receptors (BCR) are transmembrane proteins responsible for antigen recognition. A single BCR consists of two immunoglobulin (Ig) heavy and two light chains, which are responsible for ligand binding. In addition, the BCR also consists of two subunits Igα and Igβ, which are responsible for signal transduction. The constant region of the heavy chain is classified as either IgM, IgD, IgG, IgA or IgE isotypes. Naïve B cells express IgD and IgM, while the other isotypes are expressed on activated and memory B cells (34).

**T cell-independent B-cell responses**

T cell-independent antigens are antigens that can activate a B cell without T cell help. T cell independent type 1 (TI-1) antigens can cause B cells to proliferate and differentiate through polyclonal stimulation. An example would be bacterial lipopolysaccharide (LPS) which can activate B cells through toll-like receptor 4 (TLR4). T cell independent type 2 (TI-2) antigens have highly repetitive structures (e.g. bacterial capsular polysaccharides), which activate B cells by crosslinking of BCRs and B cell coreceptors (CD19, CD21, CD81). BCR cross-linking activates Src-family tyrosine kinases (Blk, Fyn, and Lyn) followed by the activation of Syk tyrosine kinase which results in the activation of transcription factors NF-κB, NFAT and AP-1. This induces gene transcription that results in cell proliferation and differentiation (35). Marginal zone B cells respond to TI-1 antigens. For example, these cells would respond to the capsular polysaccharides included in 23-valent pneumococcal polysaccharide vaccine (PPV23). These cells are important for rapid response to pathogens and differentiate into plasma cells that secrete IgM (36). Memory B cells can be produced by a T cell-independent response, however, these cells do not have enhanced sensitivity upon re-stimulation with the same antigen (their response

is more similar to that of a naïve B cell rather than a memory B cell) and they are not long lived like class switched memory B cells produced during a T cell-dependent response (37).

**T cell-dependent B-cell responses**

T cell-dependent responses occur in response to antigens such as proteins and involve interactions between follicular B cells and helper T cells (CD4+). For example, responses to protein-polysaccharide conjugate vaccines such as the 13-valent pneumococcal protein-polysaccharide vaccine results in a T cell-dependent response. In order for a B cell to be activated by a T cell-dependent antigen, two signals are required. The first is from the antigen activating BCRs and the second is from a T cell that has been activated by the same antigen. T cells are initially activated by dendritic cells that have displayed a protein antigen from extracellular microorganisms as a small peptide bound to a class II major histocompatibility complex (MHC) molecules. These helper T cells differentiate into effector T cells, which migrate towards the edges of lymphoid follicles. B cells that have been stimulated by the same antigen also migrate towards the edges of lymphoid follicles due to changes in chemokine receptor expression (e.g. CCR7 and CXCR5). The B cell displays the endocytosed antigen as a class II MHC peptide. The effector T cell that is able to recognize the class II MHC peptide presented by the B cell, also expresses CD40 ligands. This activates the production of cytokines (e.g. IL-2, IL-4 etc.) which results in the initial proliferation of B cells and antibody production. Some of the T cells that were activated by the B cells migrate to adjacent follicles that have high numbers of B cells which are then called follicular helper T cells. The B cells that interacted with the follicular helper T cells migrate back into lymphoid follicles. Here they divide rapidly forming a germinal center where proliferation, isotype switching, somatic hypermutation, and

affinity maturation occurs. This produces memory B cells, long-lived plasma cells and class switched, high affinity antibodies. The memory B cells produced will be able to respond rapidly upon secondary exposure to the antigen and when stimulated will result in the production of high affinity class switched antibodies (31, 35, 38).

**Antibodies**

The five antibody isotypes have different biological roles. IgM antibodies are produced during the primary immune response to an antigen. These antibodies are polyreactive and have low affinity to an antigen because they have not undergone somatic hypermutation. However, these antibodies are able to respond rapidly to multiple antigens. IgM is superior to other isotypes when activating the classical complement pathway and is able opsonize pathogens resulting in phagocytosis. On mucosal surfaces, IgA is the most predominant antibody isotype. It is able to neutralize toxins, bind microorganisms and prevents adhesion of microorganisms to mucosal surfaces. There are two subclasses of IgA, IgA2 is the more predominant subclass on mucosal surfaces and is less susceptible to bacterial proteases than IgA1. IgG is the most predominant isotype in the body and has the longest half-life. These antibodies aid to neutralize toxins and viruses as well as opsonization of microorganisms for phagocytosis. IgG antibodies are classified into four subclasses. IgG1 and IgG3 antibodies are produced in response to a protein antigen while IgG2 and IgG4 antibodies are typically produced in response to polysaccharide antigens, upon secondary exposure to an antigen. IgG antibodies are important for classical complement pathway activation, but the affinity for C1q is not equal for all subclasses with IgG3 having the highest affinity followed by IgG1 and IgG2, while IgG4 is unable to activate the classical complement pathway. IgE is found in the lowest concentrations in

serum but binds to an antigen with high affinity. It is present during allergic reactions and in response to infection by parasitic worms. The function of circulating IgD is unclear, but it has been found to react with specific bacterial proteins (e.g. IgD binding protein of *Moraxella catarrhalis*) (39).

**Pathogens of interest: *Haemophilus influenzae* and *Streptococcus pneumoniae***

The Canadian Indigenous population has increased rates of invasive bacterial diseases such as invasive pneumococcal disease (IPD), invasive *Haemophilus influenzae* disease, invasive Group A streptococcal disease, invasive meningococcal disease, and invasive Group B streptococcal disease (2). Due to the immune dysfunction associated with CKD, they are immunocompromised and therefore at an increased risk for invasive disease caused by *Haemophilus influenzae* and *Streptococcus pneumoniae*. In the next section of this review, these two pathogens as well as epidemiology of invasive diseases they cause in Canada, and immunization against these pathogens will be discussed.

***Haemophilus influenzae***

*Haemophilus influenzae* (*H. influenzae*) is a Gram-negative, coccobacillus, which inhabits the upper respiratory, urinary and genital tracts of humans. It is categorized based on the presence or lack of a polysaccharide capsule. The encapsulated forms are classified as serotypes a - f, and the non-encapsulated are referred to as nontypeable (NTHi). *H. influenzae* can spread through respiratory droplets and have been found to be a major cause of invasive (bacteremia, meningitis, bacteremic pneumonia, epiglottitis, septic arthritis) and non-invasive infections (otitis media, sinusitis) (40, 41).

**Invasive disease caused by *Haemophilus influenzae* in Canada**

Prior to the introduction of the *H. influenzae* type b (Hib) vaccine in Canada in 1988, Hib was the most predominant serotype that caused invasive disease. Since the introduction of the Hib vaccines, invasive disease caused by Hib has decreased by 95% from 1.76 cases per 100,000 population (1979–1988) to 0.08 cases per 100,000 population (2006–2012) (42). Invasive disease caused by non-Hib strains became nationally notifiable in 2007, however not all provinces or territories reported these cases to the Public Health Agency of Canada. Based on the available data on non-Hib invasive disease, there was an average incidence of 1.36 per 100,000 population per year (range was 0.87 – 1.87 per 100,000 population per year) between 2007 and 2017 (43). Based on the limited data available, Tsang and Ulanova (2017) (44) summarized invasive *H. influenzae* disease since the implementation of publicly funded Hib vaccine programs. Of the encapsulated serotypes, invasive disease caused by H. *influenzae* type f (Hif) is the most common amongst the North American and European Caucasian infants, immunocompromised individuals and elderly adults. NTHi strains have also been major causes of invasive disease and caused more disease than Hif. *Haemophilus influenzae* type a (Hia) has replaced Hib and has become a significant cause of invasive diseases in the Indigenous peoples of North America residing in Alaska, northern Canada, southwestern US as well as Australia. Invasive Hia disease is most prevalent in children under 5 years old, and adults with immunocompromising conditions or comorbidities (44).

**Invasive *Haemophilus influenzae* disease in northwestern Ontario**

The study by Cerqueira *et al*. (2019) (45) reported the most recent epidemiological data on invasive *H*. *influenzae* disease in northwestern Ontario over seven years between 2011 – 2018 based on findings in Thunder Bay, Ontario. There were 24 cases of invasive *H. influenzae* disease in individuals between 0 – 88 years old (mean age = 51.4 years). Of these cases 15.4% occurred in children under 3 years old and 25% of all cases occurred in people of Indigenous descent. A large proportion (70.8%) of cases had major comorbidities that potentially decrease immune function such as type 2 diabetes mellitus, chronic obstructive pulmonary disease, etc. Of the isolates that went for serotype identification (22 of the 24 total cases), 9.1% were Hib, 40.9% were Hia and 50.0% NTHi. The invasive Hia cases resulted in severe disease such as bacteremic pneumonia, septic shock, and epiglottitis with a 11% fatality rate (45). This study demonstrates that there are adults at risk for invasive Hia disease that could benefit from immunization with the Hia conjugate vaccine. Eton *et al*. (2017) (46) investigated the incidence of invasive *H. influenzae* disease in Sioux Lookout, Ontario between 2010 – 2015. The local hospital Meno Ya Win Health Centre, services 28 communities in northwestern Ontario and has a total catchment population of 29,000 with 82% of patients being Indigenous. There were 10 invasive *H. influenzae* cases of which 90% occurred in Indigenous peoples aged 3 months to 71 years old (mean age = 33.4 years old). Thirty percent of these cases occurred in children aged 3 – 35 months. Of the 10 cases, information regarding potential immune disorders was available for 8 patients (low birth weight, type 2 diabetes mellitus, alcoholism, chronic lymphatic leukemia etc.). The average annual incidence rate of invasive *H. influenzae* disease was 6.25/ 100, 000 population, with the highest rate occurring in the 71 – 75 years old range (35.5/ 100,000/ year). The most common serotype was Hia which accounted for 50% of cases, 20% were NTHi, one case was Hie, one case was Hib and the last case was an unknown non-Hib strain. Fifty percent

of cases had pneumonia, of these cases one was associated with septic shock, two with sepsis and one with pericarditis. There was a case fatality rate of 10% (46). These studies emphasize that there is a growing need for development of immunizations against Hia and non-typeable *H. influenzae* strains in the Indigenous population.

## Risk factors for invasive *Haemophilus influenzae* disease in the North American Indigenous population

The study by Tsang *et al.* (2014) (41) summarized the risk factors for invasive *H. influenzae* disease. A major issue for the Indigenous population is low socioeconomic status. This is associated with poor living conditions such as overcrowding and lack of adequate amounts of clean water. Indicators of low socioeconomic status, such as indoor wood heating, poor hygiene, rodents in the home, multiple children in the family, shared childcare facilities, parental smoking and lack of breastfeeding also have an association with increased risk for invasive *H. influenzae* disease. The indicators of low socioeconomic status listed increase the transmission rates of the pathogen in these communities, resulting in a higher probability of colonization of the pathogen. These communities also have a high burden of chronic conditions such as obesity, diabetes, chronic obstructive pulmonary disease, tuberculosis and substance abuse issues that increases their risk for invasive *H. influenzae* disease (41).

## Immunization against invasive *Haemophilus influenzae* type b disease and recommendations in Canada

The Hib conjugate vaccine consists of the capsular polysaccharide polyribosylribotol phosphate (PRP) covalently linked to a carrier protein such as tetanus toxoid (PRP-T), diphtheria

toxoid (PRP-D or *Neisseria meningitidis* group B outer membrane protein (PRP-OMP) as well as Hib oligomers conjugated to non-toxic variant of diphtheria toxin CRM197 (HbOC) (41). In Canada for children under 5 years, a schedule that administers between 1 – 3 doses of Hib conjugate vaccine based on age that first dose was received is recommended. For individuals ≥ 5 years, Hib immunization is recommended for those with asplenia, hyposplenism, cochlear implant, congenital immunodeficiency, HIV, hematopoietic stem cell transplantation (HSCT) recipients, malignant hematologic disorders and organ transplant recipients. (47).

**Immunogenicity of *Haemophilus influenzae* type b conjugate vaccine in adults with CKD**

Due to the increased risk of invasive Hib disease in the Indigenous population and adults with CKD, our group has previously assessed humoral immune responses to the Hib PRP-T vaccine (Act-Hib; Sanofi Pasteur) in adults with CKD receiving hemodialysis between 24 – 80 years old in adults residing in northwestern Ontario. PRP IgG geometric mean concentrations (GMC) were determined with enzyme-linked immunosorbent assay (ELISA) and the functional abilities of Hib specific antibodies was reported as geometric mean titres (GMT) determined using the serum bactericidal assay (SBA). One-month post-immunization, IgG GMCs increased 23-fold and 97% of patients had a minimum antibody concentration of 1 μg/ mL; SBA GMTs also increased significantly. There was a correlation between the post-immunization IgG concentrations and the SBA GMTs in CKD patients which indicated the functional importance of the antibodies generated from immunization with PRP-T. IgG concentrations decreased significantly at 6 months compared to 1 month post-immunization, but did not change significantly between months 6, 9 or 12 post-immunization. However, antibody GMTs and SBA GMTs at 12 months remained significantly higher compared to pre-immunization. These results

were comparable to the healthy controls. Although this is the only study to assess antibody responses to Hib immunization in adults with CKD, this study demonstrated that adults with CKD are able to develop antibodies specific for Hib PRP capsular polysaccharide and the presence of IgG suggests that class switched memory B cells were produced in response to the PRP-T Hib conjugate vaccine (48).

**Naturally acquired immunity against *Haemophilus influenzae* type a in First Nations Adults**

Although there is no vaccine to protect against disease caused by Hia, our group has assessed naturally acquired humoral immunity against Hia in adults. This was performed in order to determine if the increased incidence of invasive disease caused by Hia in the Indigenous population of northwestern Ontario was due to lack of Hia specific antibodies. Nix *et al*. (2015) measured IgG and IgM GMCs as well as SBA GMTs specific for Hia in healthy adults and adults with CKD that self-identified as either First Nations or non-First Nations. There were no significant differences in SBA GMTs between healthy and CKD patients, but First Nations participants had significantly higher GMTs compared to non-First Nations participants in both healthy and CKD groups. There were no significant differences in IgM or IgG GMCs between all CKD and all healthy adults, First Nations CKD and First Nations healthy adults, or non-First Nations CKD and non-First Nations healthy adults. These results suggested that the higher SBA GMTs in the First Nations adults are due to increased circulation of Hia in First Nations communities (49).

Increased incidence rates of invasive Hia disease for all ages in the First Nations population has been reported from northwestern, but not from southern Ontario. To determine if

the higher Hia SBA GMTs in the Indigenous population were due to increased circulation of Hia and prevalence of invasive Hia disease in the First Nations communities of northwestern Ontario, antibody concentrations and functional abilities were assessed in First Nations adults from northwestern Ontario (FNNO) and southern Ontario (FNSO). The antibody concentrations and SBA titres of First Nations adults were compared to those of non-First Nations adults from Kenora, located in northwestern Ontario (NFNK) and non-Indigenous elderly non-frail adults (≥ 65 years old) from across Canada (NFNC). The highest SBA titres were found in the FNSO group, but both FNSO and FNNO had significantly higher SBA GMTs compared to NFNC and slightly higher GMTs compared to NFNK. Similarly, FNSO had the highest IgM GMCs followed by FNNO, NFNK and NFNC. There were no significant differences in IgG GMCs between groups, but FNNO and FNSO had a higher ratio of IgM to IgG, while NFNK and NFNC had a higher ratio of IgG to IgM. This study found that amount of naturally acquired antibodies against Hia are higher in First Nations adults and is not dependent on potential exposure to Hia. It is still unclear how these antibodies are acquired, but it is thought that they are part of the natural antibody repertoire that is present without the requirement of antigenic stimulation in the First Nations population (50).

**Virulence factors as potential vaccine candidates against non-*Haemophilus influenzae* type b strains**

Currently there is no vaccine available to protect against non-Hib strains. The study by Nix *et al*. (2018) (50) addressed two questions, whether bactericidal Hia antibodies are strain specific and what antigenic components of Hia are recognized by these antibodies. To answer the first question, serum bactericidal activity against strains that do not circulate locally and do not

belong to sequence type 23 were measured. Pooled serum of First Nations adults from northwestern Ontario showed bactericidal activity for invasive isolates from Manitoba that were not genetically related to sequence type 23 and have not caused disease in northwestern Ontario. To determine which antigenic components of Hia are recognized by antibodies that result in bactericidal activity, antigen specific antibodies were absorbed. When cross-reactive Hia capsular polysaccharide antibodies (antigenically distinct Hib or *Streptococcus pneumoniae* serotype 6B) as well as protein D specific antibodies were absorbed separately, the remaining serum had significantly decreased bactericidal activity. When Hia capsular polysaccharide specific antibodies and lipooligosaccharide (LOS) specific antibodies were absorbed individually, a significant decrease in serum bactericidal activity occurred (50). Choi *et al*. (2015) (51) also found that naturally acquired LOS antibodies significantly contribute to the serum bactericidal activity against encapsulated and non-typeable *H. influenzae* strains (51). LOS is similar to lipopolysaccharide (LPS) but lacks repeating units of O-antigen side chain. LOS stimulates the expression of antigen presenting and co-stimulatory molecules but has decreased pro-inflammatory abilities than LPS, which makes it a promising vaccine candidate (52). These studies demonstrate the importance of Hia capsular polysaccharides and LOS as potential vaccine candidates against Hia and possibly *H. influenzae* non-typeable strains.

***Streptococcus pneumoniae***

*Streptococcus pneumoniae* (pneumococcus) a Gram-positive diplococcus, encapsulated extracellular bacterium that colonizes the upper respiratory tract and spreads through respiratory droplets (53, 54). There are 98 different serotypes that have been identified through antigenic differences in the polysaccharide capsule (55). Pneumococcus can cause non-invasive disease

(e.g. otitis media, sinusitis, non-bacteremic pneumonia) and invasive disease (e.g. meningitis, septicemia, pericarditis, etc.) when it overcomes host defenses (56, 57). It is the most common cause of community acquired pneumonia (CAP) worldwide (58). Children under 5 years of age, the elderly, and immunocompromised adults have the highest incidence of invasive pneumococcal disease (IPD) and CAP (56, 59). The majority of global CAP cases are caused by *S. pneumoniae*, and 30-70 % of adult CAP cases require hospitalization (58, 60).

**Invasive pneumococcal disease and community acquired pneumonia in Canada**

There are multiple studies that discuss IPD and CAP in Canada, but the two studies described have collected data from across Canada and have the most recent data.

The streptococcus annual 2017 report by the National Microbiology Laboratory (NML) reported in 2017 there were 3006 isolates that caused IPD in Canada. The five most predominant serotypes that caused IPD were 3, 22F, 4, 9N and 8. There are two pneumococcal vaccines available in Canada, the 23-valent penumococcal polysaccharide vaccine (PPV23) and the 13-valent pneumococcal protein-polysaccharide conjugate vaccine. Of the five serotypes identified that resulted in the highest amount of IPD cases, all five are serotypes included in PPV23 and 2/5 are included in PCV13. When adult IPD cases were separated based on age, there were three groups: 15 – 49, 50 – 64 and ≥ 65 years. The five serotypes causing the highest incidence of IPD for the adult groups are: serotypes 4, 12F, 3, 7F and 8 for the 15 – 49 years group, serotypes 3, 4, 22F, 9N and 20 for the 50 – 64 years group, and serotypes 22F, 3, 15A, 9N and 23A for the ≥ 65 years group. Of the 3006 isolates, nearly half (1238 isolates) were from the ≥ 65 years group (61).

The Serious Outcomes Surveillance (SOS) of the Canadian Immunization Research Network (CIRN) investigated the incidence of hospitalized CAP cases from nine hospitals located in British Columbia, Ontario, Québec, New Brunswick and Nova Scotia in December of 2010. There were 4769 all-cause CAP cases, of these 434 had pneumococcal tests performed on blood, sputum and urine along with nasopharyngeal swabs analyzed. The majority of these cases were in adults 65 years and older with comorbidities. Of these cases, 22.1% (96/ 434) were pneumococcal CAP cases, among these cases 29.2 % were bacteremic and 70.8% were non-bacteremic. The five serotypes that caused most pneumococcal CAP cases were 7F, 3, 19A, 22F and 6A. Of these serotypes 3/ 5 are included in both PPV23 and PCV13, while serotype 22F is included in PPV23 and serotype 6A is included in PCV13 (62).

**Invasive pneumococcal disease in the First Nations Indigenous population in northwestern Ontario**

Eton *et al*. (2017) (46) investigated the incidence of IPD in northwestern Ontario through retrospective chart review of the Sioux Lookout Meno Ya Win Health Center (SLMHC). The incidence of IPD over a 5.5-year period from January 2010 – July 2015 was 23.1/ 100 000/ year, more than double the 2013 rate for all of Canada (9.0/ 100 000/ year). The age specific incidence of IPD was highest in the 71-75 years group (106.5/ 100 000/ year) (46). Eton and Ulanova (2018) (63) also investigated the incidence of IPD in Kenora, Ontario Lake of the Woods District Hospital (LTWDH). Between 2010 – 2016 there were 31 cases of IPD in individuals 3 – 90 years old (mean age = 51.3 years). Nearly half of the cases occurred in individuals that identified as Indigenous (48.4%) and 90% of cases occurred in individuals with underlying conditions. The incidence of IPD was 26.6/ 100,000/ year which at the time was almost three times the rate for

the whole Canadian population 9.0/ 100,000/ year. Serotypes that caused the highest incidence of disease were 3 (23%), 22F (10%), 20 (7%), and 15C (7%). Serotypes (35B, 33F, 19A, 16F, 15B, 15C, 12F, 11A, 9N, and 8) caused 3% of IPD cases. For 19.4% of cases the serotype was not specified. In a large proportion of adult cases (66.7%), IPD was caused by serotypes included in PPV23. For the pediatric cases (2 of the 31 total cases), all serotypes that caused IPD were not serotypes that are included in PCV13 (63). A study by our group evaluated hospitalized cases of IPD in adults residing in Thunder Bay, Ontario that occurred between 2006 and 2015. It was reported that 29.1% of patients with IPD were Indigenous, and the likelihood of immunocompromising conditions was higher amongst the Indigenous patients than in the non-Indigenous. Of the serotypes that were isolated 79.2% were included in PPV23 and 28.3% in PCV13. The incidence rate in adults 18 – 64 years old was 8.9 – 25.9/ 100,000/ year and for adults ≥ 65 years old the range was 18.5 – 60.7/ 100,00/ year (64). These studies demonstrate the increased rate of IPD in northwestern Ontario and the high proportion Indigenous peoples with comorbidities being hospitalized with IPD.

**Risk factors for invasive pneumococcal disease**

It has been found that the incidence of IPD is 3 - 5 times more common amongst Indigenous Navajo adults compared to the general US population. The risk factors for IPD include CKD, congestive heart failure, alcohol use or alcoholism, extremely high or low body max index (BMI), and unemployment are more prevalent in Navajo communities compared to the general United States population (65). Similarly, to this North American Indigenous population, Indigenous Canadians have an increased prevalence of diseases such as obesity, diabetes, circulatory diseases and cancer resulting in a decreased life expectancy (66). These

studies suggest that the increased incidence rate of IPD could be due to the higher proportion of Indigenous peoples that suffer from IPD risk factors, which result in immune dysfunction within the FN population (1).

**Pneumococcal vaccination programs and recommendations for adults**

Pneumococcal immunization programs are publicly funded in Canada for specific groups at increased risk for IPD. PPV23 was implemented for adults 65 years and older in 1996. In 2005 the 7-valent pneumococcal protein-polysaccharide conjugate vaccine (PCV7) for infants was introduced, but replaced in 2009 with PCV10, then PCV13 in 2010 (67). The two pneumococcal vaccines currently used in Canada are PPV23 and PCV13. PPV23 contains purified capsular polysaccharides from the 23 pneumococcal serotypes most commonly associated with IPD (1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19F, 19A, 20, 22F, 23F, and 33F). It is currently recommended in Canada for adults with CKD (68). PPV23 and PCV13 have 12 serotypes in common, with serotype 6A being unique to PCV13. The PCV13 consists of purified capsular polysaccharides of 13 serotypes (1, 3, 4, 5, 6A, 6B, 7F, 9 V, 14, 18C, 19A, 19F, and 23F) conjugated to a carrier protein (CRM197). PCV13 is routinely used for immunization of young children and is recommended for some categories of immunocompromised adults, such as those with asplenia or splenic dysfunction, bone marrow transplantation recipients, HIV-infected individuals etc. (69). As of 2016, the National Advisory Committee on Immunization updated pneumococcal immunization recommendations for healthy adults 65 years and older. If adults have not previously received PPV23, they are to receive one dose of PCV13 followed by one dose of PPV23. If they have been immunized with PPV23 previously, they are to receive one

dose of PCV13, but depending on age and IPD risk they may need an additional dose of PPV23 (70).

Although there are publicly funded pneumococcal immunization programs for adults at increased risk for IPD in Canada, pneumococcal vaccine uptake in adults is low. Statistics Canada reported that amongst the adult population with chronic conditions, only 17.3% (13.7 - 20.8, 95% confidence intervals, CI) of 18 – 64 year-olds and 36.5% (32.7 – 40.3, 95% CI) of 65 years and older, correspondingly, received at least one dose of PPV23 (71).

**Effectiveness and immunogenicity of PPV23**

The publicly funded pneumococcal immunization program with PPV23 for adults of 65 years and older was implemented in Canada in 1996. A study conducted by Toronto Invasive Bacterial Diseases Network (TIBDN) investigated the incidence of IPD between 1995 – 2011. Prior to the introduction of PCV7 in 2005, the incidence of IPD caused by the 23 serotypes in PPV23 decreased by 49% in older adults. The estimated vaccine efficacy of PPV23 for all eligible adults was found to be 42.2%. For other groups such as healthy adults 65 years and older, PPV23 efficacy was 68.6% and for adults of any age with immunocompromising conditions efficacy was 23.3% (72).

Immunogenicity of PPV23 in adults has been previously assessed using the opsonophagocytic assay (OPA). Shiramoto *et al.* (2015) (73) immunized healthy pneumococcal vaccine naïve adults ≥ 65 years with one dose of PPV23. The OPA GMTs were significantly higher one month post-immunization and the fold change was between 8.2 – 65.4 times higher than pre-immunization (73). Ahn *et al*. (2015) (74) also assessed OPA GMTs of pneumococcal vaccine naïve adults ≥ 65 years post PPV23 with underlying conditions (e.g. diabetes, cardiac

disease etc.). Participants were separated based on age, group 1 (65 – 74 years) and group 2 (≥ 75 years). The range of the proportions of participants that were able to have a ≥ four-fold OPA GMT increase was 53% - 93% for group 1, and 38% - 84% for group 2 (74). Ciprero *et al*. (2016) (75) assessed the response to PPV23 with ELISA and defined a response to immunization as a ≥ two-fold increase in IgG for adults ≥ 50 years. For the six serotypes tested, the range of the proportion of participants that achieved ≥ two-fold increase was 79.2% - 89.6% (75). However, in comparison to younger adults, elderly adults have been found to have decreased OPA titres compared to younger adults. Younger adults had significantly higher IgG, IgM and IgA concentrations post-PPV23 compared to pre-immunization for the two serotypes tested. The elderly participants only had higher IgM concentrations for one serotype and higher IgG concentrations for both serotypes, suggesting that PPV23 is less immunogenic in elderly adults (76). These studies demonstrate that older adults have the ability to respond to PPV23 but it is less immunogenic compared to responses in younger adults. However, the data is difficult to interpret because there is no accepted OPA titre or antibody concentration that would suggest prevention of IPD in adults.

**Immunogenicity of PPV23 in adults with CKD**

A study by Mahmoodi *et al*. (2009) (77) evaluated for antibody response to PPV23 in CKD patients 18 – 64 years and controls. Because there is no minimum antibody concentration known to prevent IPD in adults, response to immunization was based on healthy control IgG concentrations. Of the 66 vaccinated CKD patients, 21% were categorized as hyporesponsive to PPV23, while 79% responded. The hyporesponsive patients had significantly lower concentrations and fold-changes in IgG concentrations compared to the CKD patients that

responded to PPV23 and healthy controls 1-month post-immunization. There was no significant difference in IgG fold changes between healthy controls and the CKD patients that responded to PPV23. The hyporesponsive patients also had a higher incidence of pneumococcal infections (18 infectious episodes in the hyporesponsive group compared to 1 infectious episode in the responsive group) (77). Pourfarziani *et al*. (2008) (78) immunized kidney transplant recipients and stable dialysis patients with one dose of PPV23. One-month post-PPV23 antibody concentrations were significantly higher and remained higher 6 months post-immunization compared to pre-immunization concentrations. However, concentrations 1-year post-immunization were significantly lower compared to 6 months post-immunization (78). Similarly, Kazancioglu *et al*. (2000) (79) found that antibody concentrations of renal transplant patients were significantly higher 6 and 12 weeks post-PPV23 for 20/ 21 participants compared to pre-immunization concentrations however, antibody concentrations were not measured past 12 weeks post-PPV23 (79). These studies suggest that some patients with CKD and renal transplant patients are able to respond to PPV23, however the response appears to be inconsistent and suboptimal.

**Effectiveness of pneumococcal conjugate vaccines and immunogenicity of PCV13**

The effectiveness of pneumococcal conjugate vaccines has had a significant impact on the incidence of IPD in Canada. TIBDN investigated the incidence of IPD between 1995 – 2011, and since the introduction of the publicly funded pneumococcal infant immunization program with PCV7 in 2005, the incidence of IPD in Ontario has decreased significantly. Between 2001 and 2011, the incidence of IPD caused by PCV7 serotypes decreased by 88% in adults 15 – 64 years old and by 89% in adults ≥ 65 years old, but IPD caused by serotypes not included in

PCV7 increased. The incidence of PCV10 and PCV13 serotypes that caused IPD in adults increased until their implementation in 2009 and 2010 (72). Between 2010 to 2016, 3787 IPD cases in adults ≥ 65 years old from British Columbia, Saskatchewan, Manitoba, Ontario, New Brunswick, Nova Scotia, Prince Edward Island, Yukon, Northwest Territories and Nunavut were collected from public health laboratories for isotype identification at the NML. It was found that over the 6-year period, the number of IPD cases did not change significantly, but the incidence of IPD caused by serotypes included in PCV13 decreased and IPD caused by non-PCV13 vaccine serotypes increased. The incidence of IPD cases caused by all serotypes included in PCV13 decreased from 48.6% to 25.2% in Canada (80). These studies demonstrated that pneumococcal conjugate vaccines are effective in reducing the incidence of IPD in adults.

PCV13 has been shown to be immunogenic for healthy adults and those with comorbidities such as diabetes mellitus, heart disease, liver disease etc. A study by van Deursen *et al*. (2017) (81) compared responses to PCV13 and a placebo in adults ≥ 65 years old who were pneumococcal vaccine naïve. The main finding from the study was that PCV13 was able to significantly increase OPA GMTs and IgG concentrations compared to pre-immunization in immunocompetent adults. OPA GMTs and IgG concentrations remained higher than pre-immunization 2 years post-PCV13 regardless of age or comorbidity (81). Because PPV23 and PCV13 are recommended for adults in Canada, studies that have assessed the immunogenicity of PCV13 and compared responses to PPV23 will be discussed. Jackson *et al*. (2013) (82) found that in adults 60 – 64 years old immunized with PCV13 had significantly higher OPA GMTs for 9/ 13 serotypes compared to participants immunized with PPV23 (82). Shiramoto *et al*. (2015) (73) also immunized healthy adults ≥ 65 years old with either PPV23 or PCV13. One month post-immunization PCV13 immunized participants had significantly higher OPA titers for 9/ 12

shared serotypes and 6A compared to PPV23 immunized participants (73). These studies demonstrate that PCV13 is more immunogenic in older adults compared to PPV23.

PPV23 is recommended for adults ≥ 65 years old, therefore it is important to investigate the effect of previous PPV23 immunization on subsequent immunization with PCV13. Another study by Jackson *et al*. (2013) (83) assessed the immunogenicity of PCV13 in adults ≥ 70 years that were previously immunized with PPV23. Compared to adults that only received one dose of PCV13, adults that were immunized with PPV23 followed by PCV13 had significantly lower OPA GMTs for all 13 serotypes. Greenberg *et al*. (2014) (84) immunized pneumococcal vaccine naïve adults aged 60 – 64 years old with PPV23 followed by PCV13 one year later. OPA GMTs in adults immunized with PPV23 followed by PCV13 were significantly lower for all 13 serotypes compared to adults immunized with one dose of PCV13 (84). These studies suggest that previous immunization with PPV23 has a negative effect on the immunogenicity of subsequent immunization with PCV13.

**Immunogenicity of PCV13 in adults with CKD**

There have been two studies that have assessed the immunogenicity of PCV13 in adults with CKD. Mitra *et al*. (2016) (85) immunized patients with CKD ≥ 50 years old, a portion of these patients received PPV23 ≥ 5 years prior to PCV13 but the number was not specified. Patients were considered to have responded to PCV13 if IgG concentrations post-immunization were ≥ 1 μg/ mL and increased ≥ two-fold. All patients responded to at least one serotype at 2 and 12 months post-PCV13. Two months post-immunization 53% of the patients responded to 10/ 13 serotypes. At 12 months post-immunization, 24% of patients responded to 10/ 13 serotypes and 35% of participants responded to 3/ 13 serotypes. This study suggests that CKD

patients are able to respond to PCV13, but responses decrease 1-year post-PCV13 (85). The second study by Vandecasteele *et al*. (2018) (86) consisted of adults with CKD that were either pneumococcal vaccine naïve or previously immunized with PPV23. Antibody concentrations were back-transformed and reported as ELISA GMTs, OPA GMTs were also reported. At day 28, ELISA GMTs for pneumococcal vaccine naïve patients immunized with PCV13 were 1.85- to 2.34-fold higher for 6/ 13 serotypes compared to pneumococcal vaccine naïve patients immunized with PPV23. There were no significant differences in OPA GMTs between groups. At day 365, ELISA GMTs for pneumococcal vaccine naïve patients immunized with PCV13 were significantly higher for serotype 6A only compared to pneumococcal vaccine naïve patients immunized with PPV23. This suggests that in pneumococcal vaccine naïve CKD adults, PCV13 is more immunogenic compared to PPV23. Next, CKD patients previously immunized with PPV23 were compared to pneumococcal vaccine naïve patients. Pre-immunization with PCV13, ELISA GMTs were significantly lower in pneumococcal vaccine naïve patients for 2/ 13 serotypes and 4/ 13 serotypes for OPA GMTs compared to patients previously immunized with PPV23. At day 28 post-PCV13, pneumococcal vaccine naïve patients had significantly higher ELISA GTMs for 12/ 13 serotypes, and 11/ 13 serotypes for OPA GMTs. At day 28 post-PCV13, pneumococcal vaccine naïve patients had significantly higher ELISA GTMs for 10/ 13 serotypes, and 9/ 13 serotypes for OPA GMTs (86). This study suggests that immunization with one dose of PCV13 is immunogenic in adults with CKD and previous immunization with PPV23 decreases antibody responses to PCV13.

**B-cell responses to pneumococcal immunization**

A small number of studies have quantified B cell subpopulations to determine if pneumococcal specific immunological memory has been established post-immunization. Some of these studies have compared B-cell responses to PPV23 and PCV7 in healthy adults, but none in response to PCV13. The studies that have compared B-cell responses to PPV23 and PCV7 in healthy adults will be described. In the study by Baxendale *et al*. (2010) (87) healthy adults aged 52 – 74 years old and received either one dose of PPV23 or PCV7. Exclusion criteria included immunization with PPV23 ≤ 5 years, but the proportion of the participants that were previously immunized with PPV23 was not reported. There were no significant differences in pneumococcal memory B cells detected through enzyme-linked immunospot assay (ELISPOT) between healthy adults immunized with PPV23 or PCV7 day 7 or day 28 post-immunization (87). Truck *et al.* (2013) (88) immunized healthy pneumococcal vaccine naïve adults 50 – 70 years old with either one dose of PPV23 or PCV7. Memory B cells specific for pneumococcal serotypes 1 and 3 were detected via ELISPOT. One-month post-immunization numbers of memory B cells specific for serotype 1 did not change significantly in PCV7 immunized adults, but adults immunized with PPV23 experienced a significant decrease in memory B cells compared to pre-immunization numbers. Similarly, PPV23 immunized adults experienced a decrease in serotype 3 specific memory B cells compared to pre-immunization numbers while PCV7 immunized adults had no significant difference in memory B cells 1-month post-immunization (88). Clutterbuck *et al*. (2012) (89) investigated combinations of pneumococcal immunization schedules in the same adults that participated in Truck *et al*. (2013) (88). Memory B cells were quantified using both ELISPOT and flow cytometry analysis. One-month post-immunization, adults immunized with PCV7 had an increase in memory B cells while adults immunized with PPV23 had a decrease in memory B cells compared to pre-immunization. Six

months post-immunization, adults that were immunized with PCV7 experienced a decrease in the number of memory B cells back to pre-immunization amounts, and the numbers of memory B cells in the PPV23 immunized adults decreased further below pre-immunization amounts. To summarize, these studies suggest that immunization with PCV7 does not have a negative effect on the memory B cell population in healthy adults. However, the study by Truck *et al*. (2013) and Clutterbuck *et al*. (2012) suggests that immunization with PPV23 may have a negative effect on the numbers of serotype specific memory B cells.

The study by Clutterbuck *et al*. (2012) (89) also measured pneumococcal specific class switched memory B cells ($CD19^{+}CD20^{+}CD27^{hi}IgM^{-}$) for two groups of adults that received either two doses of PCV7 (PCV7-PCV7) or one dose of PPV23 followed by PCV7 (PPV23-PCV7). One month after the second immunization, PCV7-PCV7 adults had more class switched memory B cells compared to the PPV23-PCV7 group. This study also investigated the change in CD5 expression in B cells within the B1 ($CD19^{+}CD20^{+}CD27^{-}IgM^{hi}$) B cell population. Two populations were quantified, the B1a ($CD5^{int}$) and B1b ($CD5^{-}$). The B1b subpopulation was quantified because these cells have an important role in protecting mice against pneumococcus. There were no significant differences between groups in the amounts of B1a, but the PPV23-PCV7 group had less B1b cells compared to the PCV7-PCV7 group (89). Moens *et al*. (2015) (90) investigated the roles of CD5+ and CD5- B cells in the antibody responses to pneumococcal capsular polysaccharides. It was found that CD5- B cells produced in response to PPV23 have an important role in producing IgG specific for pneumococcal capsular polysaccharides. A positive association was found between the concentrations of IgG capsular polysaccharide antibodies and the number of CD5- B cells (90). In the Clutterbuck *et al*. (2012) (89) study the PPV23-PCV7 immunized adults also experienced decreased antibody concentrations also known as

hyporesponsiveness in addition to decreased memory B cells and CD5- B cells compared to PCV7-PCV7 immunized adults. The capsular polysaccharides in PPV23 stimulate a T cell-independent response of marginal zone B cells that does not result in the production of class switched memory B cells. The decreased antibody response and decline in memory B cells in adults immunized with PPV23 is thought to be due to PPV23 driving pre-existing class switched memory B cells to terminal differentiation and depletion of the memory B cell pool making the response to subsequent doses with PCV7 to be decreased (89).

**B-cell responses to pneumococcal immunization in immunocompromised adults**

B-cell responses to pneumococcal immunizations have not been previously measured in patients with CKD, therefore studies that have assessed responses in adults with immunocompromising conditions will be described. Farmaki *et al*. (2018) (91) immunized HIV positive adults with one dose of PCV13 followed by one dose of PPV23 1 year later. Patients were excluded from the study if they were previously immunized with PCV13 or PPV23 ≤ 1 year ago. Flow cytometry quantified class switched memory B cells (CD19+CD10-CD21++CD27+IgM- and IgM memory B cells (CD19+CD10-CD21++CD27+IgM+). Memory B cell numbers were not negatively affected by PCV13 after 1 month and the numbers did not significantly decrease at 12 months post-immunization. When patients received PPV23, there was a significant decrease in the IgM memory B cell pool compared to pre-immunization with PCV13. However, amounts of class switched memory B cells remained higher than pre-immunization with PCV13 (91). Papadatou *et al*. (2014) (92) immunized adults with β-thalassemia and asplenia between 19 – 48 years old that were previously immunized with one dose of PCV7 as well as 1– 4 doses of PPV23. All patients were vaccinated with one dose of

PCV13 and memory B cells were quantified using ELISPOT. It was found that as the number of PPV23 doses increased, the numbers of pneumococcal memory B cells decreased (92). These studies suggest that immunization with PPV23 has a negative effect on pre-existing memory B cells in immunocompromised adults.

**Conclusion**

Patients with CKD in northwestern Ontario are at increased risk for invasive disease caused by pneumococcus and Hia. Due to their immune dysfunction, immunization is needed to maintain immunological memory by stimulating the production of antigen specific memory B cells and antibodies. Based on previous studies on humoral immune responses to conjugate vaccines to prevent disease caused by pneumococcus and Hib, these vaccines are able to elicit a response in these patients suggesting immunological memory has been established. This also suggests that immunization with a new conjugate Hia vaccine could be successful. However, it is uncertain how long protection will last. Considering the conflicting data on responses to PCV13 in adults with CKD, it is also important to determine the effect of previous capsular polysaccharide immunization on responses to protein-polysaccharide conjugate vaccine responses in adults with CKD. More studies will need to be performed in order to establish an effective immunization schedule for adults with CKD.

## Chapter 1B: Research Rationale, Questions, Hypotheses and Objectives

Adults with CKD are immunocompromised which makes them more susceptible to infections. In northwestern Ontario, there is an increased prevalence of CKD in the First Nations population compared to non-First Nations adults. Amongst the First Nations population of northwestern Ontario, there is also an increased incidence of invasive disease caused by *H. influenzae* type a (Hia) and *S. pneumoniae* (pneumococcus). In order to maintain immunological memory in adults, stimulation of pre-existing memory is achieved through immunization. Therefore, in order to determine if patients with CKD have the ability to respond to immunization, naturally acquired humoral immunity against Hia and pneumococcus needs to be evaluated in adults with CKD. Quantification of naturally acquired B-cell subpopulations and antigen specific antibodies in Canadian adults with CKD have not been investigated previously. Immunization against pneumococcus is recommended for adults with CKD in Canada, however immunization with PPV23 is suboptimal at inducing antibody responses in adults with CKD compared to healthy adults. PCV13 is recommended for certain groups of immunocompromised adults, but there have been only two studies that have measured the immunogenicity of PCV13 in adults with CKD with conflicting results. Quantification of B-cell subpopulations, pneumococcal specific B cells and their relationship with antibody responses to PCV13 have not been reported previously. In addition, the effect of previous immunization with PPV23 in response to PCV13 on B-cell and antibody responses in adults with CKD have not been previously investigated.

**The first question that will be addressed is: are there differences in naturally acquired humoral immunity against Hia and pneumococcus in healthy adults and adults with CKD, or First Nations and non-First Nations adults of the same health status?** Due to

the immune dysfunction in adults with CKD, it is hypothesized that humoral immunity against Hia and pneumococcus could be decreased in CKD patients compared to healthy adults. The increased incidence of invasive disease in the First Nations population of northwestern Ontario, also suggests that humoral immunity in First Nations adults could be decreased compared to non-First Nations adults. Chapters 2 and 3 will address this question.

**Chapter 2 objectives**

1. Quantify the proportions and absolute numbers of B cells and subpopulations in First Nations and non-First Nations patients with CKD.
2. Quantify the concentrations of IgM and IgG Hia capsular polysaccharide specific antibodies and the functional abilities of Hia antibodies.
3. Determine if there are differences between healthy adults and adults with CKD in the proportions of B cells and B-cell subpopulations, as well as Hia antibody capsular polysaccharide specific concentrations and functionality.

**Chapter 3 objectives**

1. Compare the concentrations of naturally acquired IgG antibodies against pneumococcal serotypes 6B and 14 in pneumococcal unimmunized First Nations and non-First Nations healthy adults.
2. Compare the concentrations of naturally acquired IgG antibodies against pneumococcal serotypes 6B and 14 in healthy adults with pneumococcal unimmunized First Nations and non-First Nations adults with CKD.

**The second question that will be addressed is: are adults with CKD able to respond to PCV13 immunization and does previous immunization with PPV23 affect their ability to respond to subsequent immunization with PCV13?** Although patients with CKD are

immunocompromised, they have shown the ability to respond to the conjugate Hib vaccine, therefore it is hypothesized that they will be able to respond to PCV13. Considering that other studies have shown that previous immunization with PPV23 has a negative effect on pneumococcal specific humoral immune response to subsequent immunization with PCV13, it is hypothesized that patients with CKD that have previously been immunized with PPV23 will also have decreased humoral immune responses to immunization with PCV13. Chapters 4A and 4B will address this question by quantifying humoral immune responses to immunization with one dose of PCV13 in CKD patients that are either pneumococcal vaccine naïve or have received PPV23 $\geq$ 1 year ago.

**Chapter 4A objectives**

1. Quantify the proportions and absolute numbers of B cells and subpopulations pre-immunization and 7 days post-immunization.
2. Quantify the numbers of total IgG and antigen specific (pneumococcal serotypes 6B and 14) antibody secreting cells 7 days post-immunization.

**Chapter 4B objectives**

1. Quantify IgG, IgM and IgA concentrations pre-immunization, day 28 and day 365 post-immunization specific for seven pneumococcal serotypes (3, 6B, 9V, 14, 19A, 19F, and 23F).
2. Perform correlation analyses to determine the relationship between B cells and the fold change in antibody concentrations.

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## Chapter 2: Natural immunity against *Haemophilus influenzae* type a and B-cell subpopulations in adult patients with severe chronic kidney disease

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### Abstract

Individuals suffering from severe chronic kidney disease (CKD) are immunocompromised and therefore highly susceptible to various infections including *Haemophilus influenzae* type a (Hia), an emerging pathogen in North American Indigenous populations. Immunocompromised Indigenous adults are considered a target for a new Hia vaccine under development. In an attempt to foresee their response to Hia immunization, we studied natural immunity against Hia and B-cell subpopulations in sixty patients with CKD residing in a geographic region with noticeable presence of Hia invasive disease. Serum bactericidal activity (SBA) against Hia, concentrations of IgG and IgM antibodies specific to Hia capsular polysaccharide, and B-cell subpopulations were studied in patients with CKD and 35 healthy controls of the same age. Of the patients with CKD, proportions and absolute numbers of B-cell subpopulations were determined for 28 patients. The patients had lower SBA titres compared to controls. Although no significant differences in anti-Hia IgG or IgM antibody

concentrations between control and CKD groups were found, IgM antibody concentrations were higher in Indigenous than non-Indigenous patients. Patients with CKD had a higher proportion of B cells (CD19+), class switched memory B cells (CD19+CD27+IgM-) and a lower proportion of CD19+CD27-IgM- B cells compared to healthy controls. Non-Indigenous patients with CKD had significantly higher proportions of IgM memory B cells and CD19+CD27-IgM- B cells compared to Indigenous patients with no significant difference in absolute numbers. Because 72% of CKD patients had detectable SBA titres and 100% had detectable IgG and IgM antibodies it is possible that a portion of IgM memory B cells and class switched memory B cells are specific for Hia resulting from a natural exposure to the pathogen. The data suggest that a Hia-conjugate vaccine may be immunogenic in adult patients with CKD as it will potentially induce re-activation of immunological memory against Hia.

Key Words: B cells, *Haemophilus influenzae* type a, severe chronic kidney disease, flow cytometry analysis, serum bactericidal assay, ELISA.

## Introduction

Severe chronic kidney disease (CKD) is defined as a decreased kidney glomerular filtration rate (GFR) of less than 30 mL/ min/ 1.73m$^2$ for a minimum of 3 months, and is a serious condition characterized by high mortality rates (1, 2). Increased inflammatory responses and reactive oxygen species generation associated with uremia contribute to the development of other conditions, such as cardiovascular disease and cancer (3). Infection is the second major cause of death in these patients after cardiovascular disease (4). In patients with CKD, a high risk for septicemia and other severe infections is attributed to their compromised immune system and

increased exposure to infectious agents in dialysis units (5, 6). In North America, CKD disproportionally affects Indigenous populations. The burden of CKD among Indigenous peoples is mostly influenced by dramatically rising incidence of obesity and type 2 diabetes mellitus resulting in diabetic nephropathy as well as a high rate of glomerulonephritis (7, 8). *Haemophilus influenzae* type a (Hia) is an emerging infection in North American Indigenous populations; invasive Hia disease mostly affects young children, the elderly, and immunocompromised individuals (9-11). Our studies over the last 15 years have shown that invasive Hia disease is consistently present in Northwestern Ontario, Canada (12). This geographic region has 25.4% population of Indigenous peoples (13), and several cases of invasive Hia disease occurred in Indigenous peoples suffering from CKD (11, 14, 15). A new vaccine against Hia is currently under development in Canada (16). Because immunocompromised individuals are highly susceptible to invasive Hia disease, patients with CKD are being considered as a target population for a new vaccine.

We have recently found that healthy Indigenous adults have higher titres of serum bactericidal antibodies against Hia compared to non-Indigenous peoples living in the same region suggesting that they may have developed immunological memory against Hia as a result of exposure to the pathogen (10, 17). While long-lived plasma cells are directly responsible for antibody production, memory B cells generated during the primary immune response are able to mount rapid recall response upon secondary exposure to the same antigen (18, 19). Therefore, the effect of adult immunization is critically dependent on the presence of memory B cells. As no previous research on memory B cells in Indigenous patients with CKD had been done, we made an attempt to characterize their subpopulations in Indigenous and non-Indigenous patients. We

also tried to determine if there is an association between natural humoral immunity to Hia and B-cell subpopulations.

## Methods

### Participants

This study was approved by the research ethics boards of Lakehead University and the Thunder Bay Regional Health Sciences Centre (TBRHSC). Sixty patients with stage four or five CKD (stage 4, GFR < 30 mL/ min/ 1.73m$^2$, stage 5, GFR < 15 mL/ min/ 1.73m$^2$) (2) who received hemodialysis in the TBRHSC were recruited from May 2015- January 2018; all were required to provide informed consent before determining their eligibility for the study. Ethnicity was determined based on self-declaration. Patients with severe CKD were considered eligible if they: were over 18 years old, did not have a history of immunocompromising conditions, were not taking immunosuppressive medications for more than 14 days in the past 6 months, had not received any vaccine in the past month and were not receiving blood transfusions or blood products in the past 3 months. The majority of 35 healthy controls were recruited through the Thunder Bay 55 Plus Centre, and were considered eligible if they were over the age of 18 years old and did not have a history of taking immunosuppressive medications. Table 1 displays the demographics of study participants; supplementary table 1 describes additional information on the underlying causes of CKD and comorbidities.

### Analysis of B cells

Peripheral blood mononuclear cells were isolated using Lymphoprep (Stemcell Tecnologies, Vancouver, BC, CAN). Monocytes were removed using two consecutive

incubations in complete culture medium, i.e. RPMI 1640 medium with L-glutamine (Fisher Scientific, Whitby, ON, CAN) and 1% antibiotic-antimicotic (Life Technologies, Burlington, ON, CAN) supplemented with 20% fetal bovine serum (FBS, Fisher Scientific) in a cell culture dish for 1 hr at 37°C, 5% $CO_2$. Non-adherent cells were then washed and re-suspended in complete culture medium supplemented with 10% FBS at 2 x $10^6$ cells/ mL. From the cell suspension, 200,000 cells were immunostained with PE Mouse Anti-Human CD19, APC Mouse Anti-Human IgM, and PerCP-Cy™5.5 Mouse Anti-Human CD27 at 4°C for 1 hr. Samples were analyzed with BD FACSCalibur™ Flow Cytometer and CELLQUEST PRO software (BD Biosciences) to determine proportions of B cells (CD19+) and subpopulations: naïve (CD27-IgM+), IgM memory (CD27+IgM+), CD27-IgM-, and class switched memory (CD27+IgM-) (Figure 1). Purity of CD19+ gated B cells was verified by counterstaining cells with FITC Mouse Anti-Human CD3 and PerCP-Cy™5.5 Mouse Anti-Human CD14 (BD Biosciences).

Analysis of B cells was conducted for 28 out of 60 patients (supplementary table 2). For these patients, a complete blood count (CBC) was performed at the TBRHSC clinical lab to determine the lymphocyte count (x$10^9$ cells/ L). The absolute number of B cells was calculated by multiplying the percentage of CD19+ cells of the total gated lymphocyte population by the total lymphocyte count. Similarly, the numbers of B-cell subpopulations were determined by multiplying the percentage of each subpopulation by the absolute number of B cells (supplementary table 3).

Analysis of *Haemophilus influenzae* type a specific antibodies

Serum Bactericidal Assay (SBA) was performed as previously described (17) using Hia strain 08-191and baby rabbit complement (Pel-Freez, Rogers, AR, USA) as the exogenous

complement source. SBA titers were determined as the reciprocal serum dilution required to kill ≥ 50% of the initial bacterial inoculum (20). Titers below the lower detection limit of 16 were reported as 8 for statistical purposes.

IgG and IgM antibodies specific to Hia capsular polysaccharide were quantified by ELISA as previously described (10). Antibody concentrations were determined using our internal standard that was standardized to the Hia reference serum provided by the Centers for Disease Control and Prevention (10). The internal standard contained 1.25 µg/ mL anti-Hia IgG and 2.09 µg/ mL anti-Hia IgM. The standard curve was made using a log-log non-linear regression to produce the line of best fit. Outliers were deleted and values were considered acceptable if the coefficient of variation was below 30%.

## Statistical Analysis

Statistical analysis was performed using Graph-Pad Prism 5 (GraphPad Prism Software Inc., San Diego, CA). The means for the percentage of each B-cell subpopulation and absolute numbers of B cells were calculated as well as the geometric means for anti-Hia antibody concentrations and SBA titres with 95% confidence intervals (CI). Groups were compared either using a Student's t-test, or Mann-Whitney U test, one-way ANOVA, or Kruskal-Wallis test depending on data distribution and number of groups compared. Correlation between antibody concentrations and B-cell subpopulations was determined using Pearson or Spearman's correlation. Significance was determined at p value $< 0.05$. Outliers were identified and removed (21).

**Results and Discussion**

To assess natural immunity against Hia in patients with severe CKD, we quantified functionally active antibodies using SBA, and anti-Hia capsular polysaccharide specific IgG and IgM using ELISA. The patients had lower geometric mean SBA titres than healthy controls (145, CI: 86.53 - 244.1 *vs.* 324.7, CI: 189.4 - 556.4, Student's t-test, $p < 0.05$, figure 2A). Moreover, larger proportion of patients had SBA titres below lower detection limits compared to healthy controls, 28% (17/60) *vs*. 9% (3/35), $p < 0.05$ (Fisher's exact test). These results are in agreement with our earlier observations suggesting that CKD may negatively affect natural immunity against Hia and therefore this patient population may be at risk of invasive Hia disease (10). However, no significant difference in SBA titres between healthy controls, CKD Indigenous, and CKD non-Indigenous patients was detected when a multiple comparisons test was performed (figure 2A-B). No significant difference in anti-Hia capsular polysaccharide IgM (1.0 μg/ mL, CI: 0.8 - 1.3 *vs.* 1.2 μg/ mL, CI: 0.9 - 1.5) or IgG (0.6 μg/ mL, CI: 0.4 - 0.9 *vs.* 0.5 μg/ mL, CI: 0.4 - 0.7) concentrations between patients and controls was detected using either a Student's t-test or Mann-Whitney U test, or between healthy controls, CKD Indigenous, and CKD non-Indigenous using a multiple comparisons test ($p > 0.05$) (figure 2C-F).

While SBA geometric mean titres (GMT) in Indigenous CKD patients did not significantly differ from non-Indigenous patients (111.4, CI: 51.90 - 239.2 *vs.* 189.6, CI: 90.98 - 395.0*,* $p > 0.05$, figure 2B), the concentration of IgM antibodies specific to Hia capsular polysaccharide was significantly higher in Indigenous compared to non-Indigenous patients (1.4 μg/mL, CI:1.0 - 1.9 *vs.* 0.7 μg/mL, CI: 0.5 - 1.1, Mann-Whitney U test, $p < 0.05$, figure 2D). No difference in specific IgG concentrations between these groups was detected (figure 2F).

In all the groups, the geometric mean concentrations of Hia capsular polysaccharide-specific IgM were higher than the concentrations of specific IgG (figure 2C-F). Healthy controls had a slightly greater ratio of IgM to IgG compared to patients with CKD (2.4:1 *vs.* 1.7:1, $p > 0.05$, figure 2C-E) while the Indigenous and non-Indigenous patients had similar ratios (1.8:1 *vs.* 1.4:1, $p > 0.05$, figure 2D-F). These results confirm our previous findings on the prevalence of IgM in the anti-Hia capsular polysaccharide antibody repertoire and higher anti-Hia IgM concentrations in Indigenous than non-Indigenous adults (10, 17). Although the prevalence of IgM over IgG suggests that anti-Hia capsular polysaccharide antibodies may be part of the natural IgM antibody repertoire, this may also be an indicator of a recent exposure to the pathogen (17). Indeed, our epidemiological studies have found that during the last two decades, Hia has been consistently present in Indigenous communities of this geographic region (11, 14, 15). Naturally acquired antibodies are largely determined by the colonization, and the colonization rates may vary among different populations depending on age, race, living conditions, and socio-economic status (22, 23). However, the relationship between the development of naturally acquired antibodies and *H. influenzae* colonization history is complex, and potential effects of some cross-reactive antigens present in the environment need to be considered (17).

Severe CKD is associated with chronic inflammation (24). In other inflammatory diseases, such as systemic lupus erythematosus, rheumatoid arthritis, multiple sclerosis, and inflammatory bowel disease, B-cell subpopulations are often altered compared to healthy people (25). This suggests that patients with CKD may also have alterations in B-cell subpopulations. To determine proportions of B cells and their subpopulations, we have used flow cytometry analysis to detect the cell surface expression of CD19 as a marker for B cells and CD27 for

memory B cells (figure 1). IgM memory B cells and switched memory B cells were defined as CD19+CD27+IgM+ and CD19+CD27+IgM- cells, respectively (26).

Our analysis showed that patients with CKD had a higher percentage of B cells in comparison to healthy controls (11%, CI: 5.9 - 8.0 *vs.* 7%, CI: 8.4-13.7, Student's t-test, $p < 0.01$, table 2), with no significant differences between Indigenous and non-Indigenous patients ($p > 0.05$, table 2). There was a significant difference in the proportions of B cells between healthy controls, CKD Indigenous, and CKD non-Indigenous patients (one-way ANOVA, $p < 0.01$). Higher proportion of B cells in patients with CKD compared to healthy controls is likely due to chronic inflammation caused by uremia, and can be associated with common co-morbidities found in these patients, such as diabetes mellitus and chronic obstructive pulmonary disease (27, 28). We also found that patients with CKD had significantly higher proportions of class switched memory B cells (CD19+CD27+IgM-) (22%, CI: 16.4 - 28.5 *vs*. 16%, CI: 12.2 - 19.1, Student's t-test, $p < 0.05$) and lower proportions of CD19+CD27-IgM- B cells (16%, CI: 13.1 - 19.2 *vs*. 20%, CI: 16.4 - 28.5, Mann-Whitney U test, $p < 0.05$) compared to the controls (figure 3A and B). When B-cell subpopulations were compared between healthy controls, CKD Indigenous and CKD non-Indigenous patients, no significant differences between the groups were found except for CD19+CD27-IgM- B cells (Kruskal-Wallis test, $p < 0.001$).

The T-cell-dependent antibody response is characterized by the generation of class switched memory B cells (18). Upon re-exposure to the same antigen, these cells are able to respond rapidly resulting in the production of more antigen specific memory B cells as well as plasma cells that produce high affinity antibodies (19). An increase in proportion of class switched memory B cells in CKD patients could suggest that such patients have been exposed to pathogens more often than healthy controls. Indeed, patients with CKD undergoing hemodialysis

spend a lot of time in close proximity to other patients in the hospital environment, and experience frequent disruption of the skin barrier from needles or catheter ports. As part of the standard clinical practice these patients receive vaccines prescribed for high-risk adult populations (against pneumococcal, hepatitis B virus, and varicella zoster infections) that can potentially increase the population of class switched memory B cells.

It is more difficult to interpret a decrease in the proportion of CD19+CD27-IgM- because these cells are not well characterized in the literature. Similarly, to these cells, the origin and functional roles of Double Negative (DN) B cells (CD19+CD27-IgD-) are uncertain. Because DN B cells accumulate in the elderly, it was suggested that these cells are the exhausted terminally differentiated memory B cells (29-31). Accordingly, a higher proportion of CD19+CD27-IgM- B cells (18%, CI: 13.4 - 22.6 vs. 13%, CI: 10.8 - 13.5, Mann-Whitney U test, $p < 0.05$) in non-Indigenous compared to Indigenous patients (figure 3D) may be due to the fact that the non-Indigenous patients were significantly older ($69 \pm 3$ vs. $54 \pm 3$ years, Student's t-test, $p < 0.01$, supplementary table 2).

We have also observed higher proportions of IgM memory B cells in non-Indigenous compared to Indigenous patients (12%, CI: 7.1 - 16.24 *vs*. 7%, CI: 4.3 - 9.5, Student's t-test, $p < 0.05$) (figure 3C). The IgM memory B cells are generated in the process of the T cell-independent immune response and also increase with age (32-34) that may explain their higher proportion in the non-Indigenous group of CKD patients. It is tempting to speculate that higher IgM anti-Hia antibody concentrations found in Indigenous compared to non-Indigenous patients may potentially be associated with an increased proportion of IgM memory B cells, as previous studies demonstrated an essential role of this subpopulation in antibody response to bacterial capsular polysaccharides (26). However, our analysis did not reveal any noticeable correlation

between B-cell subpopulations and antibody levels or SBA titres (data not shown). It is not surprising because antigen-specific B cells represent a very small fraction of the total B cell population. For example, in healthy unimmunized adults, only 0.5% of total CD19+ B cells are specific to any individual pneumococcal polysaccharide antigen (35).

For the interpretation of changes in B-cell subpopulations found in our patients, it is necessary to consider that the total numbers of B-lymphocytes may be decreased in CKD patients (36). We calculated the absolute numbers of B cells and their subpopulations for 28 CKD patients (supplementary table 3). No significant differences between Indigenous and non-Indigenous patients for absolute number of any of the subpopulations were found (supplementary table 3). Because the lymphocyte counts were not available in our control group we compared the absolute numbers of B cells and their subpopulations in CKD patients with published data (supplementary table 4a and b). The analysis indicated that approximately 60% of our patients (16/28) had total lymphocyte counts within normal limits defined by the Medical Council of Canada (1.0-4.0 x $10^9$ cells/L) (37), and the remaining 12 had the counts below 1.0 x $10^9$ cells/L. In comparison to absolute values of B-cell subpopulations in healthy adults between the ages of 26 and 50 reported by Morbach *et al.* (38), our patients had lower numbers of total lymphocytes, total B cells, naïve B cells, and IgM (non-switched) memory B cells, but similar numbers of class switched memory B cells and higher numbers of CD19+CD27-IgM- compared to reported DN cells (supplementary table 4b). However, this comparison needs to be taken with caution because of age differences between our patients and their cohort of healthy adults (69 ± 3 vs. 54 ± 3 years, $p < 0.01$), as well as the use of different markers for defining naïve and IgM memory B cells (IgD+ rather than IgM+). Nevertheless, 93% (26/28 patients) of our CKD patients had numbers of B cells below the normal values reported by Morbach *et al*. (199 cells/ μL, age 26-50

years) (38) and Qin *et al*. 2016 (216 cells/ μL, age 19-80 years) (39). Similar to our findings, a previous study by Pahl *et al.* (2010) demonstrated that patients with end-stage renal disease had significantly reduced numbers of total B cells and their subpopulations including memory (CD19+CD27+) and naïve B cells (CD19+CD27-) in comparison to healthy controls (40). In that study, a decrease in the absolute numbers of B cells was found to be associated with a decrease in BAFF receptor expression in transitional B cells despite elevated levels of IL-7 and BAFF in plasma. Considering that BAFF is a key differentiation and survival factor for B cells, the authors concluded that B-cell lymphopenia in end-stage renal disease is caused by the impact of uremia on maturation of transitional B cells (40).

Because the majority of our patients had below normal absolute numbers of B cells, they may potentially exhibit a low response to immunization. Indeed, it was previously found that some CKD patients were hypo-responsive to pneumococcal polysaccharide vaccine (41). The response rates to hepatitis B vaccination in these patients are also lower in comparison to healthy controls (42). However, we have recently found that the pediatric *Haemophilus influenzae* type b (Hib) polysaccharide-protein conjugate vaccine is highly immunogenic in a group of adult patients with CKD, which is comparable to the one in present study (43). Because the proposed Hia vaccine is designed based on the same principles as Hib-conjugate vaccine, i.e. composed of the capsular polysaccharide conjugated to a protein carrier, this may predict its sufficient immunogenicity in CKD patients.

This study has several limitations. There is a nearly 20-year difference between the mean ages of the CKD Indigenous and non-Indigenous patients (Student's t-test, $p < 0.01$). This difference reflects the demographics of adults suffering from CKD in Canada, i.e. people of Indigenous descent tend to develop CKD at a younger age (7). For logistic reasons, we were

unable to include a group of healthy Indigenous participants as a control and hence could not determine their B-cell subpopulations, although the data of serum Hia antibody levels and serum bactericidal activity in healthy Indigenous adults collected by our group in the same region and using the same methodology are available (10, 17). We did not study *H. influenzae* colonization rates in study participants although our ongoing studies indicate that approximately 8% of 3 – 5 year old First Nations children carry Hia in the nasopharynx (unpublished observations). However, the development of natural immunity to common bacteria such as *H. influenzae* is a result of multiple exposures to antigens. Given the transient pattern of *H. influenzae* colonization we did not consider data on single-point carriage in adults to be of value regarding the understanding of natural immunity to Hia and we were unable to conduct a longitudinal study of carriage in our cohorts for logistic reasons. Because we were unable to determine absolute lymphocyte numbers in healthy controls we have used the relevant data from literature for analysis. Lastly, no correction was applied to the threshold of statistical significance despite the many comparisons that were made, most involving small differences between groups.

**Conclusion**

Patients suffering from severe CKD may be at an increased risk for invasive Hia disease because they have decreased titres of serum bactericidal antibodies against Hia and reduced absolute numbers of B lymphocytes. Yet, 72% of CKD patients exhibited detectable serum bactericidal activity against Hia and 100% had detectable Hia-specific IgG and IgM. However, the minimum protective antibody concentrations against Hia are still unknown, and we must also consider that patients with chronic disease, such as those with CKD might require higher antibody concentrations for protection. Considering the presence of an increased proportion of

class switched memory B cells in CKD compared to healthy controls, a fraction of memory B cells resulting from a natural exposure to the pathogen can be specific for Hia. The data would suggest that a new Hia-conjugate vaccine under development may be immunogenic in adult patients with CKD, as it will potentially induce re-activation of immunological memory specific to Hia.

**Conflict of interest statement**

All authors declare there is no conflict of interest.

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**Tables and Figures**

Table 1. Demographics of study participants.

| Group | *n* | Age (years) Mean ± SEM | Range | No. (%) Female | No. (%) Indigenous |
|---|---|---|---|---|---|
| Healthy Controls | 35 | 62 ± 2 | 36-85 | 22 (63) | 5 (14) |
| CKD Patients | 60 | 61 ± 2 | 32-85 | 26 (43) | 30 (50) |
| CKD Indigenous | 30 | 53 ± 2$^{+*}$ | 32-80 | 15 (50) | |
| CKD non-Indigenous | 30 | 70 ± 2$^{*}$ | 43-85 | 11 (37) | |

+ $p < 0.0001$, chronic kidney disease (CKD) to healthy controls; *$p < 0.01$, Indigenous to non-Indigenous CKD patients (Student's t-test). The ages were significantly different between healthy controls and both CKD groups (Indigenous and non-Indigenous) ($p < 0.0001$, one-way ANOVA).

Table 2. Percentage of CD19+ lymphocytes determined by flow cytometry.

| Group | Percentage of B cells (95% CI) |
|---|---|
| Healthy Controls | 7.0 (5.9-8.0) |
| CKD | 11.1 (8.4-13.7) ** |
| CKD Indigenous | 11.6 (7.7-15.4) ** |
| CKD non-Indigenous | 10.3 (6.2-14.3) * |

* Comparison of the mean proportions of B cells between patients with chronic kidney disease (CKD) to healthy controls (* $p < 0.05$, ** $p < 0.01$, Student's t-test). The proportions of B cells were significantly different between healthy controls and both CKD groups (Indigenous and non-Indigenous) ($p < 0.01$, one-way ANOVA).

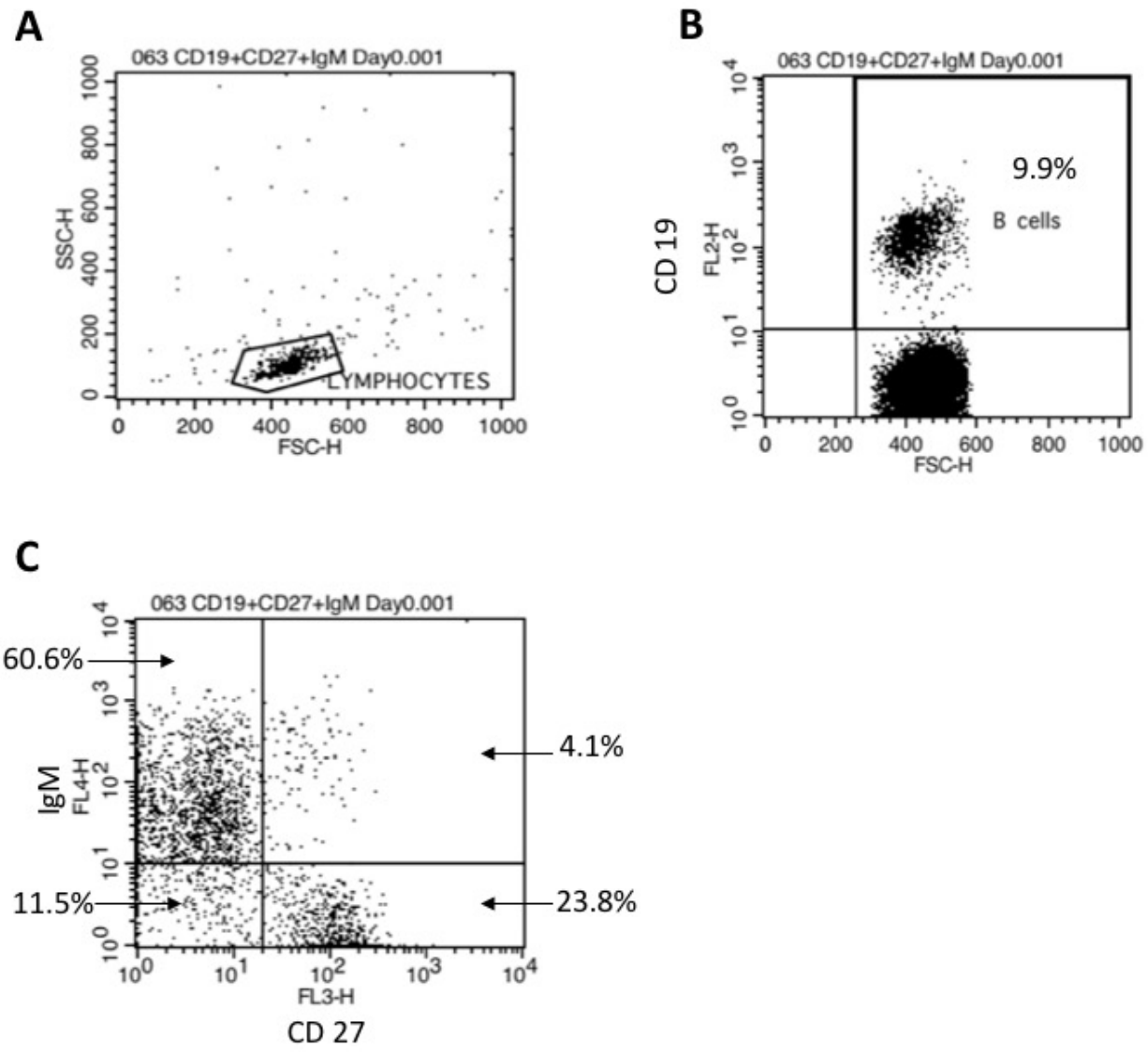


Figure 1. Flow cytometry analysis. Proportions of naïve (CD27- IgM+), IgM memory (CD27+ IgM+), CD27- IgM-, and class switched memory (CD27+ IgM-) B cells were determined by first gating the lymphocyte population (A), then gating the CD19+ lymphocytes (FL2) (B). These events were then separated based on their CD27 (FL3) and IgM (FL4) expression (C). The percent gated values were used for the proportions of B cell subpopulations were determined. One representative experiment is shown; CD19+ (9.9%), CD27-IgM+ (60.6%), CD27+IgM+ (4.1%), CD27-IgM- (11.5%), and CD27+IgM- (23.8%).

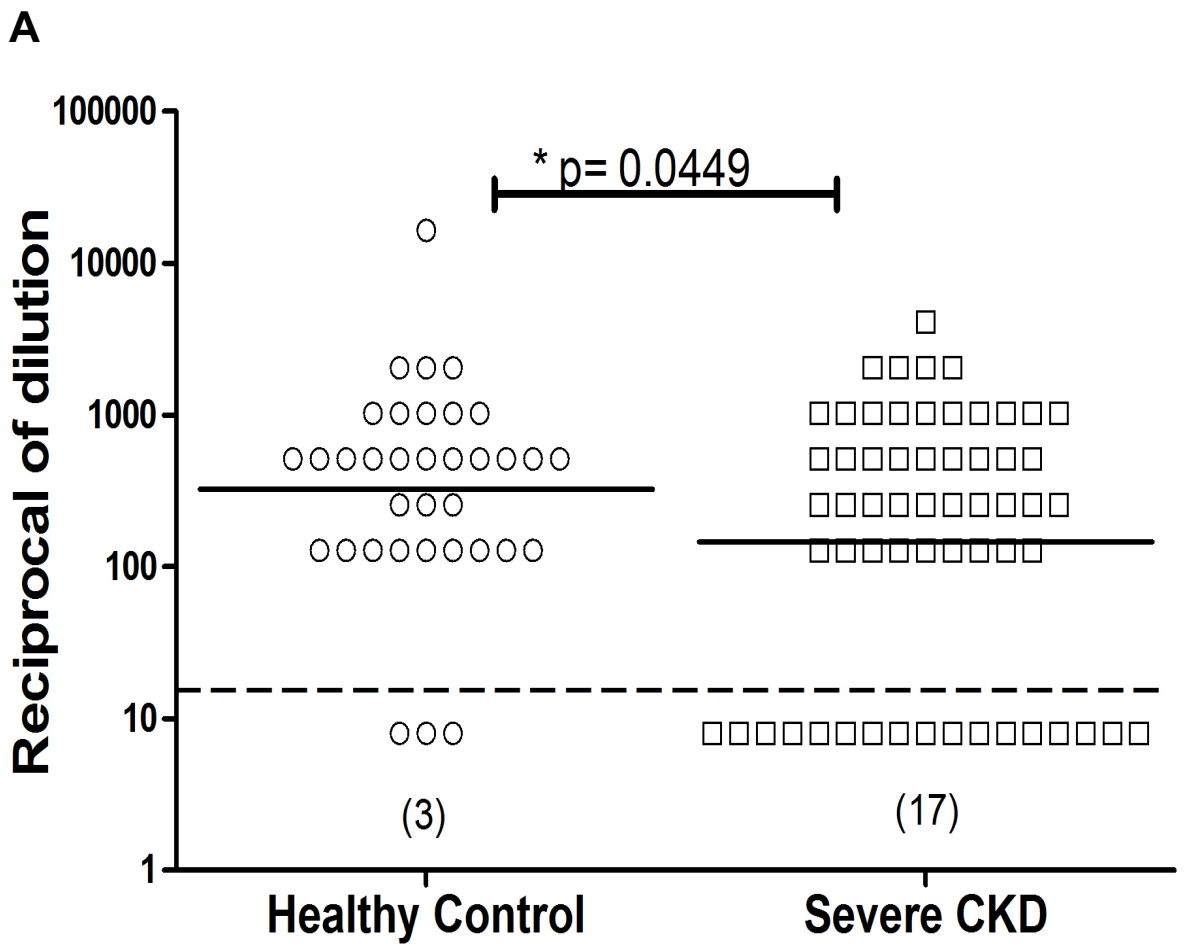


Figure 2A. Serum bactericidal antibody against *Haemophilus influenzae* type a in healthy controls and CKD patients.

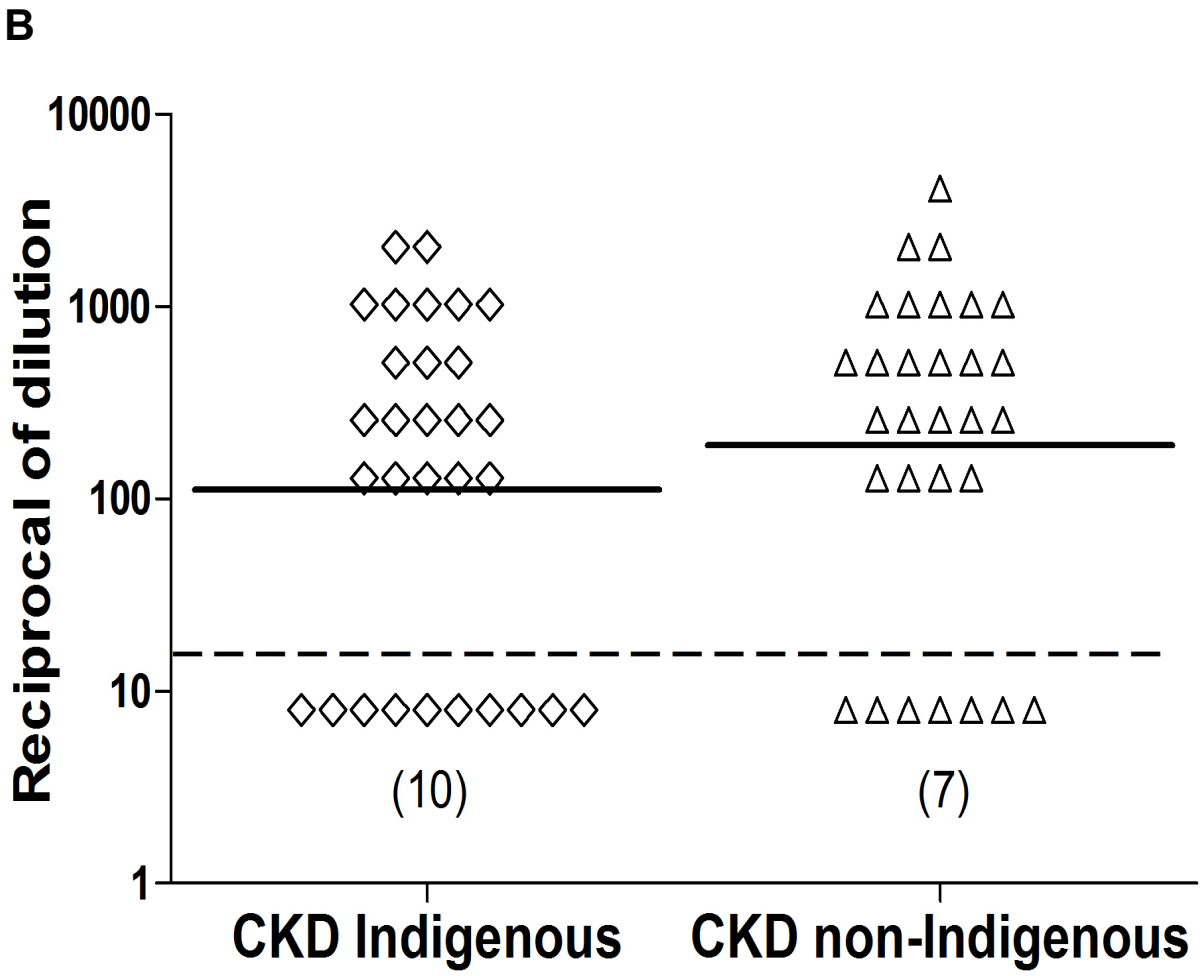


Figure 2B. Serum bactericidal antibody against *Haemophilus influenzae* type a in Indigenous vs. non-Indigenous CKD patients.

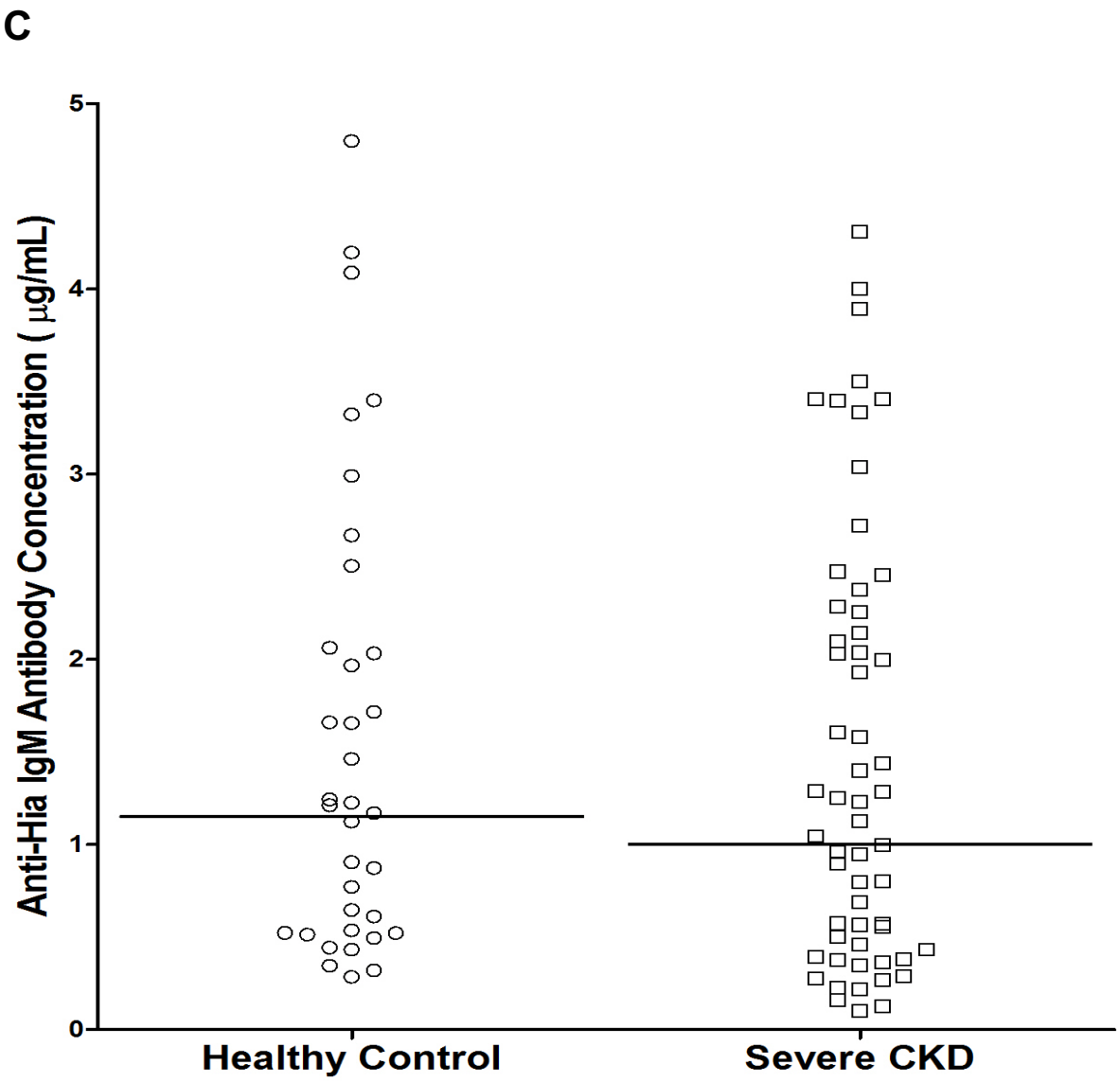


Figure 2C. The geometric mean concentrations of anti-*Haemophilus influenzae* type a capsular polysaccharide IgM in healthy controls and CKD patients.

**D**

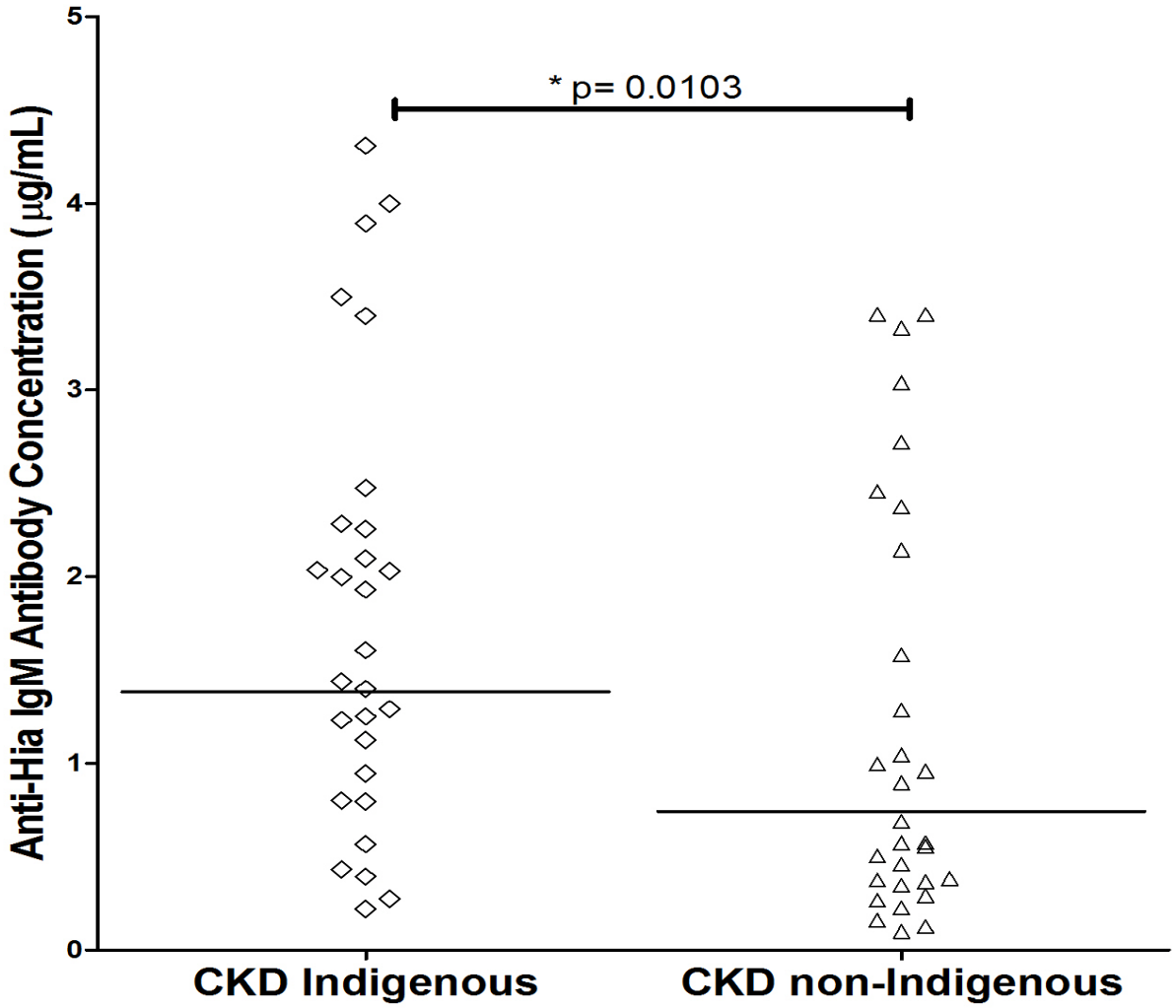


Figure 2D. The geometric mean concentrations of anti-*Haemophilus influenzae* type a capsular polysaccharide IgM in Indigenous vs. non-Indigenous CKD patients.

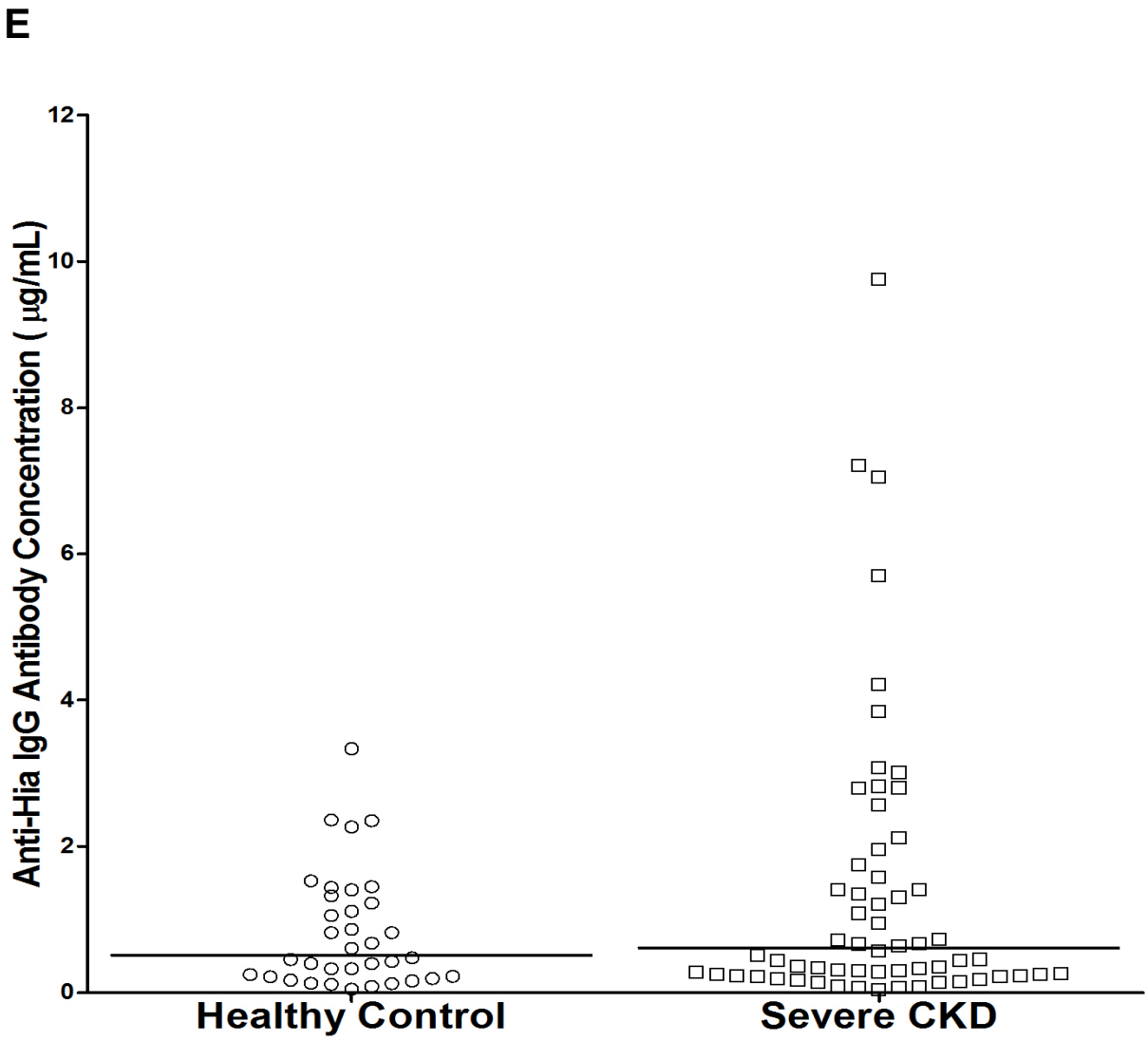


Figure 2E. The geometric mean concentrations of anti-*Haemophilus influenzae* type a capsular polysaccharide IgG in healthy controls and CKD patients.

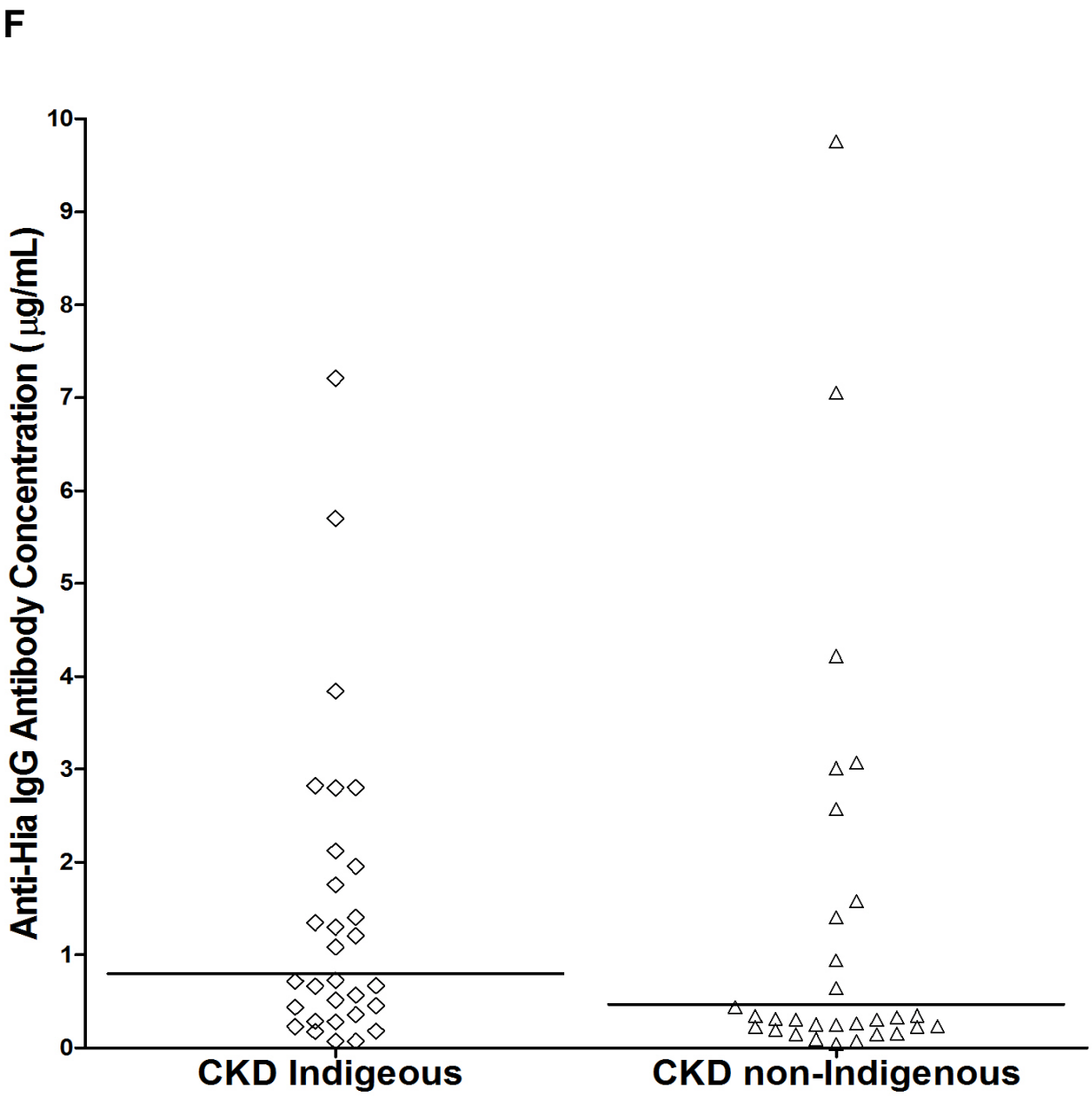


Figure 2F. The geometric mean concentrations of anti-*Haemophilus influenzae* type a capsular polysaccharide IgG in Indigenous vs. non-Indigenous CKD patients.

Figure 2. Serum bactericidal antibody against *Haemophilus influenzae* type a in healthy controls and CKD patients (A) and Indigenous vs. non-Indigenous CKD patients (B). The antibody titre was defined as the reciprocal of serum dilution resulting in ≥ 50% killing of Hia strain 08-191. The solid line indicates geometrical mean titre (GMT). The number of individual samples below the lower limit of detection (dashed line) is indicated on the graph. * $p < 0.05$, Student's t-test. The geometric mean concentrations (GMC, solid line) of anti-Hia capsular polysaccharide IgM (C-D) and IgG (E-F) in healthy controls and CKD patients (C, E) and Indigenous vs. non-Indigenous CKD patients (D, F). * $p < 0.05$, Mann-Whitney U test. No significant difference between the 3 groups (healthy control, CKD Indigenous, and CKD non-Indigenous) for GMT or GMC (IgM or IgG) was detected by one-way ANOVA or Kruskal-Wallis test.

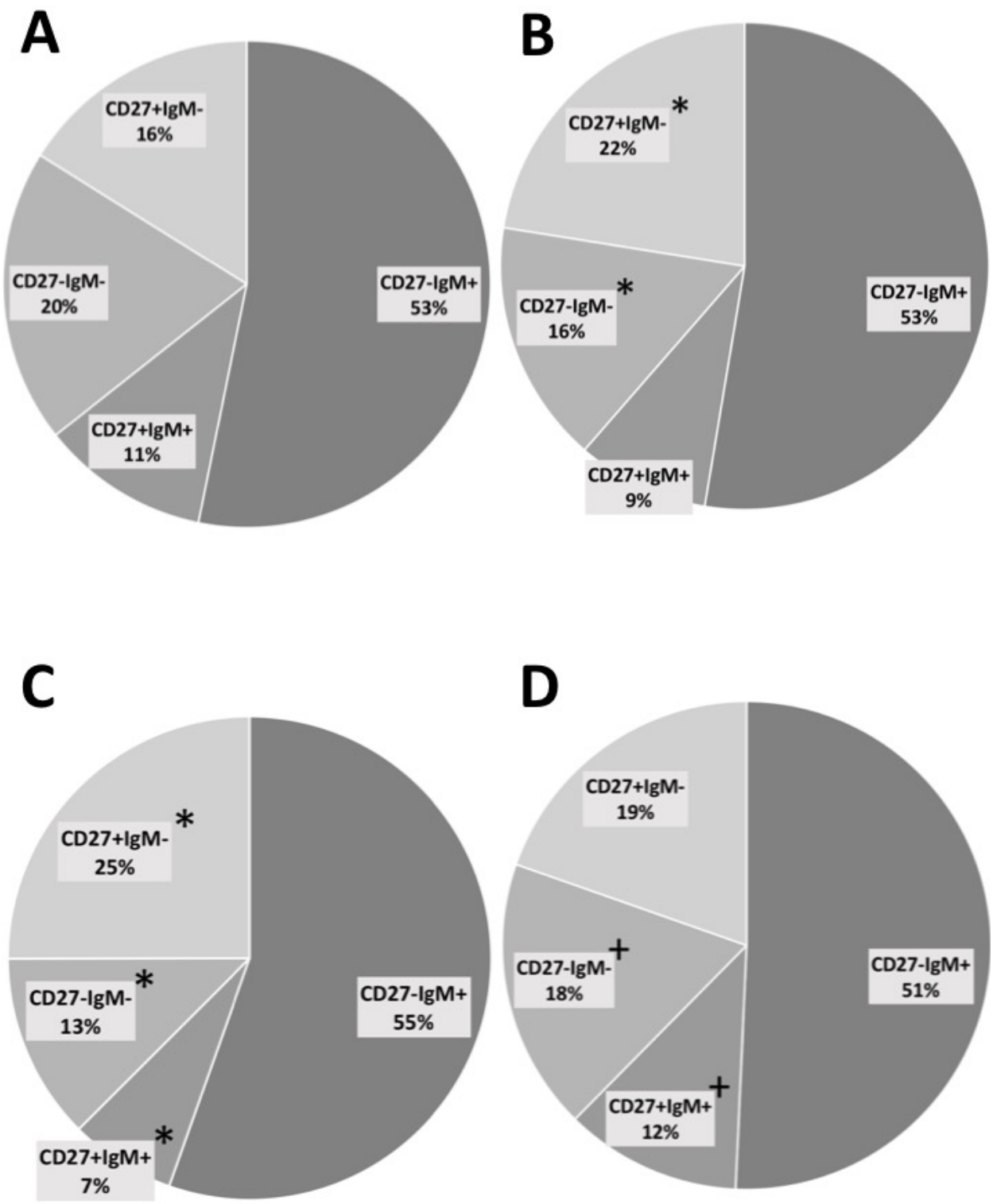


Figure 3. Proportions of B cell subpopulations were determined by flow cytometry.

Figure 3. Proportions of B cell subpopulations were determined by flow cytometry. Isolated peripheral blood mononuclear cells were immunostained for CD19, CD27, and IgM. Naïve (CD19+CD27- IgM+), IgM memory (CD19+CD27+IgM+), CD19+CD27- IgM-, and class switched memory B cells (CD19+CD27+IgM-) were determined for healthy controls (A), patients with chronic kidney disease (CKD) (B), CKD Indigenous (C) and CKD non-Indigenous (D). * $p < 0.05$ when comparing the CKD groups to healthy controls; + $p < 0.05$ comparing Indigenous and non-Indigenous patients with CKD (Student's t-test or Mann-Whitney U test). No significant differences between healthy controls, CKD Indigenous and CKD non-Indigenous patients were detected for any subpopulation except for CD19+CD27-IgM- cells, $p < 0.001$ (Kruskal-Wallis test).

**Supplementary Material**

Supplementary table 1. Causes for development of severe chronic kidney disease and comorbidities.

| **Cause of CKD** | **Patients n (%)** |
|---|---|
| Diabetic Nephropathy | 26 (43) |
| Hypertension/ Vascular disease | 6 (10) |
| Glomerulonephritis | 5 (8) |
| IgA Nephropathy | 4 (7) |
| Diabetic Nephropathy + Glomerulonephritis | 2 (3) |
| Urinary tract obstruction | 2 (3) |
| Other: | |
| Goodpasture disease | 1 |
| Nephrotic syndrome | 1 |
| Polycystic kidney disease | 1 |
| Chronic infection | 1 |
| Wegener's Granulomatosis | 1 |
| Renal agenesis | 1 |
| Acute tubular necrosis | 2 (3) |
| Interstitial nephritis | 1 |
| Unknown | 6 (10) |
| **Major comorbidities** | |
| Diabetes mellitus | 33(55) |
| Hypertension | 40 (67) |
| Cardiovascular disease | 26 (43) |
| Chronic obstructive pulmonary disease | 6 (10) |
| Cancer | 9 (15) |
| Alcohol abuse | 7 (12) |
| Dyslipidemia | 11 (18) |
| Peripheral vascular disease | 7 (12) |

Supplementary table 2. Demographics of patients with B cell analysis.

| Group | *n* | Age (years) | | No. (%) Female |
|---|---|---|---|---|
| | | Mean ± SEM | Range | |
| Chronic Kidney Disease | 28 | 60 ± 3 | 32-85 | 13 (46) |
| Indigenous | 17 | 54 ± 3 | 32-80 | 8 (47) |
| non-Indigenous | 11 | 69 ± 3$^{+}$ | 54-85 | 5 (45) |

+ $p < 0.01$, Indigenous to non-Indigenous patients with chronic kidney disease (CKD) (Student's t-test).

Supplementary table 3. Absolute numbers of lymphocytes and B cell subpopulations.

| Absolute number of cells | CKD Indigenous (mean ± SEM cells/μL) | CKD non-Indigenous (mean ± SEM cells/μL) |
|---|---|---|
| Lymphocytes | 1,091 ± 150 | 1,106 ± 107 |
| B cells (CD19+) | 108 ± 12 | 86 ± 16 |
| Naïve B cells (CD19+CD27-IgM+) | 64 ± 13 | 63 ± 18 |
| IgM memory B cells (CD19+CD27+IgM+) | 7 ± 1 | 12 ± 5 |
| CD19+CD27-IgM- B cells | 15 ± 2 | 17 ± 3 |
| Class Switched Memory B cells (CD19+CD27+, IgM-) | 23 ± 5 | 19 ± 5 |

Total of 28 patients with chronic kidney disease (CKD), 17 Indigenous and 11 non-Indigenous; no statistical significance between groups for any of the cells.

Supplementary table 4a. Proportions B cell Subpopulations.

| Group | *n* | Age | Location of Study | Total B cells (CD 19+) | Naïve B cells (CD27-IgM+) | CD27-IgM- | IgM memory B cells (CD27+IgM+) | Class Switched Memory B cells (CD27+IgM-) | Reference |
|---|---|---|---|---|---|---|---|---|---|
| CKD | 28 | 60 (32-85) | Canada | 11 | 53 | 16 | 9 | 22 | This Study |
| HC | 35 | 62 (36-85) | Canada | 7 | 53 | 20 | 11 | 16 | This Study |
| HC | 22 | 24 (18-30) | USA | -- | 61.4 | 9.8 | 14.3 | 14.4 | (1) |
| | | | | CD 19+ | CD27-IgD+ | CD27-IgD- | CD27+ IgD+ | CD27+IgD- | |
| CKD | 25 | 57 (26-74) | France | -- | 65 | 5.3 | 10 | 20 | (2) |
| HC | 27 | 49 (25-66) | France | -- | 54 | 3.6 | 16 | 27 | (2) |
| Sarcoidosis | 22 | 47/54 | USA | 3.2 | 6.7 | 4.2 | 8.4 | 13.8 | (3) |
| HC | 9 | 47/54 | USA | 5.9 | 7.1 | 2.4 | 15.1 | 16.0 | (3) |
| HC | 32 | 26-50 | Germany | 9.2 | 65.1 | 3.3 | 15.2 | 13.2 | (4) |
| HC | 41 | 72 (65-80) | China | 10.2 | -- | -- | -- | -- | (5) |

-- Information not available. Healthy control (HC), chronic kidney disease (CKD).

Supplementary table 4b. Absolute numbers of cells (cells/μL)

| Source | *n* | Age | Location of Study | Lymphocytes | Total B cells CD 19+ | Naïve B cells CD27-IgM+ | CD27-IgM- | IgM memory B cells CD27+IgM+ | Class Switched Memory B cells CD27+IgM- | Reference |
|---|---|---|---|---|---|---|---|---|---|---|
| CKD | 28 | 60 (32-85) | Canada | 1100 ± 86 | 100 ± 10 | 63 ± 10 | 15 ± 1 | 9 ± 2 | 21 ± 3 | This Study |
| | | | | | CD 19+ | CD27-IgD+ | CD27-IgD- | CD27+IgD+ | CD27+IgD- | |
| Sarcoidosis | 22 | 47/54 | USA | -- | 1149 | 312 | 10.3 | 25.7 | 29.7 | (3) |
| HC | 9 | 47/54 | USA | -- | 2346 | 337.3 | 14.8 | 44.5 | 58.3 | (3) |
| HC | 32 | 26-50 | Germany | 2364 | 199 | 131 | 7 | 35 | 29 | (4) |
| HC | 41 | 72 (65-80) | China | 1946 | 198 | -- | -- | -- | -- | (5) |
| HC | -- | -- | -- | 1000- 4000 | -- | -- | -- | -- | -- | (6) |
| | | | | | CD 19+ | CD19+CD27- | | CD19+CD27+ (all memory) | | |
| CKD | 21 | 52 | USA | 1500 | 140 | 104 | -- | 36 | | (7) |
| HC | 21 | 47 | USA | 1900 | 240 | 180 | -- | 50 | | (7) |

-- Information not available. HC, healthy control group, CKD, chronic kidney disease

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## Chapter 3: Antibodies specific for *Streptococcus pneumoniae* serotypes 6B and 14 in Canadian First Nations and Non-First Nations adults.


### Abstract

*Streptococcus pneumoniae* colonizes the upper respiratory tract and can cause non-invasive diseases such as non-bacteremic community acquired pneumonia (CAP) or invasive pneumococcal disease (IPD). The Canadian Immunization Guide recommends one dose of the 23-valent pneumococcal polysaccharide vaccine (PPV23) for adults at risk for IPD. This includes adults over the age of 65 years as well as adults with certain immunocompromising conditions such as chronic kidney disease (CKD). However, adult pneumococcal immunization rates are low in Canada. In North America, the rate of IPD in the Indigenous population is higher compared to the general population. In northwestern Ontario specifically, the First Nations (FN) population has an increased rate of IPD. We quantified pneumococcal IgG concentrations specific to serotypes 6B and 14 in 207 pneumococcal vaccine naïve adults. Participants were separated based on ethnicity and health: healthy FN, healthy non-First Nations (non-FN), FN with severe CKD (FN-CKD) and non-FN with severe CKD (NFN-CKD). Healthy FN participants had higher antibody concentrations for both serotypes 6B and 14 compared to healthy non-FN participants. FN-CKD had significantly higher serotype 14 antibody concentrations compared to NFN-CKD. Only healthy FN had significantly higher 6B antibody concentrations compared to both CKD groups. Healthy non-FN had significantly lower serotype 14 antibody concentrations compared to healthy FN and FN-CKD. Healthy participants were further separated based on community: FN from southern Ontario (FNSO), FN from northwestern Ontario (FNNO), non-FN from Thunder Bay (NFNT) and non-FN from Kenora (NFNK). Of the six groups, all three FN groups (FNSO, FNNO and FN-CKD) had higher 6B

and 14 antibody concentrations compared to non-FN groups (NFNT, NFNK, and NFN-CKD). Participants were also separated based on sex, ethnicity, and health status. Healthy FN females had significantly higher serotype 14 antibody concentrations compared to healthy FN males. There were no significant differences in either 6B or 14 antibody concentrations between healthy males and females of non-FN, FN-CKD, or NFN-CKD groups. Regression analyses revealed a positive relationship between serotype 14 antibody concentrations and age for healthy FN females. This indicates that as age increases, serotype 14 antibody concentrations also increase. There are many environmental sources that can influence naturally acquired antibodies specific to pneumococcus or antibodies that are cross reactive for other pathogens. Based on the concentrations of pneumococcal 6B and 14 IgG antibodies, the data suggest that the increased risk of IPD in the Indigenous population is due to the increased prevalence of immunocompromising conditions such as CKD. The results from this study emphasize the importance of determining a correlate of protection against pneumococcal disease in unimmunized adults.

## Introduction

*Streptococcus pneumoniae* (pneumococcus) is a Gram-positive, encapsulated, extracellular bacterium, which asymptomatically colonizes mucosal surfaces of the upper respiratory tract and spreads through respiratory droplets of colonized individuals (1, 2). Pneumococcus is capable of causing non-invasive (otitis media, sinusitis, pneumonia) and invasive (meningitis, septicaemia, pericarditis) infections, which occur when it evades the host defences and invades sterile body sites (3, 4). Children under 5 years of age, the elderly, and immunocompromised adults have the highest incidence of invasive pneumococcal disease (IPD)

and community acquired pneumonia (CAP) (3, 5). Globally, the majority of CAP cases are caused by *S. pneumoniae*, and 30-70 % of adult CAP cases require hospitalization (6, 7).

A total of 98 pneumococcal serotypes have been identified through antigenic differences in their polysaccharide capsules (8). The capsular polysaccharide is a key virulence factor in the pathogenesis of pneumococcal disease. The highly charged state of the capsule promotes colonization by allowing adherence to epithelial cells. This prevents entrapment of bacteria in mucus through repulsion of the sialic acid containing mucopolysaccharides. The capsule inhibits the classic and alternative complement pathways through prevention of complement protein binding. It also reduces opsonization by impeding interactions between bound complement proteins or antibodies with phagocyte receptors (9).

In Canada, the 23-valent pneumococcal polysaccharide vaccine (PPV23) and the 13-valent pneumococcal conjugate vaccine (PCV13) are currently used. The PPV23 contains purified capsular polysaccharides of serotypes 1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19F, 19A, 20, 22F, 23F, and 33F. The PCV13 contains polysaccharides 1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F, and 23F conjugated to a carrier protein ($CRM_{197}$). PCV13 is part of the routine infant immunization schedule and is recommended for adults with certain immunocompromising conditions. PPV23 is recommended for adults 65 years and older regardless of risk factors or previous pneumococcal immunization. This includes adults with an increased risk for IPD, such as individuals living with chronic kidney disease (CKD), or those with specific lifestyle factors such as the homeless population, people with alcoholism, people who smoke, etc. An additional dose of PPV23 is recommended for children over 2 years of age with an increased risk of IPD due to underlying medical conditions (10). However, pneumococcal vaccine uptake (PPV23) in adults is low. Statistics Canada reported that amongst

the adult population with chronic conditions, only 17.3% (13.7 - 20.8, 95% confidence intervals, CI) of 18-64 year-olds and 36.5% (32.7 – 40.3, 95% CI) of 65 years and older, correspondingly, received at least 1 dose of PPV23 (11).

The population of Thunder Bay, located in northwestern Ontario, is unique amongst other areas of Ontario because 25.4% of the population is of Indigenous descent. The proportion of Indigenous peoples is lower in other areas of Ontario such as Toronto, (located in southern Ontario) which has a proportion of 0.87% Indigenous peoples as well as northeastern Ontario where 12.7% of the population is Indigenous (12). In North America, the incidence of IPD remains higher in Indigenous populations compared to the general population despite the use of pneumococcal vaccines (13-18). Eaton *et al*. 2017 investigated the incidence of IPD in northwestern Ontario through retrospective chart review of the Sioux Lookout Meno Ya Win Health Center (SLMHC). The incidence of IPD over a 5.5-year period from January 2010 – July 2015 was 23.1/ 100 000/ year, more than double the 2013 rate for all of Canada (9.0/ 100 000/ year). The age specific incidence of IPD was highest in the 71-75 years group (106.5/ 100 000/ year) (19). A recent study by our group evaluated hospitalized cases of IPD in Thunder Bay which occurred between 2006 and 2015. It reported that 29.1% of adult patients with IPD were Indigenous, and the likelihood of immunocompromising conditions was higher amongst the Indigenous patients than in the non-Indigenous (20). CKD is an immunocompromising condition that has a high prevalence in the First Nations population of northwestern Ontario. The rate of CKD in the First Nations population of northwestern Ontario (7%) is double the rate of the general Canadian population. This high rate of CKD in combination with other conditions (e.g. diabetes) is associated with a high rate of cardiovascular disease (21). According to the National Advisory Committee on Immunization's recommendations for adult pneumococcal

immunization, adults with CKD are considered one of the groups at highest risk for IPD. People with diabetes, chronic heart disease, or lung disease are also considered at high risk for IPD (22).

The Indigenous population of northwestern Ontario has been found to have an increased susceptibility to other infectious diseases, specifically invasive disease caused by *Haemophilus influenzae* type a (Hia), which co-inhabits the nasopharynx with pneumococcus (23, 24). Our recent study (chapter 2) compared naturally acquired immunity against Hia in Indigenous and non-Indigenous Canadians. Although invasive Hia disease is most predominant in the Indigenous population, this was not due to lack of natural immunity against this pathogen. In contrast, healthy First Nations adults exhibited higher levels of Hia antibodies, as well as increased serum bactericidal activity against Hia compared to non-First Nations individuals of the same age (23). However, naturally acquired immunity against *S. pneumoniae* has not been previously assessed in Canadian adults. In this study, we determined the concentrations of pneumococcal IgG antibodies specific for serotypes 6B and 14 in both First Nations and non-First Nations adults from southern Ontario and northwestern Ontario. We also included patients with severe CKD (glomerular filtration rate (GFR), stage 4 < 30 mL/ min/ 1.73m$^2$, stage 5 < 15 mL/ min/ 1.73m$^2$) (25) receiving dialysis at the Thunder Bay Regional Health Sciences Center (TBRHSC) due to their increased risk of IPD. As these serotypes have been included in all pneumococcal vaccines, herd immunity could have a significant impact on carriage of these serotypes, which could affect the antibody concentrations in unimmunized adults.

**Materials and methods**

Ethics Statement

In designing and conducting this study, we adhered to the principles of Ownership, Control, Access, and Possession (OCAP) as defined by the National Aboriginal Health Organization (26), and the guidelines of Canadian Tri-Council Policy Statement: Ethical Conduct for Research Involving Humans (TCPS2), specifically those outlined in Chapter 9: Research Involving First Nations, Inuit and Métis Peoples of Canada (27). The study was approved by the research ethics boards of Lakehead University and TBRHSC (Thunder Bay, Ontario).

Participants

| Participant groups | |
|---|---|
| FN | Healthy First Nations adults (FNSO + FNNO) |
| Non-FN | Healthy non-First Nations adults (NFNT + NFNK) |
| FNSO | Healthy First Nations adults from southern Ontario |
| FNNO | Healthy First Nations adults from northwestern Ontario |
| NFNT | Healthy non-First Nations adults from Thunder Bay |
| NFNK | Healthy non-First Nations adults from Kenora |
| FN-CKD | First Nations adults with severe chronic kidney disease |
| NFN-CKD | Non-First Nations adults with severe chronic kidney disease |

The study included 141 healthy adults (Table 1). First Nations (FN) participants were recruited from two Ojibwa First Nations communities, one in northwestern Ontario (FNNO) and the other in southern Ontario (FNSO). Non-First Nations participants were recruited from two cities in northwestern Ontario, Thunder Bay (NFNT) and Kenora (NFNK). An additional 66 participants with CKD were recruited from the Thunder Bay Regional Health Sciences Center (THBRHSC), participants were separated into two groups: First Nations-CKD (FN-CKD) and Non-First Nations-CKD (NFN-CKD). The demographics of participants is displayed in Table 1. Healthy participants were considered eligible if they were over the age of 18 years, self-declared generally healthy, did not have a history of taking immunosuppressive medications, and declared

that they had not received a pneumococcal vaccine. Participants with CKD were considered eligible if they were over the age of 18 years, diagnosed with stage 4 or 5 CKD, had no history of immunocompromising conditions, had not taken immunosuppressive medications for more than 14 days in the past 6 months, had not received a blood transfusion or blood products within the last 3 months, had not received any vaccines in the past month, and had not received a pneumococcal vaccine previously. Ethnicity was determined based on self-declaration. Serum samples from FNSO and FNNO communities, Kenora, and some of the Thunder Bay samples were collected during January - November 2015 (23). The remaining Thunder Bay samples were collected between November 2016 and November 2017, mainly through the Thunder Bay 55 Plus Centre. CKD participants were recruited between May 2015 and February 2019 from Renal Services of the TBRHSC. Sera were collected under informed written consent and stored at -80°C prior to analysis.

Enzyme-linked immunosorbent assay (ELISA)

Amounts of IgG pneumococcal 6B and 14 antibodies were quantified using the ELISA protocol provided by the World Health Organization (28).

Statistical analysis

Statistical analyses were performed using Graph-Pad Prism 8. The lower limits of detection were determined by calculating the concentration of antibodies present in the standard serum 007sp (29) that yielded two times the assay background; the lowest serum dilution on the plate was used. All antibody values below the lower limit of detection were reported as half of the limit of detection. Data were reported as geometric means of pneumococcal antibody

concentrations with 95% confidence intervals (CI). Groups were compared using Mann-Whitney U test, Student's t- test, or Kruskal-Wallis test with Dunn's post-hoc test depending on the number of groups compared. Linear regression analysis was performed to analyze the relationship between age and antibody concentrations. Significance was determined at p value < 0.05. Outliers were identified and removed (30).

**Results**

The age range of the 207 study participants was between 18 and 87 years old (Table 1). There were no significant differences between mean ages of all healthy FN and non-FN participants. When CKD groups were compared, NFN-CKD participants had a significantly higher mean age compared to FN-CKD participants. Next, CKD and healthy participant groups were compared. FN-CKD had a significantly higher mean age than healthy FN participants. NFN-CKD had a significantly higher mean age compared to healthy groups. When participants were separated based on community and health, there was a significant difference in the ages of all six groups. The highest mean age was NFN-CKD (67 years) and the lowest mean age was FNNO (37 years). NFN-CKD had a significantly higher mean age compared to the other five groups. FNNO had a significantly lower mean age compared to FN-CKD, FNSO, and FNNO. NFNK had the highest proportion of females (74%) while NFN-CKD had the lowest (32%).

To determine if there were differences in pneumococcal antibody concentrations between healthy FN and non-FN adults, IgG specific to capsular polysaccharides of serotypes 6B and 14 were quantified (Figures 1A and 1B). Healthy FN adults had significantly higher concentrations of antibodies specific for 6B and 14 compared to healthy non-FN participants. Additionally, when participant groups were separated based on health and community, healthy FN groups

(FNSO and FNNO) had significantly higher serotype 6B and 14 concentrations compared to non-FN groups (NFNT and NFNK) and both CKD groups. There were no significant differences in 6B antibody concentrations between CKD groups, but FN-CKD had significantly higher serotype 14 antibody concentrations compared to NFN-CKD. Healthy FN participants had the highest concentrations of antibodies specific to serotypes 6B and 14. Healthy non-FN and FN-CKD participants had the lowest concentrations of serotype 6B antibodies. Healthy non-FN participants also had the lowest concentrations of antibodies specific for serotype 14. Serotype 14 antibody concentrations for NFN-CKD were significantly higher compared to healthy non-FN participants, but significantly lower compared to healthy FN participants.

Participants were separated based on health and community. Figure 2A displays the concentrations of antibodies specific for serotype 6B. Of the six groups, FNSO had the highest concertation of serotype 6B antibodies followed by FNNO, FN-CKD, and NFNK. NFNT and NFN-CKD had the same geometric mean concentrations, which were the lowest amongst the six groups. There were no significant differences in 6B antibody concentrations between healthy FN groups (FNSO *vs*. FNNO) or healthy non-FN participants (NFNT *vs*. NFNK). Serotype 6B antibody concentrations for FN-CKD were significantly lower compared to healthy FN groups, but significantly higher compared to NFNT.

Figure 2B displays the concentrations of antibodies specific for serotype 14 based on health and community. FNSO had the highest concentration of serotype 14 antibodies followed by FNNO and FN-CKD (having the second highest concentrations), NFN-CKD, NFNK, and NFNT. There were no significant differences between healthy FN groups (FNSO *vs*. FNNO) or healthy non-NF groups (NFNT *vs.* NFNK). FN-CKD had significantly higher serotype 14

antibody concentrations compared to healthy non-FN groups. NFN-CKD had significantly lower serotype 14 antibody concentrations compared to healthy FN groups.

To summarize, healthy FN participants had higher antibody concentrations for both serotypes 6B and 14 compared to healthy non-FN participants. FN-CKD had significantly higher serotype 14 antibody concentrations compared to NFN-CKD, but there were no significant differences in serotype 6B antibody concentrations. Only healthy FN participants had significantly higher 6B antibody concentrations compared to CKD groups. Healthy non-FN participants had significantly lower serotype 14 antibody concentrations compared to healthy FN participants and FN-CKD. When participants were separated based on health and community, all three FN participant groups (FNSO, FNNO, and FN-CKD) had higher serotype 6B and 14 antibody concentrations compared to non-FN participant groups (NFNT, NFNK, and NFN-CKD).

Participants were also separated based on sex; results are displayed in Supplementary Table 1. There were no significant differences in pneumococcal antibody concentrations for either serotype 6B or 14 between all healthy females and all healthy males. Healthy participants were further separated based on ethnicity and sex. FN females had significantly higher concentrations of serotype 14 antibodies compared to FN males but there were no significant differences in concentrations of serotype 6B antibodies. There were no significant differences in concentrations of either serotype 6B or 14 antibody concentrations between non-FN females and non-FN males. When participants of the same sex but different ethnicities were compared, FN females had significantly higher concentrations of both pneumococcal 6B and 14 antibody concentrations compared to non-FN females. FN males had significantly higher serotype 6B

antibody concentrations compared to non-FN males, but there were no significant differences in serotype 14 antibody concentrations.

CKD participant groups were also further separated based on sex. There were no significant differences in 6B or 14 antibody concentrations between females and males for either CKD group. There were no significant differences between FN-CKD and NFN-CKD females or males in serotype 6B or 14 antibody concentrations. Healthy participants were compared to CKD participant groups. Concentrations of serotype 6B antibodies were significantly higher for FN healthy females compared to FN-CKD females as well as FN healthy males compared to FN-CKD males. There were no significant differences in serotype 14 antibody concentrations between FN healthy and FN-CKD females or males. NFN-CKD females had a significantly higher serotype 14 antibody concentrations compared to non-FN healthy females. There were no significant differences in serotype 14 antibody concentrations between non-FN healthy and NFN-CKD males. To summarize, the differences in antibody concentrations of participants of opposite sex but same ethnicity and health status, FN females had higher concentrations compared to FN males for serotype 14. There were no significant differences in either 6B or 14 antibody concentrations between females and males of non-FN, FN-CKD or NFN-CKD groups.

To determine if antibody concentrations were dependent on age, linear regression analysis was performed (Supplementary Table 2). When all healthy participants were combined, there were no significant relationships indicating dependence of antibody concentration on age for either 6B or 14. When participants were further separated based on ethnicity, only serotype 14 antibody concentrations were dependent on age for non-FN participants. When the participants were further separated based on sex, the positive relationship between age and antibody concentration for serotype 14 was only found in the non-FN healthy female group.

There were no significant relationships indicating dependence of antibody concentration on age for either serotype 6B or 14 for either CKD group. To summarize, a positive relationship was found between serotype 14 antibody concentrations and age for healthy FN females, indicating that as age increases, serotype 14 antibody concentrations also increase.

**Discussion**

Both pneumococcal serotypes 6B and 14 have a history of antibiotic resistance in children and have been documented as poorly immunogenic (31, 32). Serotypes 6B and 14 have also been known to cause IPD in older Alaskan adults (33). Salt *et al*. (2007) observed that the least immunogenic serotypes cause the most infections in children, and serotype 6B being poorly immunogenic makes it more favourable for persistent carriage (34). This indicates the importance of their inclusion in pneumococcal vaccines. In the most recent data obtained from the National Laboratory Surveillance of Invasive Streptococcal Disease in Canada annual summary for 2017, the prevalence of IPD was less than 1% for both serotype 6B and 14 in all adult age groups (15 - 49, 50 - 64, and 65 years and over). The most common serotypes that caused IPD in all age groups were 3, 22F, 4, 9N, and 8, all of which are serotypes included in PPV23, and three of these are included in PCV13 (35). The rates of IPD in Canada caused by PCV13 serotypes have decreased since the implementation of PCV13 (2010), with 6B accounting for 0.3% of cases and 14 accounting for 0.5% of cases in the 15 - 49-year group in 2012 (36). Leblanc *et al*. (2019) investigated the incidence of serotypes causing pneumococcal CAP in Canada between 2011 and 2015 and found that serotype 14 has not caused any cases of CAP since 2011, and CAP cases caused by serotype 6B were last seen in 2012 and 2013, with both serotypes causing less than 5% of CAP cases each year. The serotypes causing the most

pneumococcal CAP cases from this 4-year period were 3, 7F, 19A, 6A, and 5, all of which are in PCV13, and four in PPV23 (37).

Previous studies have found that since the implementation of the infant immunization program with PCV7, the prevalence of serotypes included in PCV7 causing IPD has decreased over time. This could be due to serotype replacement, which results in the increased prevalence of non-vaccine serotypes causing IPD. It is also possible that the reduction in vaccine serotypes has resulted in better detection of non-vaccine serotypes, a phenomenon also known as unmasking (38, 39). This suggests that the colonization rates of these serotypes have decreased in adults. Colonization of pneumococcus is important for adults because it is an immunizing event that results in the production of anti-capsular and anti-protein antibodies. The maintenance of pneumococcal memory B cells is reliant on repeated exposure to pneumococcus (40, 41). It has also been suggested that colonization with pneumococcus throughout life results in an increased secondary immune response to pneumococcal conjugate vaccines (41). Pneumococcal colonization could also be important in boosting immunity against pneumococcus in adults for the maintenance of pneumococcal antibody concentrations. This has been found to be important for maintenance of antibodies specific for other pathogens such as *Haemophilus influenzae* type b (Hib). Makela *et al*. (2003) found that maintenance of immunological memory post- Hib conjugate vaccine was dependent on repeated exposure to both Hib and cross-reactive bacteria (42).

The differences in pneumococcal antibody concentrations between FN and non-FN participants could be due to antibodies that are cross-reactive with other pathogens or environmental antigens. Lagergard *et al*. (1983) found that pneumococcal serotype 6B antibodies are cross-reactive with Hia polysaccharide (43). Disease caused by Hia is more prominent in the

Canadian Indigenous populations, specifically in the FN population of northwestern Ontario (23). It has also been reported that pneumococcal IgG antibodies cross-react with *Streptococcus mitis*, a bacterium that commensally inhabits the oral cavity and upper respiratory tract (44). Peoples of Indigenous descent have been found to have poor oral health due to isolation, reliance on processed foods, and a lack of access to dental care (45). This could cause an imbalance in the oral environment, making it more favorable for bacterial growth (46).

Pneumococcal antibodies have been found to cross-react with *Klebsiella pneumoniae* capsular polysaccharides (47). This bacterium inhabits gastrointestinal tracts of humans and animals and is also an opportunistic pathogen that can cause disease in humans and other animals. *K. pneumoniae* has been associated with foodborne disease outbreaks, but has also been known to cause extraintestinal human infections such as pneumonia (48). Heidelberger *et al*. (1986) found that pneumococcal antibodies cross-react with *Pseudomonas aeruginosa* lipopolysaccharides (49). *P. aeruginosa* is typically found in natural water sources such as lakes and rivers (50). It has been documented that Indigenous communities have a history of having inadequate access to safe drinking water and hence could have been exposed to *P. aeruginosa* more frequently than non-FN communities (51). Exposure to plant polysaccharides for nutrition or medical purposes could also result in the production of cross-reactive antibodies. Heidelberger *et al*. (1982) investigated cross-reactions of anti-pneumococcal serum and plant polysaccharides. Anti-pneumococcal serotype 6 serum has been found to cross-react with polysaccharides of *Althaea officinalis* (marshmallow plant) as well as *Ocimum basilicum* (basil), and anti-pneumococcal serotype 14 serum has been found to cross-react with polysaccharides of linseed (flax) and sassafras. These are just a few examples of the plant polysaccharides tested; unfortunately, not many plants that typically grow in Canada were tested. Traditional Indigenous

medicine uses a wide variety of plants to treat ailments (52), and therefore it is possible that some of these plant polysaccharides induce production of natural antibodies cross-reactive with pneumococcal polysaccharides.

As mentioned previously, the FN population in northwestern Ontario not only have an increased incidence of IPD, they also have an increased incidence of disease caused by Hia (19, 53, 54). In a previous study by our group, Nix *et al*. (2018) found that both the serum bactericidal activity and IgM antibody concentrations specific to Hia are higher in the FN population compared to non-FN individuals (23). Similarly, our FN groups (FNSO, FNNO and FN-CKD) had higher IgG serotype 6B and 14 antibody concentrations compared to non-FN groups (NFNT, NFNK, and NFN-CKD). Nix *et al*. (2018) reported that FNSO had the highest serum bactericidal activity and IgM antibody concentrations specific to Hia, and in our present study, FNSO also had the highest IgG antibody concentrations specific for both pneumococcal serotypes 6B and 14. These findings suggest that there is increased carriage of Hia and pneumococcus in the FN population. Unfortunately, there is very limited information in the literature on risk factors for increased carriage in adults, and from the studies that are available, many of the factors seem to be dependent on the community being assessed. Some of these common risk factors that increase carriage include: presence of young children in the household, overcrowding in the household, lack of hygiene or clean water, living in a remote community, tobacco use, and lower socioeconomic status (55-58).

When comparing the CKD groups to the healthy participants of the same ethnicity, FN-CKD participants had significantly lower serotype 6B antibody concentrations compared to healthy FN participants, but no significant differences in serotype 14 antibody concentrations. There were no significant differences in serotype 6B antibody concentrations between NFN-

CKD and healthy non-FN participants, but NFN-CKD had significantly higher serotype 14 IgG concentrations compared to healthy non-FN participants. When comparing CKD groups, FN-CKD had significantly higher concentrations of serotype 14 antibodies compared to NFN-CKD.

It is important to consider that NFN-CKD have a significantly higher mean age compared to the four healthy participant groups. It is also important to note that the FN-CKD mean age is significantly lower compared to NFN-CKD. This is not uncommon as it has been reported previously that people of Indigenous descent have a tendency to develop CKD at a younger age (59). Five of the six groups have a mean age that is considered middle age (40 - 60 years) while the NFN-CKD participants' mean age categorizes them as elderly (≥ 65 years). This is important to consider when comparing groups because of a phenomenon known as immunosenescence, which is the decline of immune system function due to aging in the elderly. In regard to humoral immunity, elderly people have diminished B cell responses and decreased antibody levels (60). However, our NFN-CKD patients have higher serotype 14 concentrations compared to healthy non-FN, suggesting that immunosenescence has not resulted in decreased serotype 14 concentrations. We cannot fully explain why NFN-CKD have higher serotype 14 concentrations, but it is possible that the NFN-CKD patients could have increased exposure to pneumococcus or antigens in the hospital environment that resulted in the production of cross-reactive antibodies.

Patients with CKD have multiple immune abnormalities due to uremia, but in terms of humoral immunity, these patients have reduced numbers of B cells and decreased function of antibody producing plasma cells (61). Watt *et al*. (2007) reported that IPD is 3 - 5 times more common amongst Indigenous Navajo adults compared to the general US population. The identified risk factors for IPD included CKD, congestive heart failure, alcohol use or alcoholism, extremely high or low body mass index (BMI), and unemployment (62). Factors that contribute

to the progression of carriage to IPD consist of age, cigarette smoking, alcohol consumption, and comorbidities (chronic obstructive pulmonary disease, heart failure, asthma, and diabetes) (63). Indigenous Canadians have an increased prevalence of diseases such as obesity, diabetes, circulatory diseases, and cancer, resulting in a decreased life expectancy (64). This suggests that the increased IPD rate in the FN population is due to the increased prevalence of risk factors such as CKD, cardiovascular comorbidities, and diabetes mellitus in the northwestern Ontario FN population. The immune dysfunction associated with immunocompromised individuals within the FN population indicates that this could also have an impact on IPD risk (21). It has been documented that the Canadian Indigenous population has increased rates of invasive bacterial diseases such as IPD, invasive *Haempohilus influenzae* disease, invasive Group A streptococcal disease, invasive meningococcal disease, and invasive Group B streptococcal disease (65). Reisman *et al*. (2014) suggested that the increased IPD rates in Indigenous Alaskan population was due to lack of in-home water services, and that improving sanitation services and decreasing overcrowding in the household could decrease the incidence of IPD (56). A major issue as a result of the low socioeconomic status in the FN communities is inadequate access to clean water, which is associated with decreased hygiene practices such as hand washing (66). Overcrowding in households causes increased carriage of pneumococcus, which suggests that there is an increased transmission of pathogens resulting in increased rates of infections such as IPD in FN population (56, 67).

A previous study by Simell *et al*. (2008) investigated the effects of aging and sex on naturally acquired pneumococcal antibodies. Participants were separated based on sex and if they were over the age of 65 years or under 65 years old. Concentrations of anti-capsular IgG specific for serotype 6B were significantly lower in the elderly, and a larger decrease in anti-

pneumococcal capsular polysaccharide antibodies was seen in women (68). Due to a lack of elderly participants in this study, the effect on age could not be assessed. When participants of the same ethnicity but different sex were compared, the only significant difference was that healthy FN females had significantly higher serotype 14 IgG concentrations compared to FN males. Regression analyses detected only one significant relationship. A positive relationship between age and serotype 14 antibody concentrations indicates that as healthy non-FN female participants age, their serotype 14 IgG concentrations also increase. It is difficult to interpret these data without additional participant information, but serotype 14 IgG concentrations appear to have a relationship with the female sex. This could indicate that the difference between males and females could be due to differences in exposure to children who have a much higher rate of carriage of pneumococcus than adults (69). Although the previously mentioned study found that there is a negative relationship of antibody concentration and age specifically in women (68), this could be due to differences in the ages of our study participants, as well as different living conditions.

Protective antibody concentrations against pneumococcal disease have not been established for adults. To determine if the antibody concentrations of the adults included in this study were different from other adults, IgG concentrations from the literature are summarized in Supplementary Table 3. The range of serotype 6B IgG concentrations in healthy adults was 0.4-2.1 µg/ mL and for serotype 14 IgG concentrations was 0.9 - 7.4 µg/ mL. Mitra *et al*. (2016) reported a geometric mean of 0.6 µg/ mL for serotype 6B IgG antibodies and 2.1 µg/ mL for serotype 14 IgG antibodies in CKD patients. There is a wide range in the literature, and our healthy participants are within this range. The concentrations of our CKD groups are similar to those reported by Mitra *et al*. (2016) (70). The correlate of protection against IPD post-PCV

immunization for infants is 0.35 μg/ mL. However, it has been suggested that this may not be true for all serotypes, and that concentrations resulting in protection may be dependent on serotype (71).

This study has several limitations, such as not having enough elderly participants over the age of 65, and non-FN participants were not able to be recruited from southern Ontario. Carriage data was not collected, functional abilities of pneumococcal antibodies could not be assessed, and there was very limited information about participants available (e.g., number of people in household). We also did not have access to healthy participant medical records so we could not confirm that participants were pneumococcal vaccine naïve. The biggest limitation is that protective antibody titres have not been established for adults, and therefore we cannot determine the proportion of participants at increased risk for IPD.

**Conclusions**

There are many environmental factors that can influence natural antibody concentrations. Based on the concentrations of pneumococcal 6B and 14 IgG antibodies, the data suggest that the increased risk of IPD in the Indigenous population is likely due to the increased prevalence of immunocompromising conditions such as CKD, rather than decreased antibody concentrations. Determining a correlate of protection for adults would be beneficial to determine if certain adults need to receive pneumococcal immunizations before the age of 65 years old.

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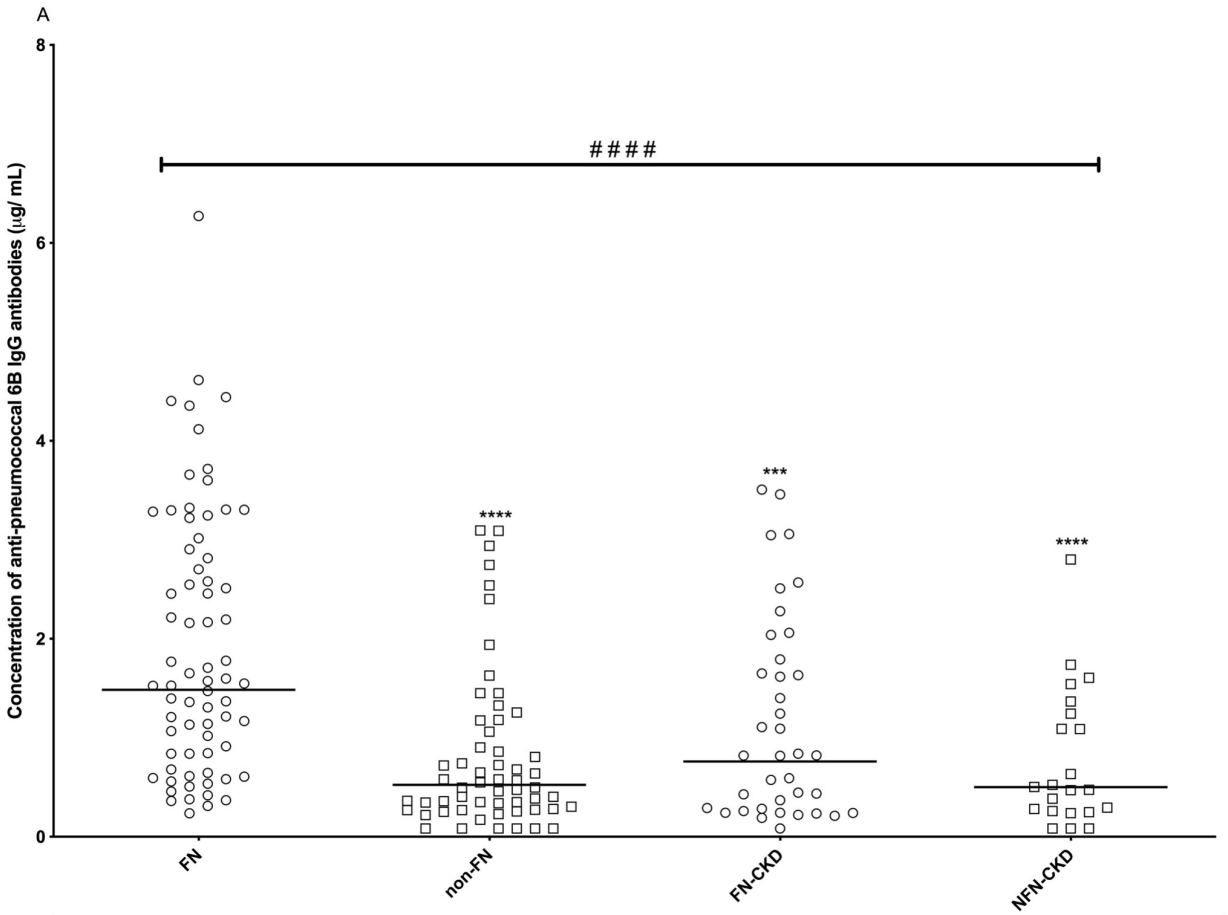
**Tables and Figures**

Table 1. Participant demographics.

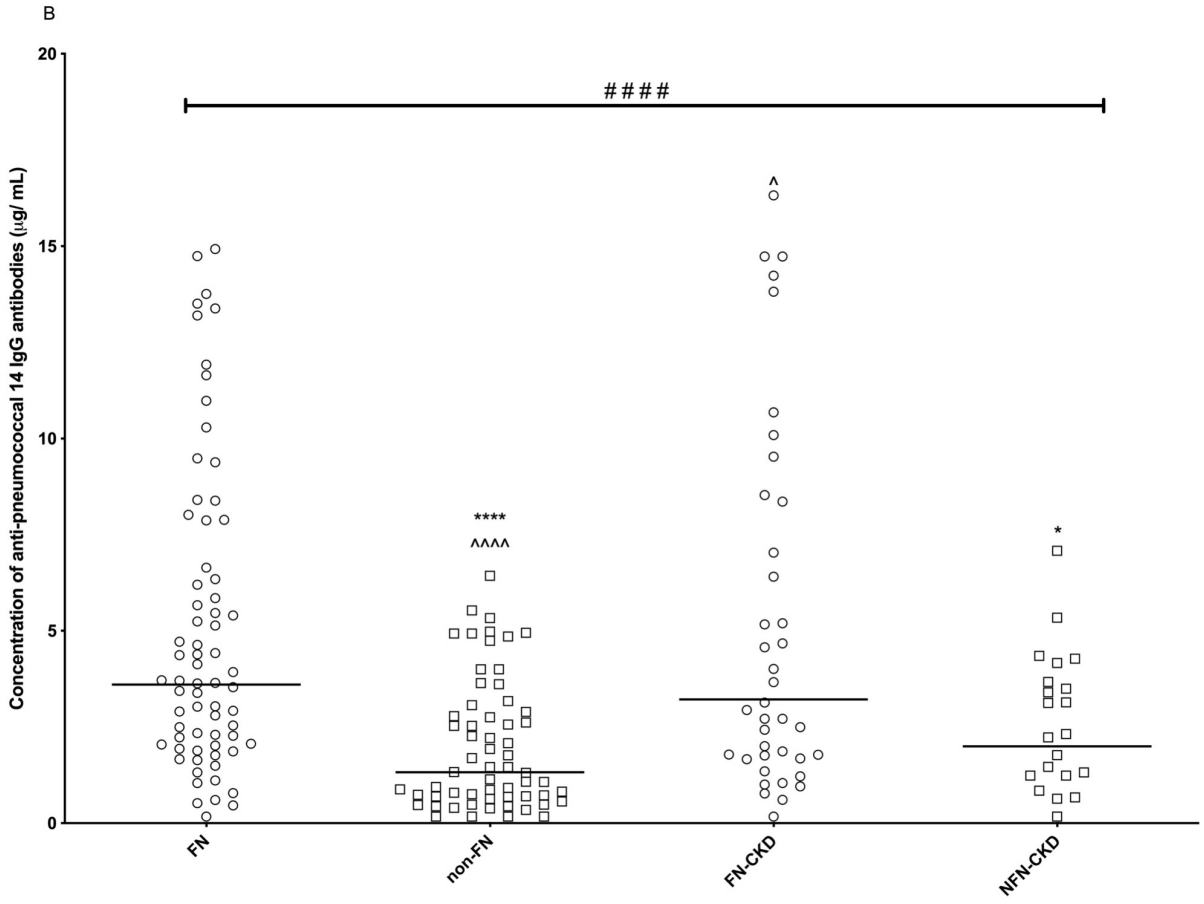
| Group | Number | Age mean (median) | Age Range | % Female |
|---|---|---|---|---|
| All healthy First Nations (FN) | 77 | 41 (39) | 18-80 | 49 |
| All healthy non-First Nations (non-FN) | 64 | 45 (52) | 20-72 | 66 |
| First Nations Southern Ontario (FNSO) | 30 | 46 (51) (2 unknown) ** | 20-80 | 50 |
| First Nations Northwestern Ontario (FNNO) | 47 | 37 (34) | 18-67 | 40 |
| Non-First Nations Thunder Bay (NFNT) | 45 | 46 (52) ** | 22-72 | 62 |
| Non-First Nations Kenora (NFNK) | 19 | 43 (48) | 20-68 | 74 (1 unknown) |
| First Nations Thunder Bay- CKD (FN-CKD) | 41 | 49 (50) *** | 20-73 | 41 |
| Non-First Nations Thunder Bay- CKD (NFN-CKD) | 25 | 67 (67) ++++, **** | 20-87 | 32 |

* compares to First Nations Northwestern Ontario, and + compares to all other groups. Mann-Whitney U test, **$p < 0.01$, *** 0.001, **** 0.0001. Kruskal-Wallis, post-hoc analysis Dunn's test $p < 0.0001$.




| Geometric Mean, 95% Confidence intervals (µg/ mL) | | | |
|---|---|---|---|
| 1.5, 1.2- 1.8 | 0.5, 0.4- 0.7 | 0.8, 0.5- 1.1 | 0.5, 0.3- 0.8 |

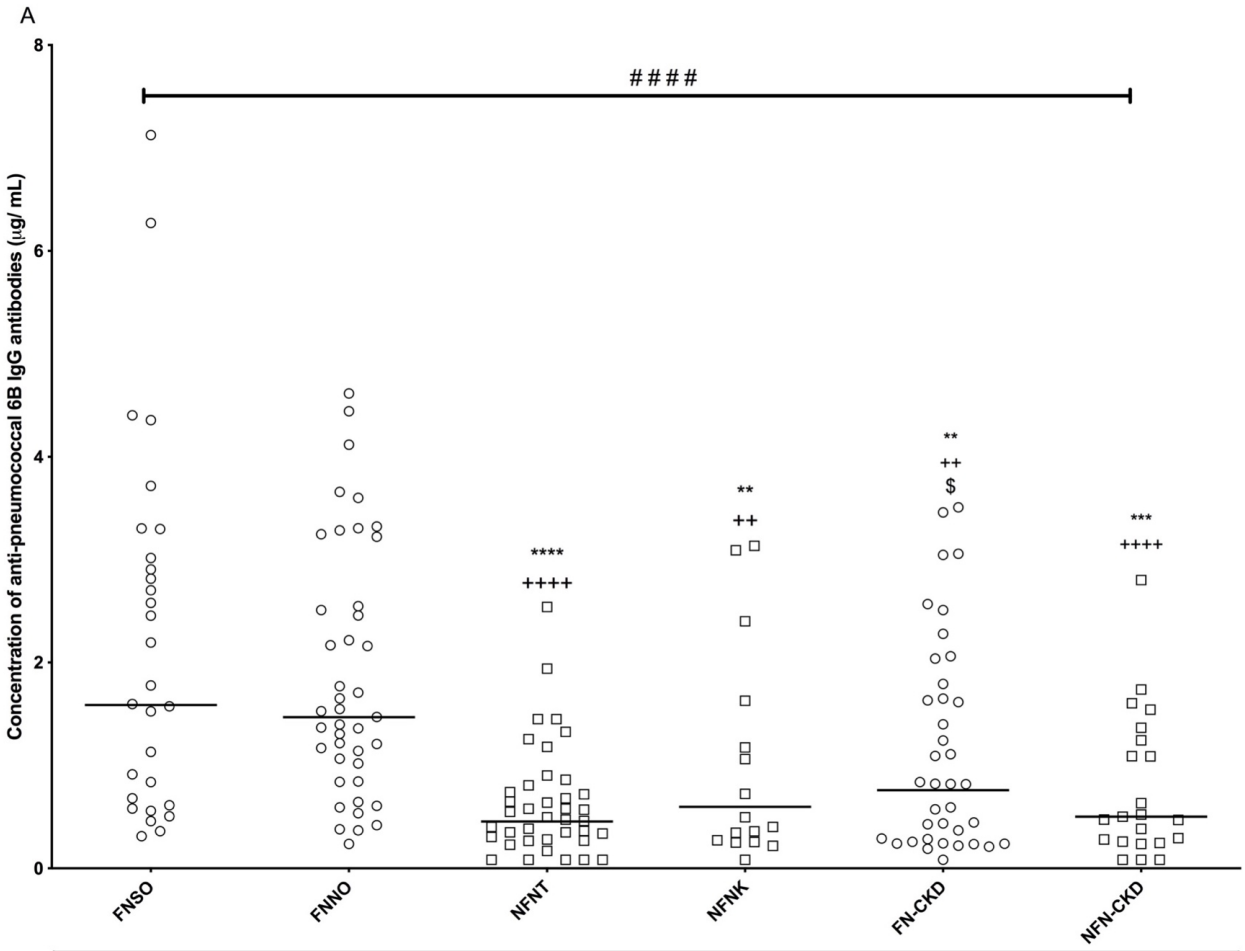
Figure 1A. Concentration of pneumococcal 6B IgG antibodies.




| Geometric Mean, 95% Confidence intervals (µg/ mL) | | | |
|---|---|---|---|
| 3.6, 2.9- 4.5 | 1.3, 1.0- 1.7 | 3.2, 2.3- 4.5 | 2.0, 1.3- 2.9 |

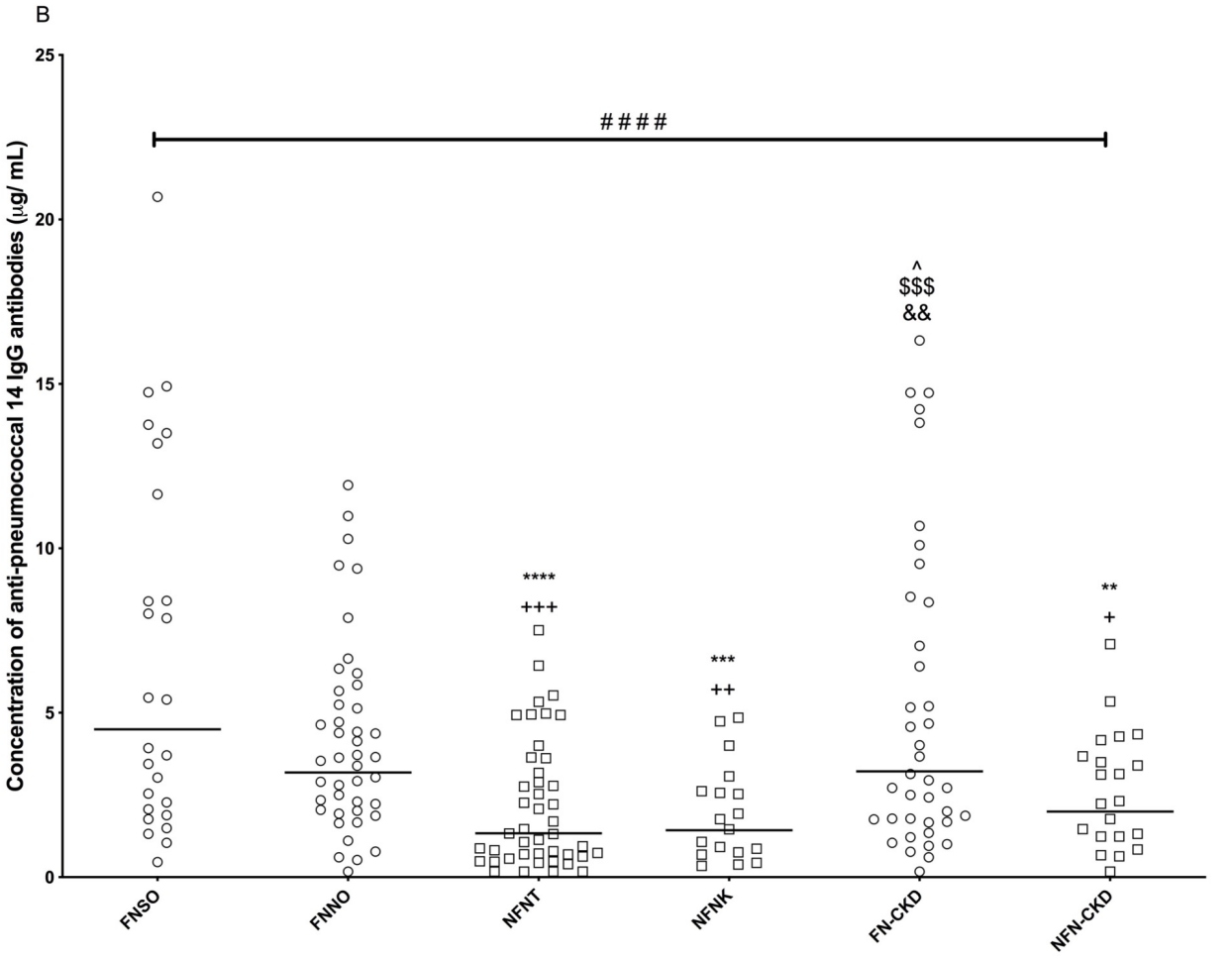
Figure 1B. Concentration of pneumococcal 14 IgG antibodies.

Figure 1. Concentration of pneumococcal IgG antibodies for healthy First Nations (FN) (n=77), healthy non-First Nations (non-FN) (n=64), First Nations with chronic kidney disease (FN-CKD) (n= 41) and Non-First Nations with chronic kidney disease (NFN-CKD) (n=25). Geometric mean antibody concentrations are displayed. Statistical significance determined by Kruskal-Wallis test, Dunn's post-hoc test (#), Mann-Whitney U test detected differences between groups except when comparing serotype 14 antibody concentrations between CKD groups, a Student's t-test was performed. * indicates significance between FN and other groups, ^ indicates significance between NFN-CKD and other groups. *$p < 0.05$, *** $p < 0.001$, **** $p < 0.0001$. Figure 1A shows the concentration of pneumococcal 6B IgG antibodies, and 1B shows the concentration of pneumococcal 14 IgG antibodies.




| Geometric Mean, 95% Confidence intervals (µg/ mL) | | | | | |
|---|---|---|---|---|---|
| 1.6, 1.1- 2.2 | 1.5, 1.2- 1.9 | 0.5, 0.3- 0.6 | 0.6, 0.3- 1.1 | 0.8, 0.5- 1.1 | 0.5, 0.3- 0.8 |

Figure 2A. Concentration of pneumococcal 6B IgG antibodies.




| Geometric Mean, 95% Confidence intervals (µg/ mL) | | | | | |
|---|---|---|---|---|---|
| 4.5, 3.0- 6.7 | 3.2, 2.4- 4.1 | 1.3, 1.0- 1.9 | 1.4, 0.9- 2.2 | 3.2, 2.3- 4.5 | 2.0, 1.3- 2.9 |

Figure 2B. Concentration of pneumococcal 14 IgG antibodies.

Figure 2. Concentration of pneumococcal IgG antibodies for each of the four communities; First Nation Southern Ontario (FNSO) (n= 30), First Nation Northwestern Ontario (FNNO) (n= 47), non-Indigenous Thunder Bay (NFNT) (n= 46), non-Indigenous Kenora (NFNK) (n= 19), First Nations with chronic kidney disease (FN-CKD) (n= 41) and Non-First Nations with chronic kidney disease (NFN-CKD) (n=25). Geometric mean antibody concentrations are displayed. Statistical significance determined by Kruskal-Wallis test, Dunn's post-hoc test (#), Mann-Whitney U test detected differences between groups except when comparing serotype 14 antibody concentrations between CKD groups, a Student's t-test was performed. * indicates significance between FNSO and other groups, + indicates significance between FNNO and other groups, $ indicates significance between NFNT and other groups, & indicates significant difference between NFNK and other groups and ^ indicates significance between NFN-CKD and other groups. *$p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. Figure 2A shows the concentration of pneumococcal 6B IgG antibodies, and 2B shows the concentration of pneumococcal 14 IgG antibodies.

**Supplementary Material**

Supplementary table 1. Sex vs. pneumococcal IgG antibody concentrations for serotypes 6B and 14.

| Group | 6B<br>GM (µg/ mL), 95% CI | 14<br>GM (µg/ mL), 95% CI |
|---|---|---|
| All healthy females vs. all healthy males | 0.9, 0.72- 1.1 vs. 1.0, 0.76- 1.4, p > 0.05 | 2.1, 1.7-2.7 vs. 1.9, 1.4- 2.5, p > 0.05 |
| First Nations healthy female vs. First Nations healthy male | 1.6, 1.3- 2.0 vs. 1.4 1.0- 1.9, p > 0.05 | **4.6, 3.7- 5.9 vs. 2.9, 2.0- 4.1, p < 0.05** |
| Non- First Nations healthy female vs. Non-First Nations healthy male | 0.47, 0.4- 0.6 vs. 0.62, 0.3- 1.1, p > 0.05 | 1.3, 1.0- 1.7 vs. 1.5, 0.9- 2.6, p > 0.05 |
| First Nations healthy female vs. Non- First Nations healthy female | **1.6, 1.3- 2.0 vs. 0.5, 0.4- 0.6, p < 0.0001** | **4.6, 3.7- 5.9 vs. 1.3, 1.0- 1.7, p < 0.0001** |
| First Nations healthy male vs. Non- First Nations healthy male | **1.4, 1.0- 1.9 vs. 0.6, 0.3- 1.1, p < 0.05** | 2.9, 2.0- 4.1 vs. 1.5, 0.9- 2.6, p > 0.05 |
| First Nations CKD female vs. First Nations CKD males | 0.8, 0.5-1.3 vs. 0.8, 0.5-1.3, p > 0.05 | 3.4, 2.0-5.9 vs. 3.1, 1.9-4.9, p > 0.05 |
| Non-First Nations CKD female vs. Non-First Nations CKD male | 0.7, 0.1-3.4 vs. 0.6, 0.4-0.9, p > 0.05 | 3.2, 2.7-3.8 vs. 1.9, 1.0-3.5, p > 0.05 |
| First Nations CKD female vs. Non-First Nations CKD female | 0.8, 0.5-1.3 vs. 0.7, 0.1-3.4, p > 0.05 | 3.4, 2.0-5.9 vs. 3.2, 2.7-3.8, p > 0.05 |
| First Nations CKD male vs. Non-First Nations CKD male | 0.8, 0.5-1.3 vs. 0.6, 0.4-0.9, p > > 0.05 | 3.1, 1.9-4.9 1.9, 1.0-3.5, p > 0.05 |
| First Nations healthy female vs. First Nations CKD female | **1.6, 1.3- 2.0 vs. 0.8, 0.5-1.3, p < 0.05** | 4.6, 3.7- 5.9 vs. 3.4, 2.0-5.9, p > 0.05 |

| | | |
|---|---|---|
| First Nations healthy male vs. First Nations CKD male | **1.4 1.0- 1.9 vs. 0.8, 0.5-1.3, p < 0.05** | 2.9, 2.0- 4.1 vs. 3.1, 1.9-4.9, p > 0.05 |
| Non- First Nations healthy female vs. Non-First Nations CKD female | 0.47, 0.4- 0.6 vs. 0.7, 0.1-3.4, p > 0.05 | **1.3, 1.0- 1.7 vs. 3.2, 2.7-3.8, p < 0.05** |
| Non- First Nations healthy male vs. Non-First Nations CKD male | 0.62, 0.3- 1.1 vs. 0.6, 0.4-0.9, p > 0.05 | 1.5, 0.9- 2.6 vs. 1.9, 1.0-3.5, p > 0.05 |

Supplementary table 2. Linear regression analysis of age and pneumococcal IgG antibody concentrations for serotypes 6B and 14.

| Group | 6B ($r^2$, p value) | 14 ($r^2$, p value) |
|---|---|---|
| All healthy participants | 0.0070, $p > 0.05$ | 0.0001, $p > 0.05$ |
| First Nations healthy | 0.0051, $p > 0.05$ | 0.0010, $p > 0.05$ |
| Non-First Nations healthy | $1.627 \times 10^{-7}$, $p > 0.05$ | **0.1181, $p < 0.01$** |
| First Nations CKD | 0.0151, $p > 0.05$ | 0.0085, $p > 0.05$ |
| Non-First Nations CKD | 0.1322, $p > 0.05$ | 0.01069, $p > 0.05$ |
| First Nations healthy female | 0.002427, $p > 0.05$ | $5.136 \times 10^{-7}$, $p > 0.05$ |
| First Nations healthy male | 0.001318, $p > 0.05$ | 0.006353, $p > 0.05$ |
| Non-First Nations healthy female | $1.723 \times 10^{-6}$, $p > 0.05$ | **0.1242, $p < 0.05$** |
| Non-First Nations healthy male | $8.377 \times 10^{-5}$, $p > 0.05$ | 0.1255, $p > 0.05$ |

Supplementary table 3. Pneumococcal 6B and 14 IgG antibody concentrations of pneumococcal vaccine naïve adults.

| Source | Study Country | 6B (µg/ mL) (95% CI) | 14 (µg/ mL) (95% CI) |
|---|---|---|---|
| All First Nations participants | Canada | 1.5 (1.2-1.8) | 3.6 (2.9-4.5) |
| All non-First Nations participants | | 0.5 (0.4-0.7) | 1.3 (1.0-1.7) |
| Southern Ontario First Nations | Canada | 1.6 (1.1-2.2) | 4.5 (3.0-6.7) |
| Northwestern Ontario First Nations | | 1.5 (1.2-1.9) | 3.2 (2.4-4.1) |
| Non-First Nations (Thunder Bay) | | 0.5 (0.3-0.6) | 1.3 (1.0-1.9) |
| Non-First Nations (Kenora) | | 0.6 (0.4-1.1) | 1.4 (0.9-2.2) |
| First Nations CKD (Thunder Bay) | | 0.8 (0.5-1.1) | 3.2 (2.3- 4.5) |
| Non-First Nations (Thunder Bay) | | 0.5 (0.3-0.8) | 2.0 (1.3-2.9) |
| 21-44 years (1) | USA | 0.7 (0.5-1.1) | 1.8 (1.0-3.3) |
| 20-45 years (1) | USA | 0.7 (0.4-1.1) | 0.9 (0.5-1.7) |
| 22-42 years (2) | Finland | 2.1 (1.5-3.1) | 1.2 (0.6-2.4) |
| 30-64 years (3) + | Finland | ~ 0.6 | ~ 1.2 |
| 30-64 years (3) # | Finland | ~ 0.4 | ~ 1.0 |
| 55-74 years (4) * | USA | 1.9 (1.6-2.3) | 5.5 (4.5-6.8) |
| 55-70 years (5) & | USA | 1.4 (0.7-2.7) | 2.6 (1.1-6.1) |
| 55-70 years (5) & | USA | 4.0 (1.6-10.1) | 3.8 (0.9-17.1) |
| 55-70 years (5) & | USA | 2.9 (1.4-6.3) | 6.6 (3.2-13.4) |
| 15-59 years (6) $ | Australia | 0.9 (0.7-1.1) | 2.5 (1.7-3.8) |
| 15-59 years (6) | Australia | 0.4 (0.3-0.6) | 1.0 (0.5-1.9) |
| 20-39 years (7) | Netherlands | ~ 0.7 | ~ 1.1 |
| 40-59 years (7) | Netherlands | ~ 0.6 | ~ 1.6 |
| 60-79 years (7) | Netherlands | ~ 0.5 | ~ 1.3 |
| 50-70 years (8) | UK | 0.4 (0.3-0.5) | 1.5 (1.2-1.9) |
| 50-70 years (8) | UK | 0.4 (0.3-0.6) | 0.9 (0.6-1.4) |
| 50-64 years (9) | USA | 1.6 (1.4-2.0) | 8.2 (7.1-9.4) |
| 65-88 years (9) | USA | 1.5 (1.3-1.9) | 7.4 (6.4-8.6) |
| 62.6 years (10) ^ | USA | 0.6 (0.3- 1.0) | 2.1 (0.8- 5.4) |

Confidence intervals (CI)
+ Female participants only
# Male participants only
*62% of participants are Indigenous
& Indigenous Alaskan participants pre-immunization concentrations (3 groups because 3 immunization groups)
$ Indigenous Australian adults
^ CKD that have not received pneumococcal vaccines in the past 5 years prior to sample collection

**References: Supplementary table 3**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# Chapter 4A: The effect of pneumococcal immunization on total and antigen-specific B cells in patients with severe chronic kidney disease

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**Abstract**

**Background:** While the 23-valent pneumococcal polysaccharide vaccine (PPV23) is routinely used in Canada and some other countries to prevent pneumococcal infection in adults with chronic kidney disease (CKD), patients develop a suboptimal antibody response to PPV23 due to their immune dysfunction. The 13-valent pneumococcal conjugate vaccine (PCV13) has superior immunogenicity in some categories of immunocompromised adults; however, its effect on the immune response in CKD patients has only been addressed by two recent studies with conflicting results. The effect of PPV23 or PCV13 on B cells in these patients has not been previously studied. We studied the absolute numbers and proportions of B cells and subpopulations in two groups of adult patients with severe CKD pre- and 7 days post-immunization with PCV13: pneumococcal vaccine naïve and previously immunized with PPV23 (over one year ago).

**Results:** PPV23 immunized patients had significantly lower proportions and absolute numbers of class switched memory (CD19+CD27+IgM-), as well as lower absolute numbers of IgM memory (CD19+CD27+IgM+) and class switched B cells (CD19+CD27-IgM-) compared to PPV23 naïve patients. Following PCV13 immunization, the differences in absolute numbers of B-cell subpopulations between groups remained significant. The PPV23 immunized group had higher proportions of CD5- B cells along with lower proportions and absolute numbers of CD5+ B cells compared to PPV23 naïve patients both pre- and post-immunization with PCV13. However, previous PPV23 immunization did not have a noticeable effect on the numbers of total IgG or serotype 6B and 14 specific antibody-secreting cells detected 7 days post-immunization with PCV13. Nevertheless, fold increase in anti-serotype 14 IgG concentrations 28 days post-PCV13 was greater in PPV23 naïve than in previously immunized patients.

**Conclusions:** The results suggest that immunization with PPV23 may result in long-term changes in B-cell subpopulations such as increased prevalence of CD5- B cells and decreased prevalence of class switched memory B cells in the peripheral blood. Because previous immunization with PPV23 in patients with CKD is associated with a significant decrease in the total class switched memory B cells in response to subsequent immunization with PCV13, this may reduce PCV13 immunogenicity in the setting of PPV23 followed by PCV13.

**Trial Registration**

Registered February 24, 2015 at ClinicalTrials.gov (NCT 02370069).

**Key words**: Chronic kidney disease (CKD), *Streptococcus pneumoniae*, B cells, memory B cells, T-cell independent response, T-cell dependent response, 23-valent pneumococcal polysaccharide vaccine (PPV23), 13-valent pneumococcal conjugate vaccine (PCV13), flow cytometry, enzyme-linked immunospot assay (ELISPOT).

## Background

Chronic kidney disease (CKD) is a common condition which affects approximately 10-15% of adults globally (1). Patients with the most advanced stages of this disease (severe CKD, or chronic renal failure) require life-saving renal replacement therapy, such as hemodialysis or kidney transplantation (2, 3). Severe CKD is characterized by high mortality rates. Adjusted for age, sex, and race, the total mortality rate for patients with CKD as of 2018 was 103.0 per 1000 patient-years, more than double the total mortality rate for people without CKD (4). Acute infections contribute substantially to the high rates of hospitalization and mortality in CKD patients, following only cardiovascular disease as a major cause of death (5). High risk of septicaemia and other severe infections are attributed to both a compromised immune system and increased exposure to infectious agents in dialysis units (5-7).

Pneumococcus (*Streptococcus pneumoniae*) is a Gram-positive, encapsulated diplococcus, which commonly colonizes the upper respiratory tract (8). Upon breaching the host defences, the microorganism can cause mucosal (otitis media, sinusitis, pneumonia) and invasive (meningitis, septicaemia, pericarditis, etc.) infections (9, 10). *S. pneumoniae* is the most common cause of community acquired pneumonia (CAP) worldwide (11). The highest incidence rates of CAP and invasive pneumococcal disease (IPD) are found in young children, elderly, and immunocompromised adults (9, 12). Patients with CKD, particularly those with nephrotic

syndrome and undergoing dialysis, are highly susceptible to pneumococcal infection, especially pneumonia (13).

To prevent pneumococcal infection in adult patients with CKD, immunization with pneumococcal polysaccharide vaccine (PPV23), which contains purified capsular polysaccharides from 23 pneumococcal serotypes most commonly associated with IPD (1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19F, 19A, 20, 22F, 23F, and 33F), is currently recommended in Canada (14). However, the effect of PPV23 in CKD patients is suboptimal because of their immune dysfunction (15, 16). The second-generation (polysaccharide-protein conjugate) vaccines, which induce T-cell dependent antibody responses to polysaccharide antigens (17), have superior immunogenicity in immunocompromised adults and the elderly (18-20). In Canada, 13-valent pneumococcal conjugate vaccine (PCV13), which consists of purified capsular polysaccharides of serotypes 1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F, and 23F conjugated to a carrier protein ($CRM_{197}$), is recommended for several categories of immunocompromised adults, such as bone marrow transplantation recipients and HIV-infected individuals (21). Although in some countries PCV13 is now used for immunization of patients with CKD, PCV13 immunogenicity in adults undergoing hemodialysis was only recently addressed by two studies with differing results. Mitra *et al.* (2016) showed that the antibody concentrations in patients with CKD declined significantly 12 months after immunization with PCV13, compared to 2 months post-immunization for 11 of the 13 serotypes tested (22). Vandecasteele *et al*. (2018) demonstrated that immunization of CKD patients with PPV23 may have a negative effect on the immune response to PCV13 (23). It was suggested that immunization with PPV23 could lead to depletion of memory B cells following exposure to purified polysaccharide antigens potentially affecting the development of antibody responses to

polysaccharide antigens administered via protein-conjugate vaccines (24). However, to the best of our knowledge, the effect of PPV23 or PCV13 on B cells in patients with CKD has not been previously studied. To address this question, we studied the proportions and numbers of B cells (CD19+), and their subpopulations: naïve (CD27-IgM+), IgM memory (CD27+IgM+), class switched (CD27-IgM-), class switched memory (CD27+IgM-), CD5+ and CD5- B cells pre- and 7 days post-immunization with PCV13. Adult patients with severe CKD were separated into two groups: one group was pneumococcal vaccine naïve and the other group was previously immunized with PPV23 (over one year ago).

**Results**

In patients with severe CKD, immunization with PCV13 did not result in any significant changes in proportions of total B cells or any B-cell subpopulation except for CD5- cells. The CD19+CD5- subpopulation increased on day 7 post-immunization (74.2, 68.5 - 80.4% *vs*. 52.0, 39.3 - 68.7%, $p < 0.05$, Table 1). No significant changes in absolute numbers of total lymphocytes, B cells, or B-cell subpopulations assessed immediately prior to immunization and 7 days post-immunization were noticed (Supplementary Table 1). There was a statistically significant positive correlation between pre- and post-immunization absolute numbers of all tested cells, except for class switched and total B cells, and proportions of all cells with the exception of class switched and CD5- B cells (Supplementary Table 2).

To determine if PPV23 immunization had an impact on B-cell subpopulations, we tested the peripheral blood immediately prior to immunization with PCV13. The patients who had previously received PPV23 had significantly lower proportions (13.7, 9.2 - 20.3% *vs.* 22.9, 17.6 - 29.8, $p < 0.05$, Figure 1A) and absolute numbers (1.3, 0.9-2.0 X $10^7$ cells/L *vs*. 2.6, 1.8 - 3.8 X

$10^7$ cells/L, $p < 0.05$, Figure 1B) of class switched memory B cells, as well as lower absolute numbers of IgM memory (4.9, 3.1 - 7.5 X $10^6$ cells/L *vs.* 9.6, 6.3 - 1.5 X $10^6$ cells/ L, $p < 0.05$) and class switched B cells (1.3, 1.0 - 1.6 X $10^7$ cells/L *vs.* 1.8, 1.8 - 4.1 X $10^7$ cells/L, $p < 0.05$) compared to PPV23 naïve patients (Figure 1B). Previous immunization with PPV23 did not have a negative effect on total lymphocytes, as no significant difference in the absolute numbers of lymphocytes prior to PCV13 immunization was found between the groups (1.0, 0.8 - 1.4 X $10^9$ cells/ L vs. 0.9, 0.8 - 1.1 X $10^9$ cells/ L, $p > 0.05$, supplementary table 3). However, PPV23 naïve patients had a slightly higher absolute number of B cells pre-immunization compared to PPV23 immunized patients (9.9, 7.0 - 13.9 X $10^7$ cells/ L vs. 8.3, 6.1 - 11.2 X $10^7$ cells/ L, $p > 0.05$, supplementary table 3).

Following the PCV13 immunization, the differences in absolute numbers of B-cell subpopulations between the groups remained significant. Those who had previously received PPV23 had lower numbers of IgM memory (4.6, 3.2 - 6.5 X $10^6$ cells/ L *vs*. 8.9, 5.7 - 14.0 X $10^6$ cells/ L, $p < 0.05$), class switched B cells (1.2, 0.9 - 1.5 X $10^7$ cells/ L *vs.* 1.6, 1.0 - 2.7 X $10^7$ cells/ L, $p < 0.05$), and class switched memory B cells (1.3, 0.9 - 2.0 X $10^7$ cells/ L *vs.* 2.6, 1.8 - 3.8 X $10^7$ cells/ L, $p < 0.05$) compared to PPV23 naïve patients (Figure 2B), although no significant differences in proportions of B-cell subpopulations between the groups were detected (Figure 2A). Both groups had the same absolute numbers of lymphocytes 7 days post-immunization (1.0, 0.7 - 1.3 X $10^9$ cells/ L *vs*. 1.0, 0.8 - 1.2 X $10^9$ cells/ L, $p > 0.05$, supplementary table 3). However, PPV23 naïve patients had slightly higher absolute numbers of total B cells compared to PPV23 immunized patients (9.3, 6.7 - 12.8 X $10^7$ cells/ L *vs*. 7.9, 5.7 - 10.9 X $10^7$ cells/ L, $p > 0.05$, supplementary table 3). The results suggest that previous

immunization with PPV23 may decrease the numbers of IgM memory, class switched, and class switched memory B-cell subpopulations both pre- and 7 days post-immunization with PCV13.

As previous studies found an association between the CD5- subpopulation of B cells and production of IgG antibodies to pneumococcal polysaccharide antigens in response to PPV23 (25), we attempted to determine if there were any differences in CD5-expressing B cells between the groups with different histories of PPV23 vaccination. Prior to immunization with PCV13, those patients who had previously been immunized with PPV23 had higher proportions of CD5- (75.5, 70.6 - 80.6% *vs.* 37.5, 20.6 - 68.3%, $p < 0.05$) and lower proportions of CD5+ cells (22.5, 18.9 - 27.2% *vs.* 37.7, 24.1 - 58.86%, $p < 0.05$) as well as CD5+ absolute numbers (2.1, 1.6 - 2.8 X $10^7$ *vs*. 3.5, 2.0 - 6.0 X $10^7$, $p < 0.05$) compared to PPV23 naïve patients (Figure 3A-B).

Seven days post-immunization with PCV13, patients previously immunized with PPV23 still had higher proportions of CD5- B cells (69.5, 55.7 - 86.9% *vs*. 37.4, 18.9 - 73.66%, $p < 0.05$) and lower proportions (16.9, 13.9 - 20.6% *vs.* 35.2, 23.0 - 53.7%, $p < 0.05$) and absolute numbers of CD5+ cells (3.0, 1.9 - 5.1 X $10^7$ cells/ L *vs*. 1.3, 0.9 - 1.9 X $10^7$ cells/ L, $p < 0.05$) compared to PPV23 naïve patients (Figure 3A-B). Although higher absolute numbers of CD5- cells were present in PPV23 immunized than in PPV23 naive patients, the difference was not statistically significant (5.2, 3.3 - 8.1 X $10^7$ cells/ L *vs*. 3.5, 1.6 - 7.7 X $10^7$ cells/ L, $p > 0.05$, Figure 3B). These results show that immunization with PPV23 may lead to in an increased prevalence of the CD5- subpopulation among circulating B cells.

To determine if the numbers of total IgG and antigen specific antibody secreting cells (ASC) on day 7 post-immunization with PCV13 depend on previous immunization with PPV23, we conducted enzyme-linked immunospot (ELISPOT) assay on peripheral blood mononuclear cells (PBMC) stimulated with *Staphylococcus aureus* Cowan strain protein A (SAC) and CpG

Oligonucleotide (ODN-2006). No statistically significant difference between PPV23 naïve and PPV23 immunized patients was found between the numbers of total IgG ASC (median ± standard deviation) (118.5 ± 93.8 *vs*. 151.0 ± 93.5, $p > 0.05$, Figure 4A), ASC specific for pneumococcal polysaccharide serotype 6B (0.5 ± 0.34 ASC *vs*. 0.5 ± 0.36 ASC), or serotype 14 (1.0 ± 0.5 ASC *vs*. 0.8 ± 0.3 ASC, $p > 0.05$, Figure 4B). After outliers were removed, the number of patients that had antigen-specific IgG ASC below the limit of detection was calculated. There were 9/16 (56%) PPV23 naïve and 16/25 (64%) PPV23 immunized patients without detectable ASC specific for 6B; for serotype 14-specific ASC, the corresponding numbers were 7/16 (44%) *vs.*13/25 (52%), $p > 0.05$ (Fisher's exact test). Hence, in this group of patients, previous PPV23 immunization did not have a noticeable effect on the numbers of total IgG ASC or serotype 6B and 14 specific ASC detected at day 7 post-immunization with PCV13.

To determine if previous immunization with PPV23 had an effect on the antibody response to PCV13, concentrations of pneumococcal 6B and 14 IgG antibodies were determined pre- and 28 days post-immunization (supplementary table 4). There were no significant differences between PPV23 naive and PPV23 immunized patients in the concentration of pneumococcal 6B antibodies pre-immunization (0.9, 0.6-1.4 µg/ mL *vs.* 1.1, 0.7 - 1.6 µg/ mL, $p > 0.05$) or post-immunization (2.1, 1.4 - 3.2 µg/ mL *vs.* 2.4, 1.4 - 3.9 µg/ mL, $p > 0.05$). Compared to PPV23 immunized patients, PPV23 naive patients had a significantly lower concentration of pneumococcal 14 antibodies pre-immunization (2.8, 1.9 - 4.2 µg/ mL vs. 5.0 3.0 - 8.1, $p < 0.05$), but there was no significant difference in post-immunization concentrations (8.0, 4.5 - 14.4 µg/ mL *vs.* 8.1, 5.0 - 14.4, $p > 0.05$). When pre- and day 28 post-immunization concentrations were compared for each patient group, both groups had a significant increase in

pneumococcal 6B antibody concentrations ($p < 0.05$), however, only PPV23 naïve patients had a significant increase in pneumococcal 14 antibody concentrations ($p < 0.01$).

While PPV23 immunized patients had a slightly smaller fold change in pneumococcal 6B antibodies compared to PPV23 naïve patients (2.3, 1.7 - 3.1 µg/ mL *vs.* 2.8, 1.8 - 4.3 µg/ mL, $p > 0.05$), they had a significantly smaller fold change in pneumococcal 14 antibodies (1.3, 1.1 - 1.6 µg/ mL *vs.* 1.9, 1.3 - 2.8 µg/ mL, $p < 0.05$). A strong negative correlation was found between the fold change of pneumococcal 14 antibodies and the pre-immunization absolute numbers of CD5- B cells in PPV23 immunized patients ($r= -0.8576$, $p < 0.001$, Pearson correlation). No other significant correlation was detected between pre-immunization or day 7 post-immunization proportions or absolute numbers of CD5- or class switched memory B-cell subpopulations and the fold change in pneumococcal 6B or 14 antibody concentrations (data not shown). These results suggest that previous PPV23 immunization negatively affects the pneumococcal 6B and 14 antibody response to subsequent immunization with PCV13 in patients with CKD.

**Discussion**

Although both types of pneumococcal vaccines induce the production of serotype-specific antibodies to *S. pneumoniae* capsular polysaccharides, PPV23 and PCV13 activate different immunological mechanisms. PPV23 contains purified capsular polysaccharides, which directly activate B cells in a T-cell independent manner. Bacterial capsular polysaccharides have multiple repeating epitopes which cross-link B cell receptors, causing the activation of Bruton's tyrosine kinase that results in activation and proliferation of antigen-specific B cells (26). The memory B cells generated from T-cell independent responses are short-lived and produce a less robust antibody response upon re-exposure to an antigen (27). Vaccines containing capsular

polysaccharides alone, such as PPV23, have poor immunogenicity in immunocompromised individuals (28). In the case of PCV13, the conjugation of polysaccharides to $CRM_{197}$, a non-toxic mutant of diphtheria toxin, which acts as a carrier protein, results in a T-cell dependent response. The protein-polysaccharide conjugate binds to B cell receptors and it is then brought into the endosome, where the protein component is processed into peptides and binds to the major histocompatibility complex class II (MHC II) molecules. Antigen presentation of the carrier protein in the context of MHC II results in the activation of CD4+ T cells. The subsequent generation of signals stimulates B cell maturation, class switching, and proliferation resulting in the production of long-lived memory B cells and secretion of polysaccharide-specific class switched high affinity antibodies (29).

It is still uncertain whether immunization with plain pneumococcal polysaccharide antigens (included into PPV23) negatively impacts antibody responses to subsequent immunization with polysaccharide-protein conjugate vaccines, such as PCV13. A study by Clutterbuck *et al.* (2012) measured the effect of PPV23 followed by the 7-valent pneumococcal conjugate vaccine (PCV7) on antigen-specific B cells in older adults. It was found that while PCV7 immunization resulted in an increase in pneumococcal serotype-specific memory B cells, immunization with PPV23 depleted these cells and resulted in attenuated memory B cell responses (24). No studies have assessed the effect of pneumococcal immunizations on B cells in patients with CKD. Vandecasteele *et al.* (2018) found that severe CKD patients previously immunized with PPV23 had lower antibody response to PCV13 compared to pneumococcal vaccine naïve patients (23). Our results agree with this study, as PPV23 immunized patients had a slightly lower fold change in pneumococcal 6B IgG antibodies, and significantly lower fold change in pneumococcal 14 antibodies in response to PCV13 immunization. Recent studies

assessed the effect of previous PPV23 immunization on the response to PCV13 or PCV7 in older adults found that previous immunization with PPV23 decreased antibody response to subsequent doses of PCV13 (20, 30, 31).

Patients with CKD are unique among other high-risk groups because they have multiple predisposing factors to pneumococcal infection, including uremia with its significant metabolic consequences, as well as the hemodialysis procedure, which causes premature aging of the immune system (6, 32). Patients with CKD also frequently have comorbidities, such as diabetes mellitus, which contribute to their immune dysfunction, and may receive immunosuppressive medications (33). These patients also have increased exposure to infectious agents in the hospital environment. Multiple immune abnormalities such as decreased bactericidal ability of neutrophils, granulocyte and macrophage phagocytic function, defective function of antigen presenting cells, reduced numbers of B cells and decreased antibody producing capacity of plasma cells, increased T- and B-cell apoptosis, decreased thymic T-cell output, and impaired activation of T-cell response have been found in CKD patients (34, 35).

Among immune abnormalities, this group has noticeable alterations in B cells and B-cell function. We have recently found that patients with CKD had decreased absolute numbers of B cells and B-cell subpopulations compared to healthy controls (36). Pahl *et al*. (2010) also found decreased numbers of B cells in CKD patients, suggesting that B-cell lymphopenia in patients with CKD was due to uremia (37). It is possible that epigenetic changes caused by CKD affect the production of lymphoid cells at the hematopoietic stem cell level, resulting in decreased numbers of circulating B and T cells (38, 39).

In this study, we found that immunization with PPV23 may have a long-term effect on B-cell subpopulations in patients with CKD. Compared to vaccine naïve patients, PPV23

immunized patients of a similar age had significantly lower proportions of class switched memory B cells pre-immunization, and significantly lower absolute numbers of these cells both pre- and 7 days post-immunization with PCV13. In the literature, class switched memory B cells are defined as CD19+CD27+IgD- or CD19+CD27+IgM- cells (40, 41). These cells respond rapidly when re-exposed to the same antigen, resulting in the production of class switched, high affinity antibodies, and hence mediate long-lived humoral immunity (42, 43).

Although pneumococcal-specific B cells are only a small proportion of the total B cell population (0.5% of the total B cell population pre-immunization and 2.0-2.75% 7 days post-immunization with PPV23) (41), our results suggest that immunization with PPV23 results in a significant decrease of total class switched memory B cell population. This has not been previously reported as most studies focus on antigen-specific memory B cells. Chovancova *et al*. (2011) did not detect a significant change in the proportion or absolute number of class switched memory B cells defined as CD19+CD27+IgD-, between pre- and 7 days post-immunization with PPV23 in adults with common variable immunodeficiency (CVID) (44). It is possible that immunization with pneumococcal polysaccharides results in polyclonal stimulation of B cells, leading to terminal differentiation of memory B cells followed by their depletion. Polyclonal stimulation of memory B cells, which causes their proliferation and differentiation into plasma cells, helps to maintain serological memory throughout life (45). Polyclonal B cell activation by capsular polysaccharides of *Neisseria meningitidis* was previously reported by Oliveira *et al*. (1996) (46).

The role of CD5+ and CD5- B cells in humans is not completely understood. Due to the lack of somatic hypermutations in CD5+ B cells, it is thought that these cells are naïve B cells (25, 47). The expression of CD5 decreases with age and is associated with an increase in CD27

expression, indicating a shift in B-cell subpopulations towards an increase in the population of memory B cells (48). A study by Moens *et al*. (2015) determined that after immunization with PPV23, ASC originating from CD5- B cells primarily produce pneumococcal anti-capsular polysaccharide IgG antibodies (25). In agreement with this study, we found significantly higher proportions of CD5- B cells in PPV23 immunized patients compared to PPV23 naïve ones. Hence immunization with PPV23 favors the generation of CD5- pneumococcal IgG memory B cells even in these immunocompromised adults. We found a strong negative correlation between the pre-immunization absolute numbers of CD5- B cells and the fold change of pneumococcal 14 IgG antibodies in PPV23 immunized patients. Hence, although previous immunization with PPV23 favours the generation of CD5- B cells, it has a negative effect on the antibody response to subsequent immunization with PCV13 in patients with CKD.

In an attempt to assess antigen-specific ASC, we used PBMC collected 7 days post immunization, given that ASC can be detected in the peripheral blood as early as 5 days after vaccination or infection and their numbers peak at day 7 (49). The cells were stimulated with B-cell polyclonal activators, i.e. SAC, which induces crosslinking of surface immunoglobulins (50) and CpG ODN-2006, which activates TLR 9 that stimulate the differentiation of memory B cells (51). We measured the B-cell responses to pneumococcal capsular polysaccharides of serotypes 6B and 14 because they have a significant impact on the global epidemiology of IPD (52).

As we detected only small numbers of ASC, this may reflect poor functional ability of B cells from CKD patients to respond to polyclonal stimulation. In addition, capsular polysaccharides of serotypes 6B and 14 have low immunogenicity (52). As various modifications of ELISPOT exist, including different methods of B cell stimulation, it is difficult to compare our results to other studies. One study determined that the more doses of PPV23

asplenic adults with β-thalassemia received, the lower the amount of capsular polysaccharide-specific memory B cells in response to PCV13 was detected (53). They also found that longer interval between PPV23 and PCV13 vaccinations was associated with greater numbers of antigen-specific memory B cells (53). In that study, the range of capsular polysaccharide-specific IgG ASC per 1,000,000 PBMC was 0-24; in comparison, our patients had a maximum of two ASC specific to 6B and 14 per 200,000 PBMC. Such discrepancies could be due to differences in age and immune abnormalities of participants. When we attempted to measure numbers of plasma cells using ELISPOT, no plasma cells specific for 6B or 14 were detected (data not shown). Similarly, in the study by Chovancova *et al.* (2011), CVID patients had no detectable IgG ASC specific for 23 pneumococcal capsular polysaccharides due to the lack of terminal B cell differentiation (44). This could potentially explain the lack of B-cell response in CKD patients as it was demonstrated that such patients have decreased B cell activating factor receptor expression in transitional B cells, resulting in their decreased survival (37).

Our study has several limitations, including small sample size that could contribute to lack of statistical significance in comparisons between groups. The patients received PPV23 at different times prior to PCV13 immunization (although always over 1 year before) that could contribute to the variability in results; we were unable to measure their immediate response to the PPV23 vaccine. Due to the large diversity among the CKD patients, we could not match our study participants for underlying conditions. We did not have healthy aged-matched controls that would have helped in the interpretation of responses by CKD patient groups. We were unable to calculate the fold change in antibody concentration for all participants due to sample collection issues such as participant withdrawal, etc. Our ongoing study of antigen-specific antibody responses in these two groups will aid in the clarification of the effect of previous immunization

with PPV23 on B cells and their ability to respond to subsequent immunization with PCV13. In addition, our work will help determine the longevity of the antibody response to PCV13 in patients with CKD.

**Conclusions**

Immunization with PPV23 may result in long-term changes in B-cell subpopulations such as an increased prevalence of CD5- B cells and decreased prevalence of class switched memory B cells in the peripheral blood. Because previous immunization with PPV23 in patients with CKD is associated with a significant decrease in the total class switched memory B cell population as well as a decreased pneumococcal antibody response to subsequent immunization with PCV13, this may reduce PCV13 immunogenicity in the setting of PPV23 followed by PCV13. Our findings emphasize the need for further studies to optimize pneumococcal immunization for adults with CKD.

**Methods**

Patient Recruitment and Eligibility

Sixty-one patients with stage 4 or 5 severe CKD (glomerular filtration rate (GFR), stage 4 < 30 mL/ min/ 1.73m$^2$, stage 5 < 15 mL/ min/ 1.73m$^2$) (54) who were receiving hemodialysis at the Thunder Bay Regional Health Sciences Centre (TBRHSC) were recruited between May 2015 and August 2018. All the patients were over 18 years old, did not have a history of immunocompromising conditions, were not taking any immunosuppressive medications for more than 14 days in the past 6 months, and had not received any vaccines in the past month or blood transfusions in the past 3 months. Patients were separated into two groups: 25 PPV23 naïve

patients (mean age 59 years, range 20 - 87 years, 32% female) and 36 previously immunized with PPV23 > 1 year ago (mean age 60 years, range 32 - 87 years, 47% female). This study was registered at ClinicalTrials.gov (NCT 02370069) and approved by the research ethics boards of Lakehead University and TBRHSC.

Immunization protocol and sample collection

All patients received a single dose of PCV13 (Prevnar13, Pfizer, lots: H22520, J77978, M573833, M73833, R56653, and T24279) administered intramuscularly in the deltoid region during the dialysis procedure. Ten milliliters of peripheral blood for B-cell analysis were collected into BD Vacutainer™ tubes with sodium heparin (BD Biosciences, Baltimore, MD, USA) immediately prior to immunization for flow cytometry analysis and 7 days post-immunization for flow cytometry and ELISPOT. Blood was collected for complete blood counts (CBC) just before immunization and day 7 post-immunization. An additional 10 mL of peripheral blood for serum antibody analysis was collected into BD Vacutainer™ Venous Blood Collection Tubes: SST™ Serum Separation Tubes: Hemogard (BD Biosciences) pre- and 28 days post-immunization, serum was stored at -80 °C.

Analysis of B cells

PBMC were isolated by density gradient centrifugation using Lymphoprep (Stemcell Technologies, Vancouver, BC, CAN). Monocytes were removed using two consecutive incubations in RPMI 1640 medium with L-glutamine (Thermo Fisher Scientific, Mississauga, ON, CAN) supplemented with 1% antibiotic-antimicotic (Life Technologies, Burlington, ON, CAN) and 20% fetal bovine serum (FBS, Fisher Scientific, Whitby, ON, CAN) (20%

supplemented medium) in a BD Falcon™ 100 X 20 mm cell culture dish (Fisher Scientific) for 1 hr at 37°C, 5% $CO_2$. Non-adherent cells were washed with RPMI 1640 medium with L-glutamine supplemented with 1% antibiotic-antimicotic and 10% FBS (10% supplemented medium) and re-suspended at 2 X $10^6$ cells/ mL in 10% supplemented medium. From the cell suspension, 200,000 cells were immunostained with PE Mouse Anti-Human CD19, APC Mouse Anti-Human IgM, PerCP-Cy™5.5 Mouse Anti-Human CD27, and FITC Mouse Anti-Human CD5 (BD Biosciences) at 4°C for 1 hr. Samples were analyzed with BD FACSCalibur™ Flow Cytometer and CELLQUEST PRO software (BD Biosciences) to determine proportions of B cells (CD19+) and subpopulations: naïve (CD27-IgM+), IgM memory (CD27+IgM+), class switched (CD27-IgM-), class switched memory (CD27+IgM-), CD5+ and CD5- B cells as previously described (36). Purity of CD19+ gated B cells was verified by counterstaining cells with FITC Mouse Anti-Human CD3 and PerCP-Cy™5.5 Mouse Anti-Human CD14 (BD Biosciences).

To determine the absolute numbers of B cells, a CBC was performed at the TBRHSC clinical lab. The absolute number of B cells was calculated by multiplying the percentage of CD19+ cells of the total gated lymphocyte population by the total lymphocyte count. The numbers of B cell subpopulations were determined by multiplying the percentage of each subpopulation by the absolute number of B cells.

Enzyme-linked immunospot (ELISPOT) assay

Preparation of ELISPOT plates

Multi-screen IP 96-well PVDF membrane filter plates (Millipore Canada Ltd, Etobicoke, ON, CAN) were coated with either goat anti-human IgG (20 μg/ mL) (Cedarlane, Burlington,

ON, CAN), or pneumococcal capsular polysaccharides of serotypes 6B, or 14 (10 μg/ mL) (Cedarlane) conjugated with methylated human serum albumin (mHSA, 10 μg/ mL) (National Institute for Biological Standards and Control, Hertfordshire, UK). Protocol for conjugation of the pneumococcal polysaccharides to mHSA for coating plates was provided by the Oxford Vaccine Group. Wells containing solution of mHSA (10 μg/ mL) in PBS (Fisher Scientific) served as "no coating control". Plates were incubated at 37°C, 5% $CO_2$ for 5 hrs then stored at 4°C overnight. The next day, plates were washed with PBS then blocked with 2% skim milk (Thermo Fisher Scientific) in PBS for 3 hrs at room temperature. The plate was washed again with PBS before cells were added.

Detection of memory B cells

Following removal of monocytes, 2 X $10^6$ PBMC were plated in a 6-well plate in 10% supplemented medium with SAC (5 μg/ mL) (Sigma Aldrich, Oakville ON, CAN) ODN-2006 (3 μg/ mL) (Cedarlane, Burlington, ON, CAN). After 6 days of stimulation at 37 °C, 5% $CO_2$, the cells were harvested, washed with 10% supplemented medium, re-suspended in the same medium and counted. Cells were plated at 200,000 cells per well in the 96-well coated plate at 37 °C, 5% $CO_2$ for 18 hrs. After the incubation, the plate was washed using PBS with 0.01% Tween 20 (Fisher Scientific) before the HRP conjugated mouse anti-human IgG was added (0.13 μg/ mL) (Hybridoma Reagent Laboratory, Baltimore, MD, USA) for 2 hrs at room temperature. To visualize the spots, KPL TrueBlue™ Peroxidase Substrate (Mandel Scientific, Guelph, ON, CAN) was added for 10 min and then washed for 5 min with distilled water. After washing, the plate was left to dry for a minimum of 1 day. Once dry, the spots were visualized using 2X magnification (Nikon Eclipse 80i).

## ELISPOT Counting

To determine the numbers of ASC, two lab members counted the spots per well individually, and a mean of the counts was calculated. Counts over 200 were considered above detection limits and assigned a value of 210; counts of 0 were assigned a value of 0.5 for statistical purposes.

## Enzyme-linked immunosorbent assay (ELISA)

Amounts of IgG anti-pneumococcal 6B and 14 antibodies were quantified using the ELISA protocol provided by the World Health Organization (55).

## Statistical Analysis

Statistical analysis was performed using Graph-Pad Prism 5 (GraphPad Prism Software Inc., San Diego, CA). The geometric means for the percentage of each B-cell subpopulation, absolute numbers of B cells, concentration, and fold change of antibody concentration with 95% confidence intervals (CI) and the medians of ASC with standard deviation were calculated. Outliers were identified and removed prior to statistical analysis (56). Groups were compared either using a Student's t-test or Mann-Whitney U test, and correlation analyses performed using Pearson or Spearman analysis based on the distribution of the data. A p value of $< 0.05$ was reported as statistically significant.

## Declarations

Ethics approval and consent to participate

This study was approved by the research ethics boards of Lakehead University (approval reference number: 085 14-15) and the Thunder Bay Regional Health Sciences Centre (approval reference number: 2014124). Written informed consent was obtained from each participant prior to determination of eligibility.

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**Tables and Figures**

Table 1. B-cell subpopulations in patients with severe chronic kidney disease.

| B cells | Pre-immunization % (CI) | Post-immunization % (CI) | p value |
|---|---|---|---|
| Total B cells (CD19+) | 8.6 (7.0 - 10.6) | 7.7 (6.9 - 8.5) | > 0.05 |
| Naïve (CD27-IgM+) | 46.3 (38.8 - 55.3) | 42.5 (36.7 - 49.3) | > 0.05 |
| IgM memory (CD27+IgM+) | 7.0 (5.2 - 9.5) | 7.8 (6.5 - 9.4) | > 0.05 |
| Class Switched (CD27-IgM-) | 14.3 (12.6 - 16.3) | 11.6 (10.1 - 13.3) | > 0.05 |
| Class Switched memory (CD27+IgM-) | 17.6 (13.5 - 23.0) | 21.4 (18-0 - 25.6) | > 0.05 |
| CD19+CD5+ | 30.2 (23.9 - 38.1) | 24.9 (19.7 - 31.5) | > 0.05 |
| CD19+CD5- | 52.0 (39.3 - 68.7) | 74.2 (68.5 - 80.4) | < 0.05 |

Immunophenotype of peripheral blood B cells was determined by flow cytometry pre- and 7 days post-immunization with PCV13. Geometric mean (GM) of proportions with 95% confidence intervals (CI) are shown. For analysis of total B cells, naïve, IgM memory, class switched and class switched memory B cells, 33 pre-immunization and 60 post-immunization samples were studied. CD5 expression analysis, 33 pre-immunization and 39 post-immunization samples were studied. Statistical significance was determined by Mann-Whitney U test.

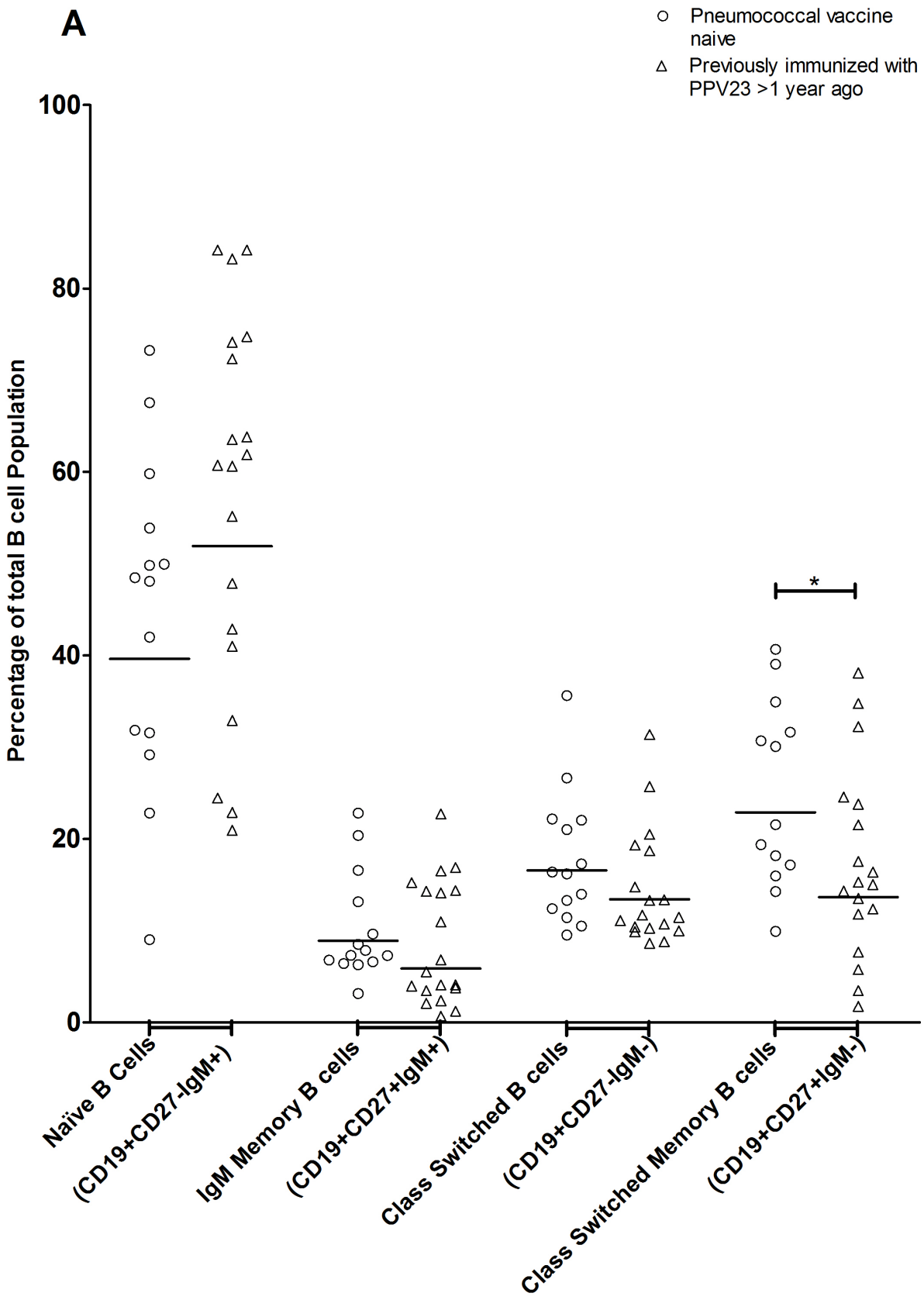
A
Pneumococcal vaccine naive
Previously immunized with PPV23 >1 year ago
Percentage of total B cell Population
100
80
60
40
20
0
*
Naïve B Cells (CD19+CD27-IgM+)
IgM Memory B cells (CD19+CD27+IgM+)
Class Switched B cells (CD19+CD27-IgM-)
Class Switched Memory B cells (CD19+CD27+IgM-)

Figure 1A. B-cell subpopulations in pneumococcal vaccine naïve (n=14) and previously immunized with 23-valent pneumococcal polysaccharide vaccine (PPV23) (n=19) patients with severe chronic kidney disease prior to immunization with 13-valent pneumococcal conjugate vaccine. Isolated peripheral blood mononuclear cells were stained for CD19, CD27, and IgM and analyzed by flow cytometry. The geometric mean of each subpopulation proportion is displayed. Statistical significance determined by Student's t-test (*$p < 0.05$).

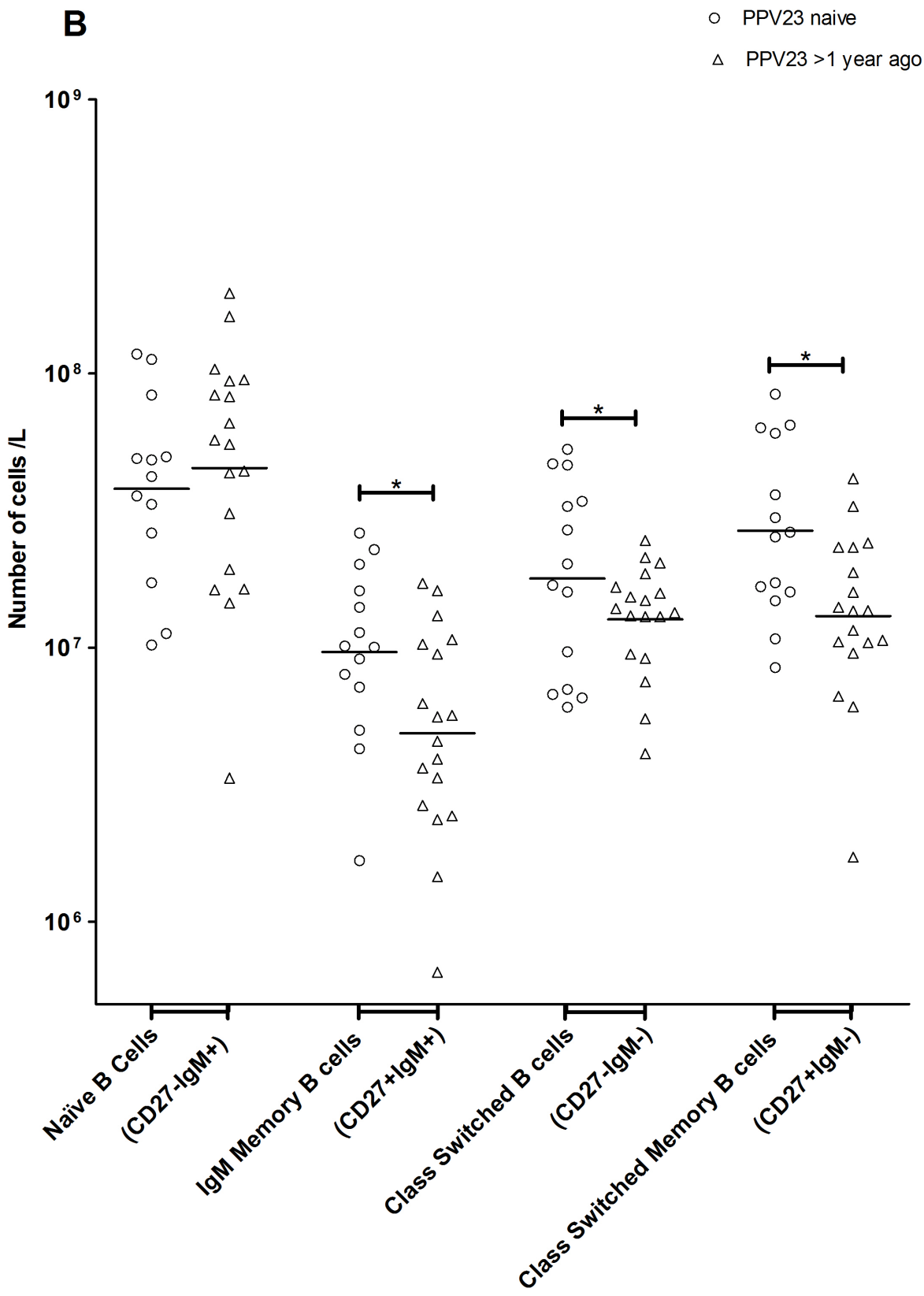
B
PPV23 naive
PPV23 >1 year ago
Number of cells /L
10⁹
10⁸
10⁷
10⁶
*
*
*
Naïve B Cells (CD27-IgM+)
IgM Memory B cells (CD27+IgM+)
Class Switched B cells (CD27-IgM-)
Class Switched Memory B cells (CD27+IgM-)

Figure 1B. Absolute numbers of B-cell subpopulations in pneumococcal vaccine naïve (n=14) and previously immunized with 23-valent pneumococcal polysaccharide vaccine (PPV23) (n=19) patients with severe chronic kidney disease prior to immunization with 13-valent pneumococcal conjugate vaccine. Isolated peripheral blood mononuclear cells were stained for CD19, CD27, and IgM. Geometric means of absolute numbers of B-cell subpopulations were determined by multiplying the proportions of B-cell subpopulations by the absolute number of lymphocytes. Statistical significance *$p < 0.05$ (Student's t-test for IgM memory and class switched B cells, Mann-Whitney U test for class switched memory B cells).

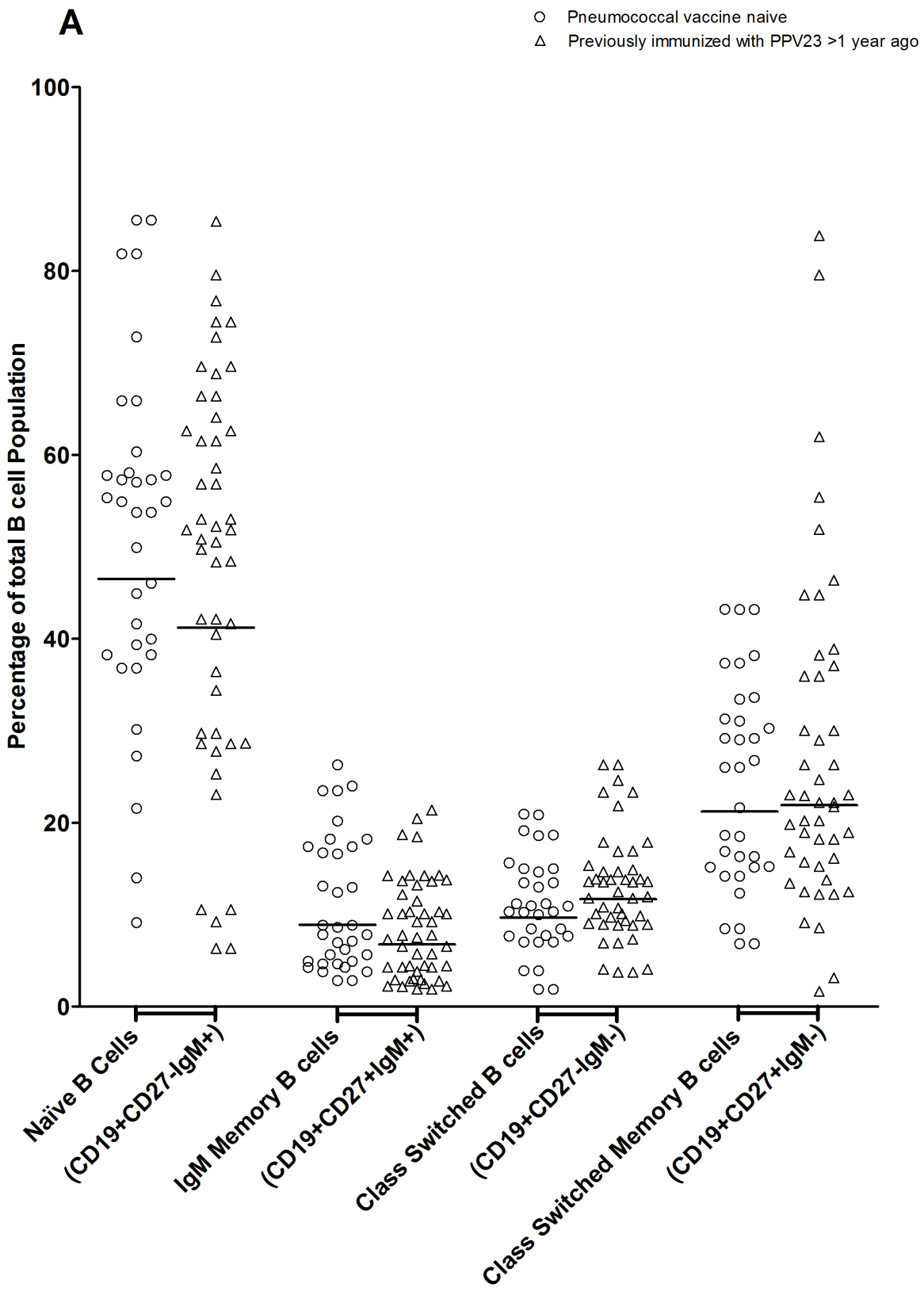
A
Pneumococcal vaccine naive
Previously immunized with PPV23 >1 year ago
Percentage of total B cell Population
100
80
60
40
20
0
Naïve B Cells (CD19+CD27-IgM+)
IgM Memory B cells (CD19+CD27+IgM+)
Class Switched B cells (CD19+CD27-IgM-)
Class Switched Memory B cells (CD19+CD27+IgM-)

Figure 2A. B-cell subpopulations in pneumococcal vaccine naïve (n=25) and previously immunized with 23-valent pneumococcal polysaccharide vaccine (PPV23) (n=35) patients with severe chronic kidney disease on day 7 post-immunization with 13-valent pneumococcal conjugate vaccine. Isolated peripheral blood mononuclear cells were stained for CD19, CD27, and IgM and analyzed by flow cytometry. The geometric mean of each subpopulation proportion is displayed. No statistically significant difference was detected between the groups.

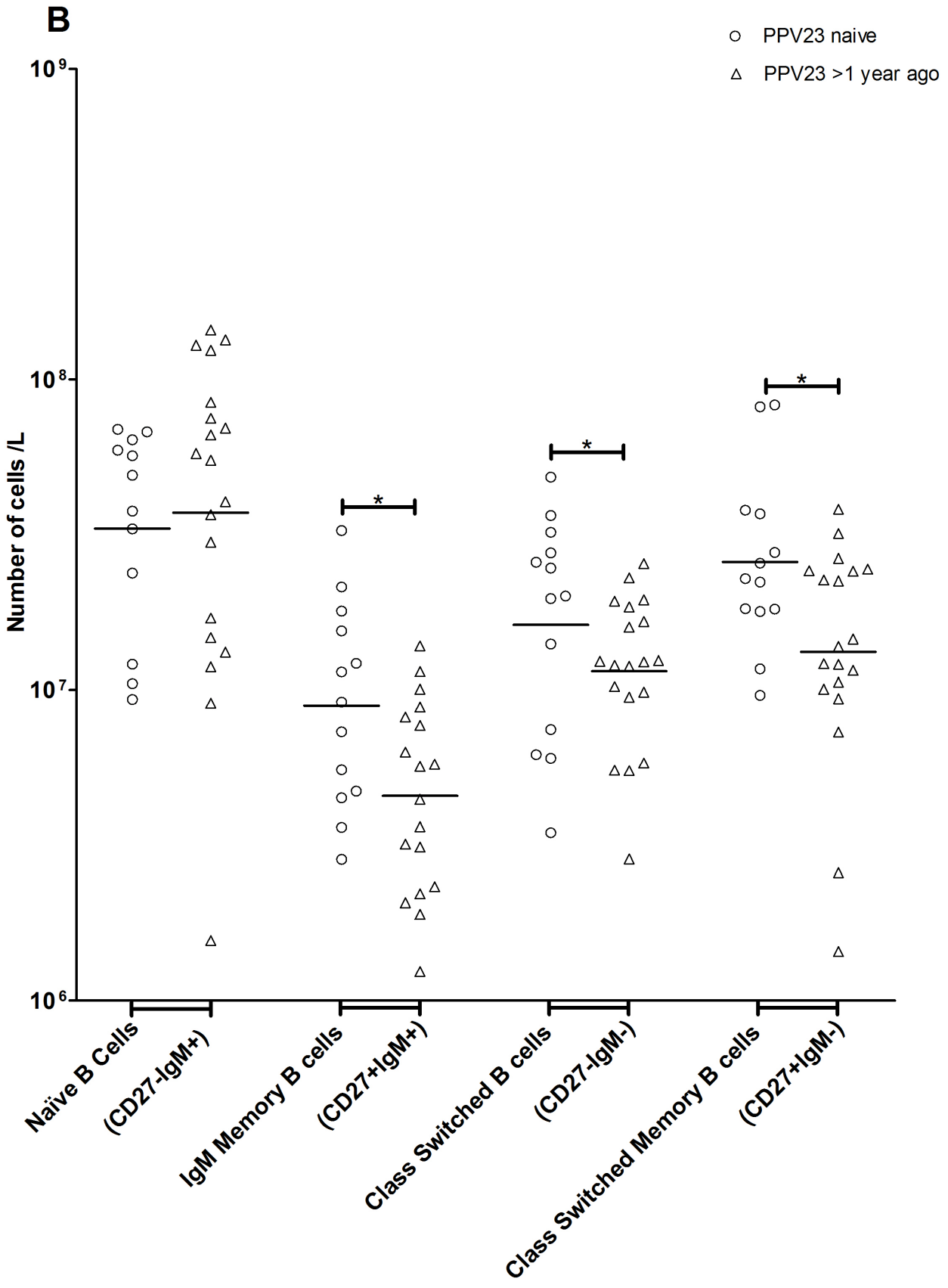
B
PPV23 naive
PPV23 >1 year ago
Number of cells /L
10^9
10^8
10^7
10^6
*
*
*
Naïve B Cells (CD27-IgM+)
IgM Memory B cells (CD27+IgM+)
Class Switched B cells (CD27-IgM-)
Class Switched Memory B cells (CD27+IgM-)

Figure 2B. Absolute numbers of B-cell subpopulations in pneumococcal vaccine naïve (n=14) and previously immunized with 23-valent pneumococcal polysaccharide vaccine (PPV23) (n=19) patients with severe chronic kidney disease 7 days post-immunization with 13-valent pneumococcal conjugate vaccine. Isolated peripheral blood mononuclear cells were stained for CD19, CD27, and IgM. Geometric mean absolute numbers of B-cell subpopulations were determined by multiplying the proportions of B-cell subpopulations by the absolute number of lymphocytes. Statistical significance *$p < 0.05$ (Student's t-test for class switched B cells, Mann-Whitney U test for IgM memory and class switched memory B cells).

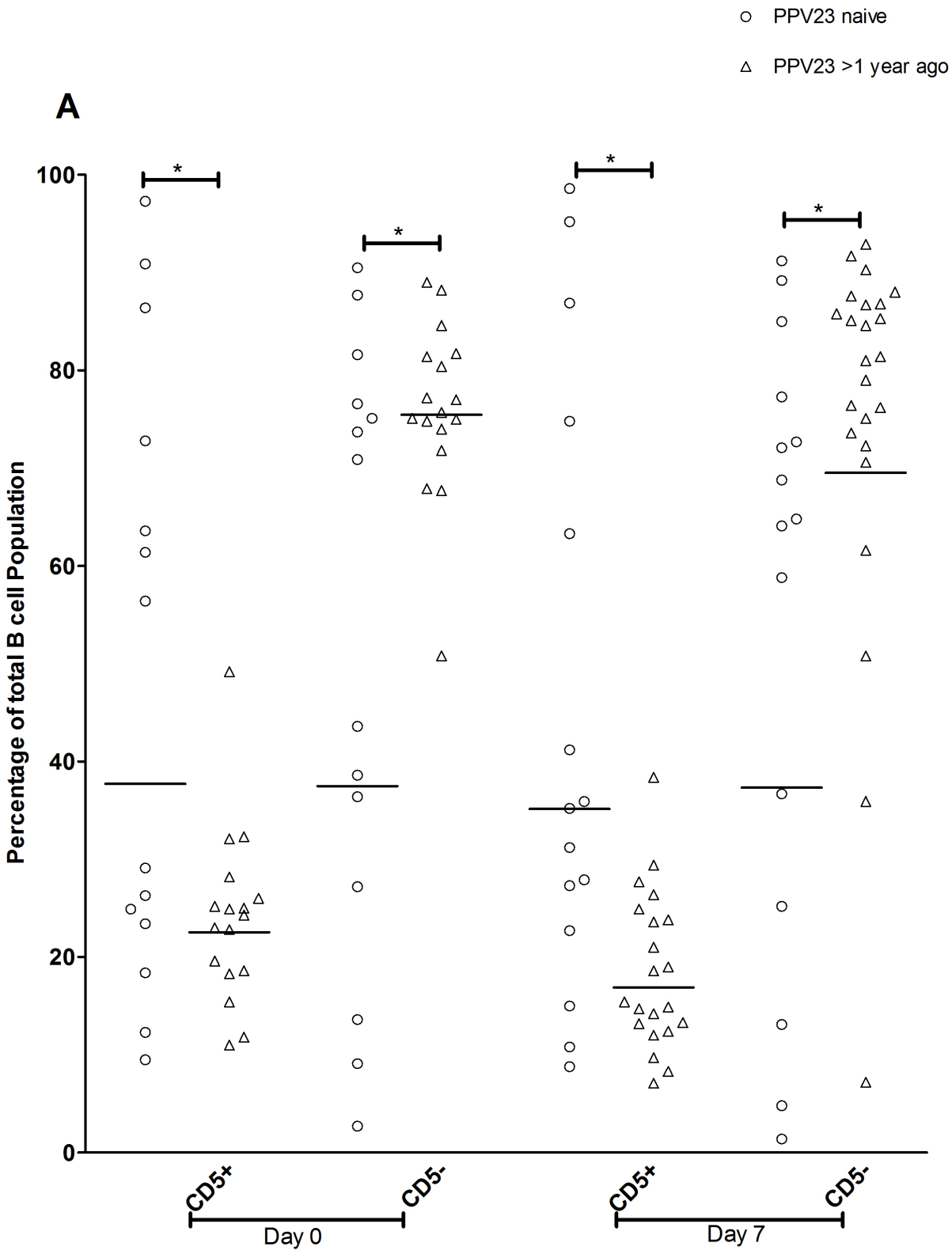
PPV23 naive
PPV23 >1 year ago
A
Percentage of total B cell Population
100
80
60
40
20
0
*
*
*
*
CD5+
CD5-
CD5+
CD5-
Day 0
Day 7

Figure 3A. CD5+ and CD5- B-cell subpopulations pre-immunization (day 0) and post-immunization (day 7) with 13-valent pneumococcal conjugate vaccine in patients with severe chronic kidney disease. Peripheral blood mononuclear cells were stained for CD19 and CD5 and analyzed by flow cytometry. The geometric means are displayed. CD5+ and CD5- B-cell subpopulations are compared between pneumococcal vaccine naïve (day 0, n=14; day 7, n=15) and previously immunized with 23-valent pneumococcal polysaccharide vaccine (PPV23) (day 0, n=19; day 7, n=24) patients. Statistical significance *$p < 0.05$ (Mann-Whiney U test for day 0 CD5+, CD5- and day 7 CD5- B cells, Student's t-test for day 7 CD5+ B cells).

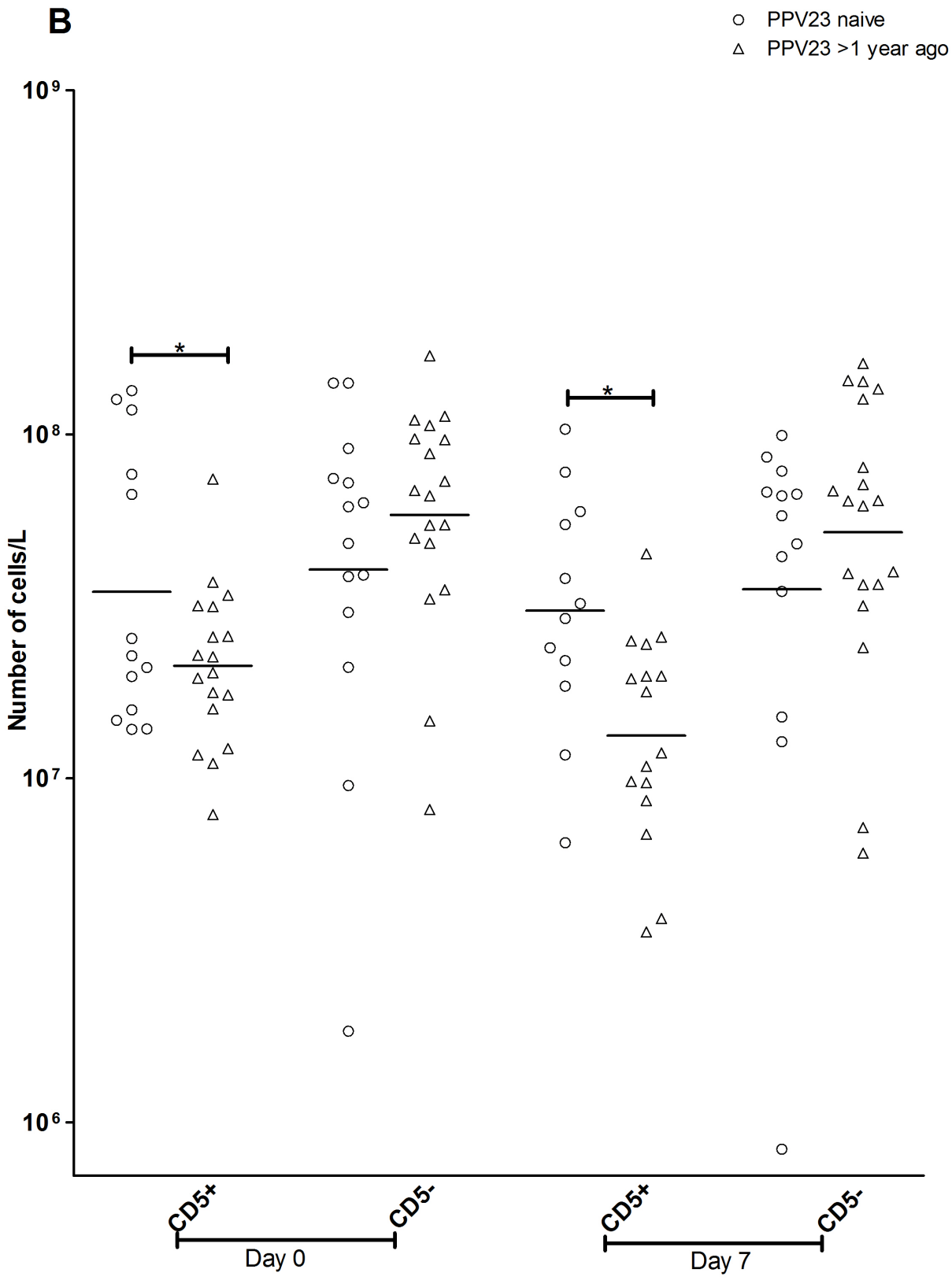
B
PPV23 naive
PPV23 >1 year ago
$10^9$
$10^8$
$10^7$
$10^6$
Number of cells/L
*
*
CD5+
CD5-
CD5+
CD5-
Day 0
Day 7

Figure 3B. Absolute numbers of CD5+ and CD5- B-cell subpopulations pre-immunization (day 0) and post-immunization (day 7) with 13-valent pneumococcal conjugate vaccine in patients with severe chronic kidney disease. Absolute numbers of B-cell subpopulations were determined by multiplying the proportions of B-cell subpopulations by the absolute number of lymphocytes. The geometric means are displayed. Comparison between pneumococcal vaccine naïve (day 0, n=14; day 7, n=15) and previously immunized with 23-valent pneumococcal polysaccharide vaccine (PPV23) (day 0, n=19; day 7, n=24) patients is displayed. Statistical significance *$p < 0.05$ (Student's t-test for day 0 CD5+ B cells, Mann-Whitney U test for day 7 CD5+ B cells).

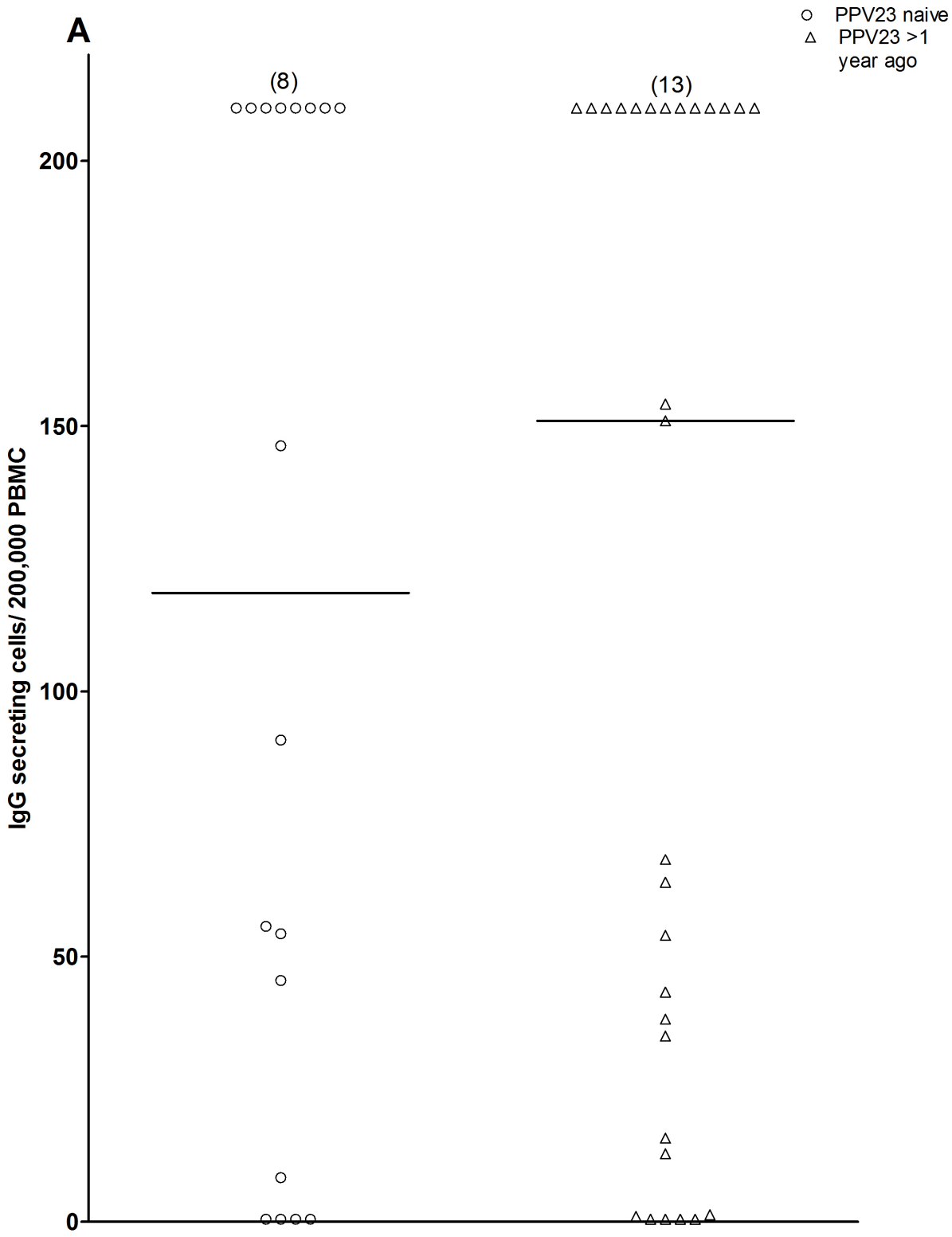
A
PPV23 naive
PPV23 >1 year ago
(8)
(13)
IgG secreting cells/ 200,000 PBMC
200
150
100
50
0

Figure 4A. Numbers of total IgG antibody secreting cells (ASC) per 200,000 peripheral blood mononuclear cells (PBMC) pneumococcal vaccine naïve (n=18) and previously immunized with 23-valent pneumococcal polysaccharide vaccine (PPV23) (n=29) patients with severe chronic kidney disease on day 7 post-immunization with PCV13. The median is displayed counts above the upper limit of detection (200) were assigned a value of 210 (8 pneumococcal vaccine naïve and 13 previously immunized with PPV23). If no spots were detected, a value of 0.5 was assigned for statistical purposes. PPV23 naïve and PPV23 immunized each had 4 values below the limit of detection. Control wells coated with methylated human serum albumin were not displayed (all values were below the limit of detection).

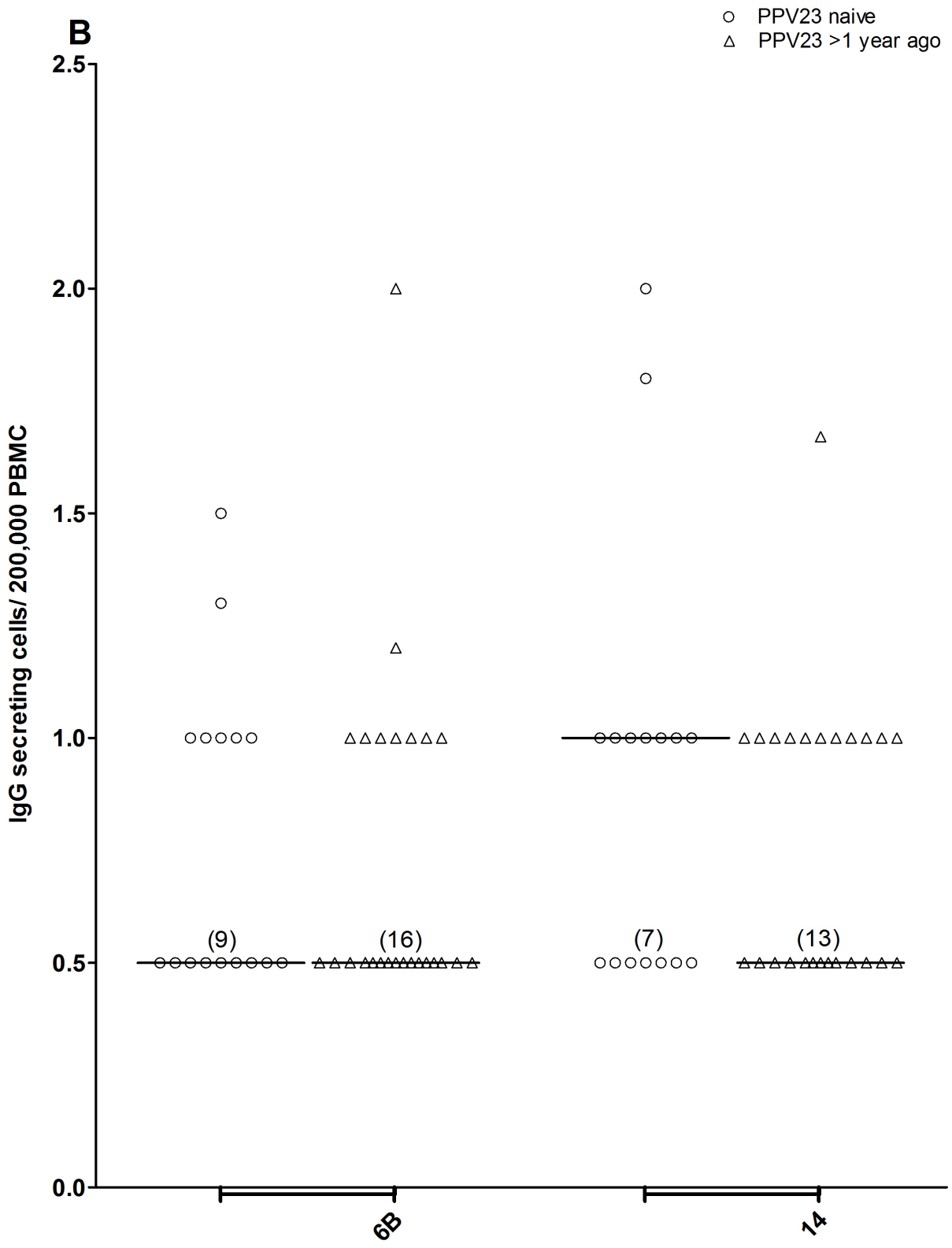
B
PPV23 naive
PPV23 >1 year ago
IgG secreting cells/ 200,000 PBMC
2.5
2.0
1.5
1.0
0.5
0.0
(9)
(16)
(7)
(13)
6B
14

Figure 4B. Numbers of IgG antibody secreting cells (ASC) specific for pneumococcal capsular polysaccharides of serotypes 6B or 14 per 200,000 peripheral blood mononuclear cell (PBMC) in pneumococcal vaccine naïve (n=18) and previously immunized with 23-valent pneumococcal polysaccharide vaccine (PPV23) (n=31) patients with severe chronic kidney disease 7 days post-immunization with PCV13 (median is shown). If no spots were detected a value of 0.5 was assigned for statistical purposes. For serotype 6B, 9 pneumococcal vaccine naïve and 16 previously immunized with PPV23 patients had ASC below the lower limit of detection. For serotype 14, 7 pneumococcal vaccine naïve and 13 previously immunized with PPV23 patients had ASC below the lower limit of detection. Control wells coated with methylated human serum albumin were not displayed (all below the limit of detection).

**Supplementary Material**

Supplementary table 1. Absolute numbers of lymphocytes and B cells in patients with severe chronic kidney disease.

| Cells | Pre-immunization Absolute number of cells/L (GM, CI) | 7 days post-immunization Absolute number of cells/L (GM, CI) | p value |
|---|---|---|---|
| Lymphocytes | 1.0 (0.8-1.4 X $10^9$) | 1.0 (0.8-1.1 X $10^9$) | > 0.05 |
| Total B cells | 9.2 (7.4-11.5 X $10^7$) | 8.4 (6.7-10.5 X $10^7$) | > 0.05 |
| Naïve (CD27-IgM+) | 4.2 (3.0-5.9 X $10^7$) | 3.7 (2.6- 5.4 X $10^7$) | > 0.05 |
| IgM memory (CD27+IgM+) | 6.6 (4.8-9.0 X $10^6$) | 5.7 (4.3-7.5 X $10^6$) | > 0.05 |
| Class Switched (CD27-IgM-) | 1.3 (1.1-1.7 X $10^7$) | 1.3 (1.0-1.6 X $10^7$) | > 0.05 |
| Class Switched memory (CD27+IgM-) | 1.5 (1.2-2.0 X $10^7$) | 1.6 (1.2-2.1 X $10^7$) | > 0.05 |
| CD19+CD5+ | 2.2 (1.8-2.7 X $10^7$) | 2.2 (1.6-3.0 X $10^7$) | > 0.05 |
| CD19+CD5- | 5.0 (3.5-7.0 X $10^7$) | 4.3 (2.9-6.3 X $10^7$) | > 0.05 |

Absolute numbers of lymphocytes and B cells (geometric means, GM with 95% confidence intervals, CI) in 33 patients with severe chronic kidney disease pre- and 7 days post-immunization with PCV13. Absolute numbers of B cells were determined by multiplying the proportion of CD19+ peripheral blood mononuclear cells by the absolute number of lymphocytes obtained via complete blood counts.

Supplementary table 2. Correlation of absolute numbers and proportions of B cells and B-cell subpopulations pre- and 7 days post-immunization.

| | Correlation of absolute numbers of cells | | Correlation of proportions of cells | |
|---|---|---|---|---|
| B cells | r | p | r | p |
| Total B cells (CD19+) | 0.6275 Spearman | < 0.0001 | 0.3414 Spearman | > 0.05 |
| Naïve (CD27-IgM+) | 0.8230 Spearman | < 0.0001 | 0.8680 Pearson | < 0.0001 |
| IgM memory (CD27+IgM+) | 0.7601 Spearman | < 0.0001 | 0.8937 Pearson | < 0.0001 |
| Class Switched (CD27-IgM-) | 0.0222 Pearson | 0.9090 | 0.5030 Spearman | 0.0064 |
| Class Switched memory (CD27+IgM-) | 0.6759 Spearman | < 0.0001 | 0.8492 Spearman | < 0.0001 |
| CD19+CD5+ | 0.6940 Spearman | < 0.0001 | 0.7384 Spearman | < 0.0001 |
| CD19+CD5- | 0.4501 Spearman | 0.0126 | 0.7259 Spearman | < 0.0001 |

Correlation between pre- and post-immunization absolute numbers or proportions of total B cells (CD19+), naïve (CD27-IgM+), IgM memory (CD27+IgM+), class switched (CD27-IgM-), class switched memory (CD27+IgM-), CD5+ and CD5- B cells.

Supplementary table 3. Absolute numbers of lymphocytes and B cells in patients with severe chronic kidney disease that are pneumococcal vaccine naïve or previously immunized with PPV23 > 1 year ago.

| Cells | PPV23 naïve Absolute number of cells/L (GM, CI) | PPV23 > 1 year ago Absolute number of cells/L (GM, CI) | p value |
|---|---|---|---|
| Total lymphocytes pre-immunization | 1.0 (0.8-1.4 X $10^9$) | 0.9 (0.8-1.1 X $10^9$) | > 0.05 |
| Total lymphocytes 7 days post-immunization | 1.0 (0.7-1.3 X $10^9$) | 1.0 (0.8-1.2 X $10^9$) | > 0.05 |
| Total B cells pre-immunization | 9.9 (7.0-13.9 X $10^7$) | 8.3 (6.1-11.2 X $10^7$) | > 0.05 |
| Total B cells 7 days post-immunization | 9.3 (6.7-12.8 X $10^7$) | 7.9 (5.7-10.9 X $10^7$) | > 0.05 |

Absolute numbers of lymphocytes and B cells (geometric means, GM with 95% confidence intervals, CI) in severe chronic kidney disease patients that are pneumococcal vaccine naïve (n=14) or previously immunized with PPV23 > 1 year ago (n=19) pre- and 7 days post-immunization with PCV13.

Supplementary table 4. Concentrations and fold change of pneumococcal 6B and 14 IgG antibodies pre- and 28 days post-immunization.

| Serotype | Group | GMC, 95% CI (µg/ mL) Day 0 pre-immunization | GMC, 95% CI (µg/ mL) Day 28 post-immunization | Fold change |
|---|---|---|---|---|
| 6B | PPV23 naive | 0.9 (0.6- 1.4) | 2.1 (1.4- 3.1) * | 2.8 |
| | PPV23 > 1 year ago | 1.1 (0.7- 1.6) | 2.4 (1.4- 3.9) * | 2.3 |
| 14 | PPV23 naive | 2.8 (1.9- 4.2) + | 8.0 (4.5- 14.4) ** | 1.9 # |
| | PPV23 > 1 year ago | 5.0 (3.0- 8.1) | 8.1 (5.0- 13.3) | 1.3 |

Geometric mean concentration (GMC) with 95% confidence intervals (CI) in severe chronic kidney disease patients who are pneumococcal vaccine naïve (n=22) or previously immunized with PPV23 > 1 year ago (n=34) pre- and 28 days post-immunization with PCV13. The fold change represents the response to PCV13 immunization. * compares day 0 pre-immunization and day 28 post-immunization antibody concentrations for each serotype and patient group, + compares the pre-immunization concentrations between patient groups, # compares the fold change for each serotype between groups. Statistical significance determined by Mann- Whitney U test, * $p < 0.05$, ** $p < 0.01$.

## Chapter 4B: Association of pneumococcal antibody levels with B-cell subpopulations in the peripheral blood of patients with CKD


### Abstract

To determine the response to pneumococcal immunization, the assessment of antigen specific antibodies post-immunization is achieved by either quantifying the amounts or functional capacities of antibodies. The 23-valent pneumococcal polysaccharide vaccine (PPV23) is currently recommended in Canada for patients with chronic kidney disease (CKD) but due to their immune dysfunction, their responses are suboptimal. The 13-valent pneumococcal protein-polysaccharide conjugate vaccine (PCV13) has been recommended for other groups of immunocompromised adults due to its superior immunogenicity. However, antibody responses to PCV13 in CKD patients have only been assessed by two previous studies with conflicting results. In this study, enzyme-linked immunosorbent assay (ELISA) quantified IgG, IgM and IgA concentrations pre, 28- and 365- days post- PCV13 in PPV23 naïve and PPV23 immunized (> 1 year ago) CKD patients. Correlation analyses were preformed between absolute numbers and proportions of class switched memory (CD19+CD27+IgM-) or CD5- B cells and the fold change in IgG concentrations between day 0 – 28 and day 0 – 365. PPV23 immunized patients had significantly higher IgG concentrations pre-immunization for serotypes 14 and 19A, day 28 for serotype 19A, and day 365 for serotype 23F. The fold change in IgG concentrations for PPV23 naïve patients were significantly higher between day 0 – 28 for serotype 14, and day 0 – 365 for serotype 3. IgG fold changes were slightly higher for all serotypes except 23F for PPV23 naïve patients between both day 0 – 28 and day 0 – 365. There were no significant differences in IgM concentrations between groups pre- or post-immunization.

PPV23 naïve patients had a significantly higher IgM fold change between day 0 – 28 for serotype 23F as well as higher IgA concentrations pre- and day 28 for serotype 23F. PPV23 immunized patients had a significantly higher IgA fold change day 0 – 365 for serotype 14. Response to immunization has been previously defined in CKD patients as a concentration of ≥ 1 μg/ mL and a ≥ two-fold antibody concentration increase. When these criteria were applied, PPV23 naïve patients had a higher proportion of patients that responded to more serotypes day 28 for IgG, IgM and IgA. Correlation results were challenging to analyze due to the small sample size, but general trends were similar for both CKD groups. There was a tendency towards a positive correlation for both proportions and absolute numbers of class switched memory B cells and the fold change in IgG concentrations between day 0 – 28 and day 0 – 365. For the absolute numbers and proportions of CD5- B cells, there was a tendency towards a negative correlation with the fold change in IgG concentrations between day 0 – 28 and day 0 – 365. Our previous study (chapter 4A) reported that PPV23 naïve patients had a higher prevalence of class switched memory B cells but a lower prevalence of CD5- B cells. Opposite trends were found for PPV23 immunized patients, suggesting that previous immunization with PPV23 favored the production of CD5- B cells. Our findings suggest that PPV23 immunization has a negative effect on the humoral immune response that not only results in long-term changes in B-cell subpopulations but also decreases antibody responses to subsequent immunization with PCV13 in patients with CKD. Although a higher proportion of PPV23 naïve patients responded to PCV13 based on the concentrations and fold changes day 28 for IgG, IgM and IgA, our findings emphasize the need for further studies to optimize a pneumococcal immunization schedule for adults with CKD.

## Introduction

Successful long-term protection against *Streptococcus pneumoniae* that is formed through immunization, is predicted by quantifying the humoral immune response. To determine if immunological memory has been established, amounts of memory B cells and antigen specific antibodies post-immunization are measured (1). Chapter 4A focused on one aspect of the humoral immune response, by measuring B-cell responses to the 13-valent pneumococcal protein-polysaccharide conjugate vaccine (PCV13) in two groups of severe chronic kidney disease (CKD) patients. One group was immunized with the 23-valent pneumococcal polysaccharide vaccine (PPV23) more than 1 year prior to participating in the study (PPV23 immunized), and the other was pneumococcal vaccine naïve (PPV23 naïve). This was achieved by collecting peripheral blood mononuclear cells (PBMC) pre and 7 days post-immunization with PCV13. These B cells (CD19+) were identified as: naïve (CD27-IgM+), class switched (CD27-IgM-), IgM memory (CD27+IgM+) or class switched memory (CD27+IgM-).

B cell receptors (BCR) are transmembrane proteins responsible for antigen recognition. A single BCR consists of two immunoglobulin heavy and two light chains, which are responsible for ligand binding. In addition, the BCR also consists of two subunits, immunoglobulin alpha and beta, which are responsible for signal transduction. The constant region of the heavy chain is classified as either IgM, IgD, IgG, IgA and IgE isotypes. Naïve B cells express IgD and IgM, while the other isotypes are expressed on activated and memory B cells (2). Isotype switching typically occurs in response to proteins, also known as T cell-dependent antigens. In the case of PCV13, pneumococcal capsular polysaccharides are conjugated to a carrier protein called CRM197 that acts as a T cell-dependent antigen. A T cell-dependent response occurs in the germinal center of lymphoid follicles though interactions between CD4+ T cells and B cells. T

cell-dependent responses result in follicular B cells proliferating and differentiating into memory B cells and plasma cells that undergo somatic hypermutation and affinity maturation to produce high affinity class switched antibodies. T cell-independent (TI) antigens have the ability to activate B cells through crosslinking of the BCRs. Marginal zone B cells respond to TI-1 antigens (e.g. lipopolysaccharides) and TI-2 antigens (e.g. polysaccharides), similarly to the capsular polysaccharides included in PPV23. These cells are important for rapid response to pathogens and differentiate into plasma cells that secrete IgM. The antibodies produced through T cell-dependent or independent pathways have the same affinity as the antigen recognition portion of the BCR (3).

To fully assess the humoral immune response to PCV13 in CKD patients, concentrations of IgG, IgM and IgA antibodies specific to pneumococcal serotypes 3, 6B, 9V, 14, 19A, 19F and 23F were measured pre-immunization, 28- and 365-days post immunization due to their inclusion in both PPV23 and PCV13. Responses to immunization are reported as the fold change and were calculated between day 0 - 28 as well as day 0 - 365 post-immunization. Correlation analyses were performed to determine if there was a relationship between B cells and the fold change in IgG concentrations between day 0 - 28 as well as day 0 - 365 post-immunization. This was performed for the absolute numbers and proportions of class switched memory and CD5- B cells. The correlation of these B-cell subpopulations and the fold change in IgG antibodies were analyzed because class switched memory B cells respond to secondary exposure to an antigen. When class switched memory B cells are activated, this results in the production of high affinity class switched antibodies, which have an important role in mediating humoral immunity (4, 5). The CD5- B cell population was chosen for correlation analyses because it has been found that

immunization with PPV23 results in the generation of CD5- B cells that differentiate into antibody secreting cells, which primarily produce anti-capsular IgG antibodies (6).

This chapter has two objectives, first to determine if there are differences in antibody responses to PCV13 in PPV23 naïve and PPV23 immunized CKD patients, and the second is to determine if there is a relationship between B cells and antibody responses post PCV13 immunization in CKD patients.

**Methods**

Of the 61 patients that we studied for analysis of B cells, antibody analyses were performed for 56 patients (22 PPV23 naïve and 34 PPV23 immunized). Amounts of pneumococcal antibodies were quantified using the ELISA protocol provided by the World Health Organization (7). Statistical analyses were performed using Graph-Pad Prism 8. The lower limits of detection were determined by calculating the concentration of antibodies present in the standard serum (007) (8) that yielded two times the assay background; the lowest serum dilution on the plate was used. All antibody values below the lower limit of detection were reported as half of the limit of detection. Data were reported as geometric means of pneumococcal antibody concentrations with 95% confidence intervals (CI) determined pre-, 28 days and 365 days post-immunization with PCV13. The fold change in antibody concentrations, between day 0 – 28 as well as day 0 – 365 was determined by dividing the post-immunization concentrations by the pre-immunization concentrations for each participant. Groups were compared either using Mann-Whitney U test or Kruskal-Wallis test with Dunn's post-hoc test depending on number of groups compared. Spearman correlation was performed between IgG concentration fold change between day 0 – 28 and day 0 – 365 and the proportions or absolute

numbers of class switched memory B cells or CD5- B cells. Significance was determined at p value < 0.05. Outliers were identified and removed prior to analysis (9).

## Results

Due to the large amount of data and analyses performed, only significant results will be reported in this section. Participant demographics are the same as described in chapter 4A.

### IgG antibody response

The geometric mean concentrations of IgG antibodies pre-immunization, 28- and 365-days post- PCV13 are displayed in table 1a. Pre-immunization, serotype (ST) 14 and 19A concentrations were higher in PPV23 immunized compared to PPV23 naïve patients. Only ST 19A concentrations remained higher day 28, while ST 23 concentrations were higher day 365 for PPV23 immunized compared to PPV23 naïve patients.

Compared to pre-immunization, PPV23 naïve patients had higher ST 6B, 9V, 14, and 19F concentrations day 28 as well as higher ST 19F concentrations day 365. PPV23 naïve patients had lower concentrations day 365 compared to day 28 for STs 3 and 9V. There were significant differences between day 0, 28 and 365 concentrations for ST 3, 6B, 9V, 14 and 19F for PPV23 naïve patients. Compared to pre-immunization concentrations PPV23 immunized patients had higher ST 6B, 9V, 19F and 23F concentrations day 28 as well as higher ST 3, 6B and 23F concentrations day 365. PPV23 immunized patients had lower antibody concentrations day 365 compared to day 28 for ST 3, 9V and19A. There were significant differences between day 0, 28 and 365 concentrations for ST 3, 6B, 9V, 19F and 23F.

The geometric means of the IgG concentration fold changes between day 0 – 28 as well as day 0 – 365 post- PCV13 are displayed in table 1b. PPV23 naïve patients had a higher fold change for ST 14 between day 0 – 28, as well as significantly higher fold change for ST 3 between day 0 – 365 compared to PPV23 immunized patients. Compared to the fold change between day 0 – 28, PPV23 naïve patients had a lower fold change between day 0 – 365 for STs 3, 9V, and 19A. PPV23 immunized patients had lower fold changes between day 0 – 365 for STs 3, 6B, 9V, 19A and 23F compared to the fold change between day 0 – 28.

**IgM antibody response**

The geometric mean concentrations of IgM antibodies pre-immunization, 28- and 365-days post- PCV13 are displayed in table 2a. There were no significant differences in concentrations for any serotype pre- or post-immunization between PPV23 naïve and PPV23 immunized patients. Compared to pre-immunization concentrations, PPV23 naïve patients had higher ST 3, 6B and 9V concentrations day 28. PPV23 naïve patients had no significant differences in concentrations day 365 compared to day 28 for any ST. There were significant differences in concentrations for ST 6B between day 0, 28 and 365 for PPV23 naïve patients. For PPV23 immunized patients, there were no significant differences between: pre-immunization and either day 28 or 365 concentrations, day 28 and day 365 concentrations, or between day 0, 28 and 365 when a multiple comparisons test was performed.

The geometric mean of the IgM antibody concentration fold change between day 0 and 28 as well as day 0 – 365 post- PCV13 are displayed in table 2b. PPV23 naïve patients had a higher fold change in ST 23F between day 0 – 28 compared to PPV23 immunized patients. There were no significant differences in fold change specific to any ST between PPV23 naïve

and PPV23 immunized patients between day 0 – 365. PPV23 naïve patients had lower fold changes for STs 6B and 9V between day 0 – 365 compared to the fold change between day 0 – 28. Both PPV23 naïve and immunized patients had significantly lower fold changes for ST 14 between day 0 – 365 compared to the fold change between day 0 – 28.

**IgA antibody response**

The geometric mean concentrations of IgA antibodies pre-immunization, 28- and 365-days post- PCV13 are displayed in table 3a. Compared to PPV23 immunized patients, PPV23 naïve patients had higher concentrations of ST 23F pre-immunization and day 28. PPV23 naïve patients had higher concentrations of STs 6B, 9V, 19F, and 23F day 28, but lower concentrations of ST 14 day 365 compared to pre-immunization concentrations. PPV23 naïve patients had lower concentrations day 365 compared to day 28 of STs 3, 6B, 9V, 14, 19F and 23F. When comparing day 0, 28 and 365 concentrations, there were significant differences in STs 6B, 9V, 14, 19F and 23F for PPV23 naïve patients. Compared to pre-immunization concentrations, PPV23 immunized patients had higher concentrations of ST 6B, 9V, 19A, 19F and 23F day 28. There were no significant differences between day 365 and pre-immunization concentrations for PPV23 immunized patients for any ST. PPV23 immunized patients had lower concentrations day 365 compared to day 28 for STs 3, 6B, 9V, 14, and 19F. When comparing day 0, 28 and 365 concentrations, there were significant differences for all STs except 14 and 19A for PPV23 immunized patients.

The geometric means of the IgA antibody concentration fold change between day 0 – 28 as well as day 0 – 365 post- PCV13 are displayed in table 3b. There were no significant differences in fold changes between PPV23 naïve and immunized patients for any ST between

day 0 – 28. PPV23 immunized patients had a higher fold change between day 0 – 365 for ST 14 compared to PPV23 naïve patients. Both PPV23 naïve and PPV23 immunized patients had a lower fold changes for all STs between day 0 – 365 compared to day 0 – 28.

**Correlation of the fold change in IgG concentrations and B-cell subpopulations**

The comparisons used for correlation analyses are displayed in Figure 1a. Correlations that have been described as significant are defined as $r \geq 0.5$ and $p < 0.05$, and a weak correlation as $r = 0.4 - 0.5$ even though $p > 0.05$. A meaningful correlation was used to describe correlations that were defined as either significant or weak, if $r < 0.4$, no meaningful correlation was found. The correlation between the proportions or absolute numbers of class switched memory (CS memory) B cells and CD5- B cells and the fold change in IgG concentrations between day 0 – 28 as well as day 0 – 365 are displayed in tables 4a and b. Unfortunately, due to the small group sizes and large variability, significance was not reached in many cases. However, we considered even weak correlations ($r = 0.4 - 0.5$) to determine the general trends between B cells and the fold change in IgG concentrations. Figure 1b displays the comparisons used for each correlation analyses and the general relationship trend that was determined based on r values. Because weak and significant correlations were considered and the same trend may have not been observed for every serotype, correlation results were described as a tendency towards either a positive or negative correlation based on the majority of the trends.

Day 0 CS memory B cell proportions had a tendency towards a positive correlation with the fold change between day 0 – 28 for both patient groups, but only PPV23 immunized had a significantly positive correlation for ST 9V. Although neither group had significant correlations for any ST between day 0 CS memory B cell absolute numbers and the fold change between day

0 – 28, PPV23 immunized patients had a tendency towards a positive correlation, while PPV23 naïve patients had a tendency towards negative correlation. Day 0 CS memory B cell proportions had a tendency towards a positive correlation with the fold change between day 0 – 365 for PPV23 naïve patients but no significant correlation was found for any ST. PPV23 immunized patients had no meaningful correlations for any ST between day 0 CS memory B cell proportions and the fold change between day 0 – 365, indicating that there is no relationship. Day 0 CS memory B cell absolute numbers had a tendency towards a positive correlation with the fold change between day 0 – 365 for PPV23 naïve patients, but no significant correlation was found for any ST. When the same comparison was made for PPV23 immunized patients, there was a tendency towards a negative correlation, and there was a significantly negative correlation with the fold change between day 0 – 365 for ST 19A. To generally summarize, there appears to be a tendency towards a positive correlation between pre-immunization CS memory B cells and the antibody fold change post- PCV13 in patients with CKD.

Day 7 CS memory B cell proportions had no meaningful correlations with the fold change between day 0 – 28 for any ST for either patient group. PPV23 immunized patients had significantly positive correlations between the CS memory B cell absolute numbers day 7 and the fold change between day 0 – 28 for STs 3, 6B, and 19F. PPV23 naïve patients had no meaningful correlations with the fold change between day 0 – 28 and the absolute numbers of CS memory B cells day 7. PPV23 naïve patients had a significantly positive correlation between day 7 CS memory B cell proportions and the fold change between day 0 – 365 for ST 9V. PPV23 immunized patients had no meaningful correlations with the fold change between day 0 – 365 and the proportions of CS memory B cells day 7. PPV23 naïve patients showed a tendency towards a positive correlation for absolute numbers of CS memory B cells day 7 and the fold

change between day 0 – 365, but no significant correlation was found for any ST. PPV23 immunized patients showed a tendency towards a negative correlation for absolute numbers of CS memory B cells at day 7 and the fold change between day 0 – 365, but no significant correlation was found for any serotype. To summarize, there appears to be a slight tendency towards a positive correlation between day 7 post-immunization class switched memory B cells and the antibody fold change post- PCV13 in patients with CKD.

Day 0 proportions of CD5- B cells for PPV23 naïve patients showed a tendency towards a negative correlation with the fold change between day 0 – 28, and a significantly negative correlation for ST 3. PPV23 immunized participants had no meaningful correlation between day 0 proportions of CD5- B cells and the fold change between day 0 – 28 for any serotype, however day 0 absolute numbers and the fold change between day 0 – 28 had a significantly negative correlation for STs 9V and 14. In PPV23 naïve patients, there was a tendency towards a negative correlation between day 0 absolute numbers of CD5- B cells and the fold change between day 0 – 28. Day 0 proportions of CD5- B cells for PPV23 naïve patients showed a tendency towards a negative correlation with the fold change between day 0 – 365, and a significantly negative correlation for ST 23F. PPV23 immunized patients showed a tendency towards a positive correlation with the fold change between day 0 – 365, and a significantly positive correlation for ST 23F. Day 0 proportions of CD5- B cells for PPV23 naïve patients showed a tendency towards a positive correlation with the fold change between day 0 and 365, while PPV23 immunized patients showed a tendency towards a negative correlation, but neither had significant correlations for any ST. To summarize, there appears to be a tendency towards a negative correlation between pre-immunization CD5- B cells and the antibody fold change post- PCV13 in patients with CKD.

Day 7 proportions of CD5- B cells for both PPV23 naïve and PPV23 immunized patients showed a tendency towards a negative correlation with the fold change between day 0 – 28, and both had a significantly negative correlation for ST 3. Similarly, both PPV23 naïve and PPV23 immunized patients showed a tendency towards a negative correlation with the fold change between day 0 – 28 and the absolute numbers of day 7 CD5- B cells. PPV23 naïve patients had significantly negative correlations for STs 3 and 23F, but PPV23 immunized patients had no significant correlations for any ST. Day 7 proportions of CD5- B cells for both PPV23 naïve and PPV23 immunized patients showed a tendency towards a negative correlation with the fold change between day 0 – 365. PPV23 naïve patients had a significantly negative correlation for ST 9V, but PPV23 immunized patients had no significant correlations for any STs. PPV23 naïve patients showed a tendency towards a negative correlation with the fold change between day 0 – 365 and the absolute numbers of day 7 CD5- B cells, there was a significantly negative correlation for ST 19F. PPV23 immunized patients showed a tendency towards a positive correlation with the fold change between day 0 – 365 and the absolute numbers of day 7 CD5- B cells, but there was no significant correlation for any ST. To summarize, there appears to be a tendency towards a negative correlation between day 7 post-immunization CD5- B cells and the antibody fold change post- PCV13 in patients with CKD.

**Discussion**

The three antibody isotypes measured in this study have different biological roles. IgM antibodies are produced during the primary immune response to an antigen by naïve B cells. IgM antibodies are polyreactive and have low affinity to an antigen because they have not undergone somatic hypermutation. Because of this, IgM antibodies are able to respond rapidly to multiple

antigens. IgA concentrations in the serum are much lower than IgG but higher than IgM. On mucosal surfaces, IgA is the most predominant antibody isotype and is key for neutralization of toxins and microorganisms as well as preventing adhesion of microorganisms attempting to bind to mucosal surfaces. There are two subclasses of IgA, IgA2 is the more predominant subclass on mucosal surfaces and is less susceptible to bacterial proteases than IgA1. IgG has the most predominant isotype in the body and has the longest half-life. IgG antibodies are involved in neutralization of toxins and viruses as well as opsonization of microorganisms for phagocytosis. IgG antibodies are classified into 4 subclasses. During the secondary response, IgG1 and IgG3 antibodies are produced in response to a protein antigen while IgG2 and IgG4 antibodies are typically produced in response to polysaccharide antigens. IgG antibodies are important for classical complement pathway activation, but the affinity for complement component 1q (C1q) is not equal for all subclasses with IgG3 having the highest affinity followed by IgG1 and IgG2, while IgG4 is unable to activate the classical complement pathway (10).

Two studies have measured the antibody response to PCV13 in CKD patients, both reporting the response to immunization based on IgG antibodies. Mitra *et al*. (2016) reported ELISA results as the geometric mean concentrations. Exclusion criteria included receiving a pneumococcal immunization ≤ 5 years, but the proportion of patients that received a pneumococcal immunization ≥ 5 years was not reported. The second study by Vandecasteele *et al*. (2018) measured the antibody response to PCV13 in CKD patients that were PPV23 naïve and patients that had received PPV23 either > 4 years ago or < 4 years ago. Antibody responses were reported as geometric mean titres (the mean of log transformed antibody concentrations).

In the study by Mitra *et al*. (2016) response to immunization was reported as a post-immunization IgG antibody concentration of at least 1 μg/ mL and a ≥ two-fold antibody

concentration increase. These criteria were applied to our study and the results are displayed in supplementary table 1a. Comparisons between our study and the studies by Mitra *et al*. (2016) and Vandecastele *et al.* (2018) are shown in supplementary tables 2a and b. The proportion of our patients that responded to PCV13 based on IgG concentrations and fold change between day 0 – 28, was higher for the PPV23 naïve patients for all STs except for 23F compared to PPV23 immunized patients. Although the proportions of our patients that responded by day 365 decreased for both groups, a higher proportion of PPV23 naïve patients responded to all STs except ST 23F compared to PPV23 immunized patients.

Mitra *et al*. (2016) reported that antibody concentrations decreased significantly by 12 months compared to 2 months post-immunization for 6/ 7 STs. Significantly lower IgG concentrations between day 28 and day 365 were found for 2/ 7 STs in our PPV23 naïve and 3/ 7 STs for our PPV23 immunized patients. It is difficult to determine if the decreases in antibody concentrations by day 365 compared to day 28 are of concern clinically because there are not any established protective antibody concentrations in the literature for adults. A few studies have used a minimum concentration of 1.3 µg/ mL of serum IgG post-PPV23 immunization as an indicator of protection (11, 12). The correlate of protection against IPD post-PCV immunization for infants is reported as 0.35 µg/ mL of serum IgG. However, it has been suggested that this may not be true for all serotypes and that concentrations resulting in protection may be dependent on serotype (13).

Vandecasteele *et al*. (2018) found no significant differences in pre-immunization titres between PPV23 immunized and PPV23 naïve patients for any of the seven STs. However, PPV23 naïve patients had significantly higher day 28 titres for all seven STs, on day 365 they remained significantly higher for 5/7 STs compared to PPV23 immunized patients. Our PPV23

immunized patients had significantly higher pre-immunization concentrations for 2/ 7 serotypes compared to PPV23 naïve patients. On day 28, our PPV23 naïve patients had significantly lower concentrations for 1/7 STs, and significantly lower day 365 concentrations for 1/7 STs compared to PPV23 immunized patients.

In comparison to both studies, our patients had similar responses to PCV13 as the CKD patients from Mitra *et al.* (2016). Compared to day 28, PPV23 naïve had significantly lower concentrations for 2/ 7 STs and PPV23 immunized patients had significantly lower concentrations for 3/ 7 STs day 365. Unlike the participants from Vandecasteele *et al.* (2018), our PPV23 naïve did not have significantly higher concentrations compared to PPV23 immunized patients post-PCV13 immunization. However, it should be noted that although only statistically significant for serotype 19A ($p < 0.05$), PPV23 naïve patients had slightly higher fold changes in IgG concentrations between day 0 and 28 for all STs except for 23F. Similarly, PPV23 naïve patients had a significantly higher fold change for ST 3 IgG concentrations between day 0 – 365, and slightly higher fold changes for all STs except for 23F.

There have been a limited number of studies that have evaluated the response to pneumococcal immunization based on serotype specific capsular polysaccharide antibody concentrations. The majority of studies quantify the responses to pneumococcal immunization using opsonophagocytic activity (OPA) titres. There is no direct relationship between antibody concentrations and OPA titres to make studies comparable. The studies that have reported antibody concentrations post-PCV13 are summarized in supplementary table 3a. There is a lot of heterogenicity in antibody concentrations between studies, making it difficult to compare concentrations between adults with different health conditions. It was clear for all studies displayed in supplementary table 3a that the lowest concentrations were for ST 3 (0.19 – 2.5 µg/

mL) and the majority of studies had the highest concentrations for ST 19A (3.5 – 27.9 μg/ mL). This shows the wide range of concentrations between different participant groups, and the differences in immunogenicity between STs. This suggests that one concentration cannot be used for all STs to determine if protection has been established against IPD in adults in response to a pneumococcal vaccine. Because there is no accepted value or fold change to indicate response to PCV13, we chose to only discuss the studies that have reported the proportion of participants that responded to immunization based on either a minimum fold change in IgG concentrations or a minimum IgG concentration (supplementary table 3b).

Studies by van Deursen and Peterson that involved healthy adults used a ≥ four-fold increase in IgG concentrations as a response to PCV13. Both reported that the lowest response to PCV13 was to serotype 3 (32.7% and 17.1%). van Deursen *et al*. (2017) reported their highest response 1 month post-PCV13 was 59.5% for ST 9V, and Peterson *et al*. (2019) reported their highest response at 2 months post-PCV13 was 46.2% for ST 6B (14, 15). There were two studies involving immunodeficient adults, Nived *et al.* (2015) assessed responses to PCV13 in adults with asplenia 4 – 6 weeks post-PCV13 and Belmonti *et al*. (2019) for HIV adults 5 years post-PCV13. Both studies measured responses based on IgG concentrations that were ≥ 0.35 μg/mL and ≥ 1.0 μg/ mL. Nearly all HIV adults had concentrations of ≥ 0.35 μg/mL, the lowest proportion that responded was 88% for 2/ 7 serotypes. Using IgG concentrations of ≥ 1 μg/mL as a definition for response to PCV13, the range of patients that responded was between 33% - 98% (16). The proportion of adults with asplenia that had concentrations of ≥ 0.35 μg/mL ranged from ~ 55% - 100%, while the proportion of participants that had concentrations of ≥ 1 μg/mL ranged from ~ 27% - 95%. It is difficult to compare responses to PCV13 between different studies due to the different response criteria used. It is also important to consider that

using one specific concentration or increase in fold change for all serotypes is not indicative of IPD prevention. The immunogenicity of capsular polysaccharides are different for each serotype (e.g. STs 6A and 6B are some of the least immunogenic capsular polysaccharides) therefore a higher concentration or larger fold change may be required for protection against certain STs (17).

No studies have measured the correlation between B cells and the antibody responses to pneumococcal immunizations in CKD patients. B cells from HIV-positive adults have also been quantified through flow cytometry and reported the correlation between antibody concentrations in response to PCV13 immunization. Farmaki *et al*. (2018) immunized HIV-positive adults with PCV13 and measured the correlation between polysaccharide specific CS memory B cells (CD19+CD10-CD27+CD21++IgM-) and IgG concentrations against pneumococcal STs 3 and 14. Groups contained both PPV23 naïve and PPV23 immunized > 1 year ago. There was a positive correlation between pre-immunization absolute numbers of polysaccharide specific class switched memory B cells and IgG concentrations 1-month post-PCV13 immunization for both STs 3 and 14 (18). Similarly, both of our participant groups also showed a tendency towards a positive correlation between total CS memory B cells and the fold change in IgG between both day 0 – 28 and day 0 – 365.

As it is described in chapter 4A, PPV23 immunized patients had significantly lower proportions and absolute numbers of CS memory B cells compared to PPV23 naïve patients pre-immunization with PCV13. This suggests that immunization with PPV23 may result in long-term changes such as a decreased prevalence of CS memory B cells in peripheral blood. Following PCV13 immunization, the differences in absolute numbers of class switched memory B cells between groups remained significant. Although statistical significance was not reached

for most serotypes, there was a greater increase in IgG concentration fold changes for all serotypes except for 23F for PPV23 naïve patients between day 0 – 28 and day 0 – 365. There were not many statistically significant correlations between B cell subpopulations and the fold change in IgG concentrations, but general trends were found. There was a tendency towards positive correlation between class switched memory B cells pre- and day 7, and IgG concentrations day 0 – 28 and day 0 – 365 for both PPV23 naïve and PPV23 immunized patients. This indicates that the higher pre-immunization proportion and absolute numbers, as well as the higher day 7 absolute numbers of CS memory B cells for PPV23 naïve patients resulted in a greater increase in IgG antibody concentrations between day 0 – 28 and day 0 – 365 compared to PPV23 immunized patients. The decreased proportions and absolute numbers of CS memory B cells pre- PCV13 suggests that PPV23 immunization could result in pneumococcal memory B cells to terminally differentiate, which caused a decreased IgG response to PCV13. Clutterbuck *et al*. (2012) demonstrated that elderly adults immunized with the 7-valent pneumococcal protein-polysaccharide conjugate vaccine (PCV7) had increased serotype specific memory B cells, but immunization with PPV23 decreased the frequency of memory B cells. In comparison to participants immunized with PCV7 alone, PPV23 immunized participants that received a subsequent dose of PCV7 had decreased memory B cell responses (19). Although Clutterbuck *et al*. (2012) did not measure the antibody responses in the setting of PPV23 followed by PCV7, our PPV23 immunized patients also had a decreased response to PCV13 compared to PPV23 naïve patients based on day 0 – 28 fold changes in IgG, IgM and IgA. The decreased fold change in IgG antibodies (both day 0-28 and 0-365) and the decreased absolute numbers of CS memory B cells pre-immunization and day 7 post-PCV13 in PPV23 immunized patients suggests that

immunization with PPV23 reduces the immunogenicity of PCV13 when followed by PPV23 in CKD patients.

The role of CD5- B cells in humans is not completely understood, but the decrease of CD5+ B cells and increase of CD27+ B cells with age suggests that CD5- B cells represent a proportion of the memory B cell population (20). A study by Moens *et al*. (2015) demonstrated that immunization with PPV23 favors the generation of CD5- B cells specific for pneumococcal capsular polysaccharides that primarily produce IgG (6). Similarly, our PPV23 immunized patients had higher proportions of CD5- B cells compared to PPV23 naïve patients both pre- and post-immunization with PCV13. However, there was a tendency towards a negative correlation between CD5- B cells pre- and day 7 and the IgG fold changes between day 0-28 and day 0-365 for both PPV23 naïve and PPV23 immunized patients. The lower IgG fold increase in PPV23 immunized patients compared to PPV23 naïve patients may suggest that the production of CD5- B cells from PPV23 results in a decreased IgG response to subsequent immunization with PCV13 in CKD patients.

The IgM and IgA responses to PCV13 are difficult to interpret because responses to PCV13 alone or PPV23 followed by PCV13 have not been previously quantified for these isotypes in adults. There were no significant differences in IgM concentrations pre-immunization, 28 or 365 days between PPV23 naïve and PPV23 immunized participants. The fold changes in IgM concentrations were similar for PPV23 naïve and PPV23 immunized patients, but ST 23F concentrations were significantly higher for PPV23 naïve patients between day 0 and 28. The criteria to define a response to immunization described by Mitra *et al*. (2016), were applied to IgM concentrations and fold changes (supplementary table 1b). Based on IgM post-immunization concentrations and fold changes, a higher proportion of PPV23 naïve patients

responded to all serotypes day 28 post-immunization compared to PPV23 immunized patients. The proportions of PPV23 naïve patients that responded was between 13.6% - 40.9% and the proportions of PPV23 immunized patients that responded was between 2.9% - 17.6%. Considering that there were no significant differences in naïve B cells between patient groups pre or post-immunization, the differences in the number of participants that met the response criteria could be due to the significantly larger absolute numbers of IgM memory B cells in PPV23 naïve patients pre- and 7-days post-PCV13 immunization (chapter 4A). This suggests that previous immunization with PPV23 could have also resulted in depletion of the total memory B cell pool and not just class switched memory B cells. It has been shown that stimulation with a T cell-dependent antigen results in germinal center dependent differentiation of IgM memory B cells that have high affinity BCRs which have undergone somatic hypermutation similarly to IgG memory B cells in a murine model (21). The antibodies produced from IgM memory B cells could be important for early responses to a pathogen as they have higher affinity for the antigen compared to IgM antibodies produced by naïve B cells.

However, IgM concentrations day 365 were higher for a larger proportion of PPV23 immunized patients responded to 6/ 7 STs. The proportion of PPV23 immunized patients that responded ranged from 10.7% - 39.3% and the proportion of PPV23 naïve patients that responded was between 5.9% - 35.3%. The higher proportion of PPV23 immunized patients that responded day 365 compared to day 28 days for 6/ 7 STs suggests that some of the PPV23 immunized patients were potentially exposed to pneumococcus shortly before the collection of day 365 samples. Because IgM antibodies are polyreactive, it is also possible that these antibodies could have been produced in response to another pathogen but are cross reactive for pneumococcal capsular polysaccharides (10). For example, Lagergard *et al*. (1983) reported that

pneumococcal serotype 6B antibodies are cross reactive with *H. influenzae* type a polysaccharide (22). In addition, Heidelberger *et al.* (1978) found that that pneumococcal capsular polysaccharide antibodies cross-react with *Klebsiella pneumoniae* capsular polysaccharides (23).

When IgA concentrations were compared between patient groups, the only significant differences were found for pre- and day 28 concentrations for ST 23F, which were significantly higher for PPV23 naïve patients. The only significant difference between IgA fold changes between groups was between day 0 – 365, which was significantly higher for ST 14 for PPV23 immunized patients. The criteria to define a response to immunization described by Mitra *et al*. (2016), were applied to IgA concentrations and fold changes (supplementary table 1c). A higher proportion of PPV23 naïve patients responded to 4/7 serotypes day 28, with proportions of patients responding ranging from 27.3% - 59.1%. The range of the proportion of PPV23 immunized patients that responded was similar to PPV23 naïve and ranged from 20.6% - 50.0%.

However, day 365 a higher proportion of PPV23 immunized patients responded to 4/ 7 serotypes which had a proportion of patients that responded ranging from 3.6% - 25.0%, while the proportion of PPV23 naïve patients that responded ranged from 0% - 29.4%. The IgA response to PCV13 was quite similar between PPV23 naïve and PPV23 immunized participants. When taking into consideration the high amounts and importance of IgA at mucosal surfaces (24), differences in IgA concentrations at mucosal surfaces between CKD patient groups may be a better indicator of protection against IPD. Since the role of the capsule is to increase adherence to mucosal epithelial surfaces as well as prevent complement activation, assessing the differences in pneumococcal capsular polysaccharide antibodies may suggest if disease caused by pneumococcus would be prevented (25).

Our limitations are the same as those listed in chapter 4A, but the ones that seem to have a significant impact are mentioned. A major limitation of our study is small sample size which likely contributed to lack of statistical significance in some comparisons between PPV23 naïve and PPV23 immunized patients. The loss of participants due to study withdrawal or death also contributed smaller groups sizes when quantifying the antibody fold changes, particularly at day 365. Because PPV23 is recommended for CKD patients, one of our patient groups received PPV23 prior to recruitment, which meant that we could not assess immediate responses to PPV23 in our CKD patients. Unfortunately, we do not have opsonophagocytosis assay (OPA) antibody titers available to determine the functionality of the antibodies produced post PCV13, as well as determine if there was any correlation with antibody concentrations or B-cell subpopulations. Another limitation is that there are no established antibody concentrations or titres that would indicate protection against IPD in adults making it difficult to determine the clinical significance of antibody data.

**Conclusions**

In conclusion, PPV23 naïve patients had significantly lower pre-immunization concentrations for STs 14 and 19, day 28 for ST 19A, and day 365 for ST 23 IgG concentrations, but also had slightly lower IgG concentrations pre, day 28 and day 365 for all STs (except day 28 for ST 9V). However, PPV23 naïve patients had significantly higher IgG fold change day 28 ST 14, and slightly higher fold changes in IgG concentrations between day 0-28 and day 0-365 for all STs except for ST 23F. Using the criteria to define response to immunization by Mitra *et al*. (2016) higher proportions of PPV23 naïve patients responded to 6/ 7serotypes day 28 and 365 post- PCV13 based on IgG concentrations and fold changes. On day 28 a higher proportion of

PPV23 naïve patients responded to more serotypes post-PCV13 immunization based on IgM and IgA concentrations and fold changes, even though there were few significant differences in antibody concentrations between PPV23 naïve and PPV23 immunized patients. Our previous study also suggested that PPV23 naïve patients had a greater response to PCV13 immunization which was indicated by an increased prevalence of CS memory B cells pre and day 7. This was confirmed when correlation analyses determined that there was a tendency towards a positive correlation between proportions and absolute numbers of CS memory B cells and IgG fold changes between day 0 – 28 and day 0 – 365. It was also demonstrated in the previous chapter that immunization with PPV23 favors the generation of CD5- B cells, as PPV23 immunized patients had a higher prevalence of CD5- B cells which had a tendency towards a negative correlation with IgG fold changes. Our findings suggest that PPV23 immunization has a negative effect on the humoral immune response that not only results in long-term changes in B cell subpopulations but also decreases antibody responses to subsequent immunization with PCV13 in patients with CKD. Our findings emphasize the need for further studies to optimize pneumococcal immunization for adults with CKD.

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**Tables and Figures**

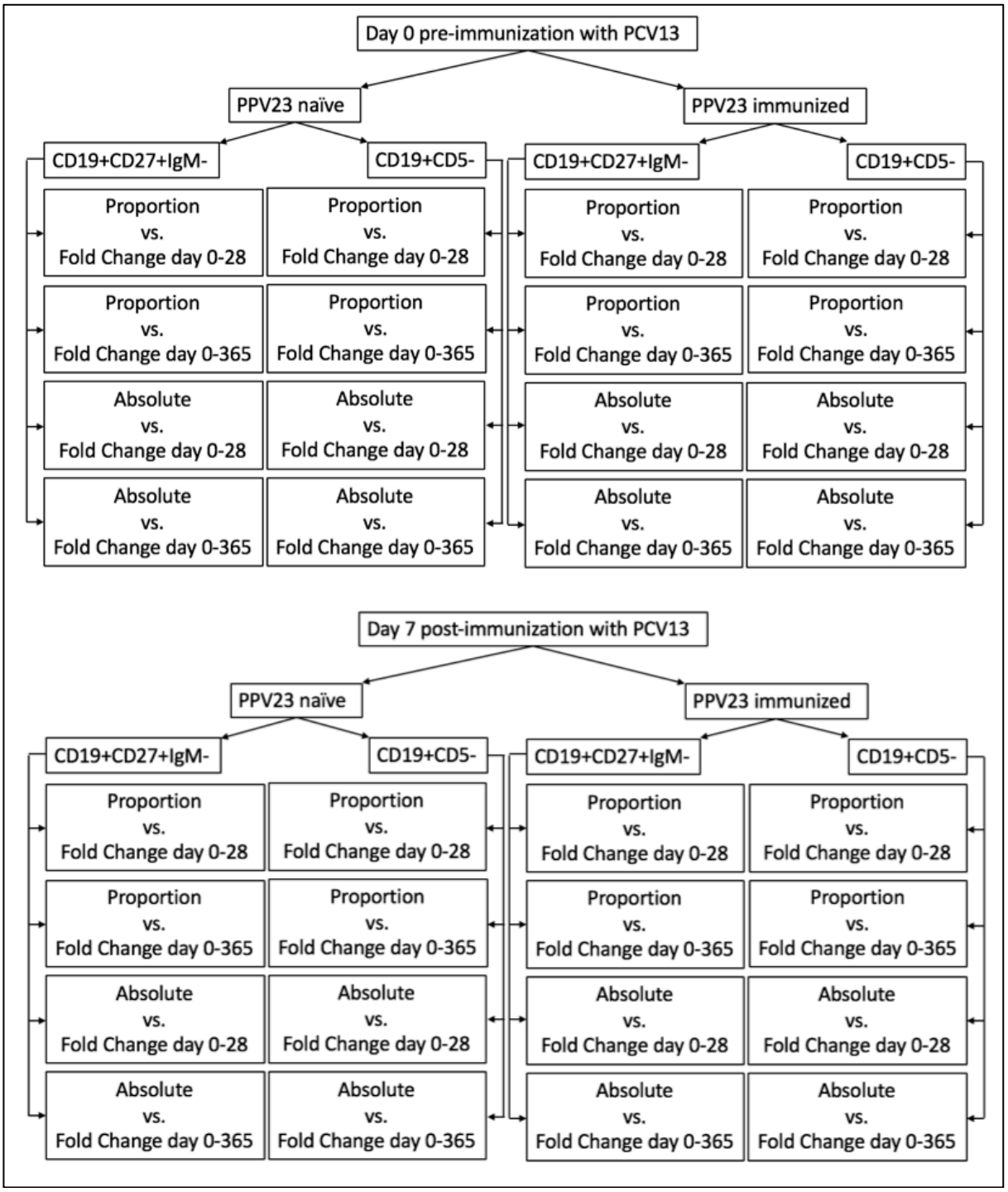


Figure 1a. A graphic representation of the correlation analysis performed between B-cell subpopulations and the fold change in IgG concentrations.

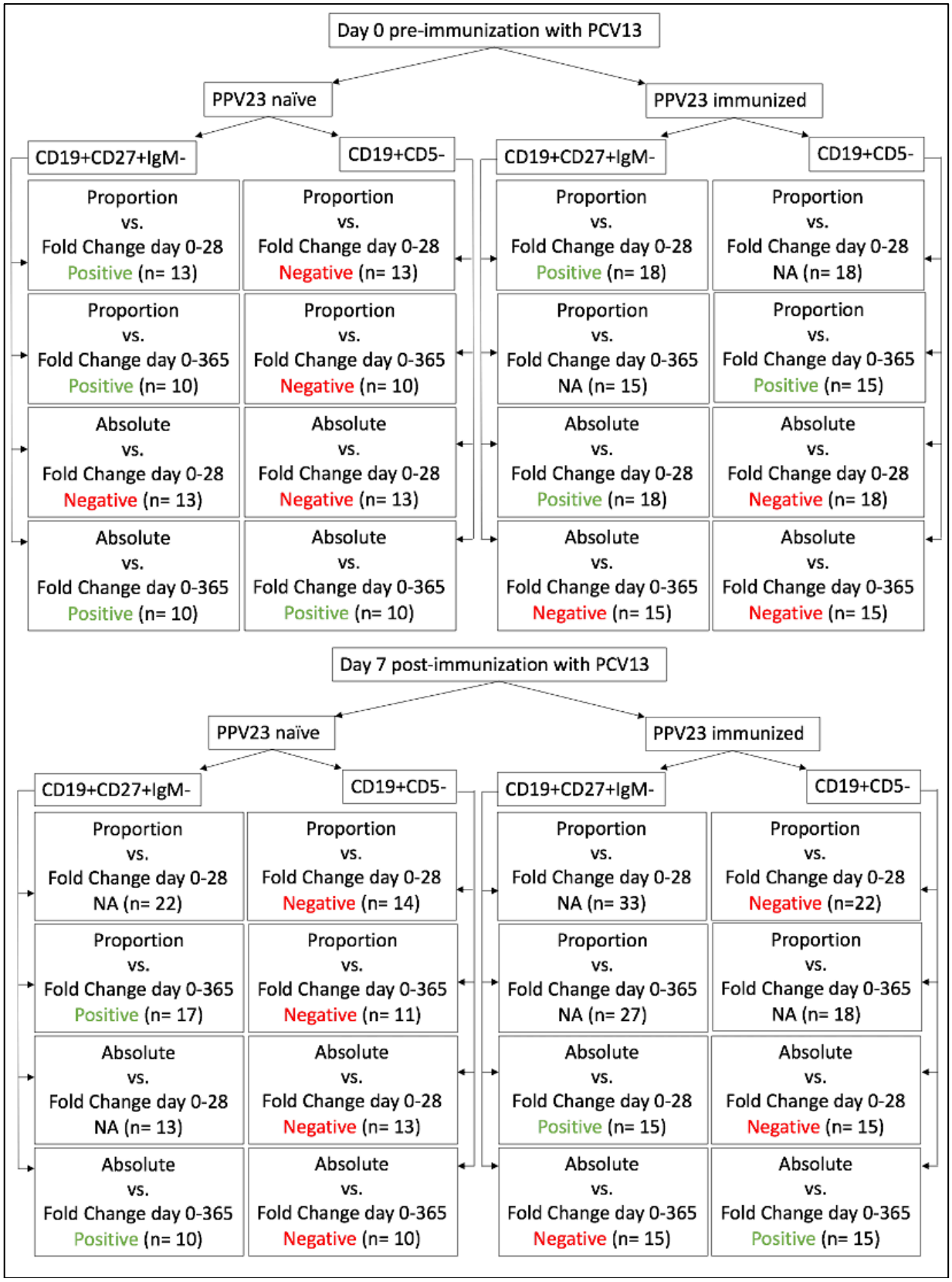
Day 0 pre-immunization with PCV13
PPV23 naïve
PPV23 immunized
CD19+CD27+IgM-
CD19+CD5-
CD19+CD27+IgM-
CD19+CD5-
Proportion vs. Fold Change day 0-28 Positive (n= 13)
Proportion vs. Fold Change day 0-28 Negative (n= 13)
Proportion vs. Fold Change day 0-28 Positive (n= 18)
Proportion vs. Fold Change day 0-28 NA (n= 18)
Proportion vs. Fold Change day 0-365 Positive (n= 10)
Proportion vs. Fold Change day 0-365 Negative (n= 10)
Proportion vs. Fold Change day 0-365 NA (n= 15)
Proportion vs. Fold Change day 0-365 Positive (n= 15)
Absolute vs. Fold Change day 0-28 Negative (n= 13)
Absolute vs. Fold Change day 0-28 Negative (n= 13)
Absolute vs. Fold Change day 0-28 Positive (n= 18)
Absolute vs. Fold Change day 0-28 Negative (n= 18)
Absolute vs. Fold Change day 0-365 Positive (n= 10)
Absolute vs. Fold Change day 0-365 Positive (n= 10)
Absolute vs. Fold Change day 0-365 Negative (n= 15)
Absolute vs. Fold Change day 0-365 Negative (n= 15)
Day 7 post-immunization with PCV13
PPV23 naïve
PPV23 immunized
CD19+CD27+IgM-
CD19+CD5-
CD19+CD27+IgM-
CD19+CD5-
Proportion vs. Fold Change day 0-28 NA (n= 22)
Proportion vs. Fold Change day 0-28 Negative (n= 14)
Proportion vs. Fold Change day 0-28 NA (n= 33)
Proportion vs. Fold Change day 0-28 Negative (n=22)
Proportion vs. Fold Change day 0-365 Positive (n= 17)
Proportion vs. Fold Change day 0-365 Negative (n= 11)
Proportion vs. Fold Change day 0-365 NA (n= 27)
Proportion vs. Fold Change day 0-365 NA (n= 18)
Absolute vs. Fold Change day 0-28 NA (n= 13)
Absolute vs. Fold Change day 0-28 Negative (n= 13)
Absolute vs. Fold Change day 0-28 Positive (n= 15)
Absolute vs. Fold Change day 0-28 Negative (n= 15)
Absolute vs. Fold Change day 0-365 Positive (n= 10)
Absolute vs. Fold Change day 0-365 Negative (n= 10)
Absolute vs. Fold Change day 0-365 Negative (n= 15)
Absolute vs. Fold Change day 0-365 Positive (n= 15)

Figure 1b. General trends in the correlation analyses between B-cell subpopulations and the fold change in IgG concentrations. Correlations with $r \geq 0.4$ for a serotype were considered a positive correlation, $r \geq -0.4$ for a serotype were considered a negative correlation, if no correlation was found for any serotype NA is stated in the figure.

Table 1a. IgG antibody concentrations in CKD patients immunized with PCV13

| Serotype | Group | Day 0<br>GM (95% CI) | Day 28<br>GM (95% CI) | Day 365<br>GM (95% CI) |
|---|---|---|---|---|
| 3 | PPV23 naive | 0.64 (0.40-1.03) | 0.89 (0.60- 1.31) | 0.37 (0.25- 0.54) // # |
| | PPV23 immunized | 0.97 (0.61- 1.54) | 1.04 (0.68- 1.59) | 0.52 (0.41- 0.68) ++ /// ### |
| 6B | PPV23 naive | 0.92 (0.59- 1.44) | 2.13 (1.44- 3.16) + | 1.73 (0.98- 3.08) # |
| | PPV23 immunized | 1.07 (0.72- 1.61) | 2.34 (1.39- 3.92) + | 2.47 (1.43- 4.27) + # |
| 9V | PPV23 naive | 1.50 (1.17- 1.92) | 3.43 (2.49- 4.71) +++ | 1.55 (1.09- 2.22) // ### |
| | PPV23 immunized | 1.64 (1.20- 2.24) | 2.70 (2.03- 3.59) + | 1.71 (1.21- 2.42) / # |
| 14 | PPV23 naive | 2.81 (1.87- 4.21) | 8.03 (4.49- 14.36) ++ | 5.29 (3.00 -9.31) # |
| | PPV23 immunized | 5.05 (3.15- 8.12) * | 8.25 (5.15- 13.22) | 7.88 (5.36- 11.58) |
| 19A | PPV23 naive | 4.60 (2.86- 7.40) | 6.77 (4.59-9.98) | 5.98 (3.83- 9.33) |
| | PPV23 immunized | 8.45 (5.82- 12.27) * | 12.13 (8.55- 17.20) ** | 8.79 (6.45- 11.99) / |
| 19F | PPV23 naive | 1.37 (0.92- 2.04) | 4.47 (2.62- 7.62) +++ | 3.05 (1.97- 4.73) + ## |
| | PPV23 immunized | 2.44 (1.57- 3.80) | 5.30 (3.43- 8.18) ++ | 4.27 (2.85- 6.41) # |
| 23F | PPV23 naive | 0.66 (0.38- 1.15) | 1.20 (0.68- 2.47) | 0.93 (0.54- 1.60) |
| | PPV23 immunized | 1.05 (0.73- 1.50) | 2.34 (1.34- 4.09) + | 1.83 (1.17- 2.86) * + # |

Geometric mean (GM), 95% confidence intervals (CI). * compares groups for each serotype at each collection, + compares day 0 to day 28 and day 365 for each group and serotype, / compares day 28 to day 365, # compares days 0, 28, 365 for each serotype for each group. Day 0 and 28; PPV23 naïve n= 22, PPV23 immunized n= 34, day 365; PPV23 naïve n=17, PPV23 immunized n=29.

Table 1b. Fold change of IgG antibody concentrations in CKD patients immunized with PCV13

| Serotype | Group | Fold Change 0-28 | Fold Change 0-365 |
|---|---|---|---|
| 3 | PPV23 naive | 1.31 (1.06- 1.61) | 0.70 (0.41- 1.18) ^ |
| | PPV23 immunized | 1.03 (0.94- 1.13) | 0.33 (0.27- 0.42) * ^^^^ |
| 6B | PPV23 naive | 2.77 (1.78- 4.32) | 1.37 (0.86- 2.16) |
| | PPV23 immunized | 2.23 (1.67- 2.97) | 1.20 (0.88- 1.62) ^^ |
| 9V | PPV23 naive | 1.76 (1.26- 2.46) | 0.87 (0.60- 1.25) ^^ |
| | PPV23 immunized | 1.36 (1.20- 1.55) | 0.78 (0.61- 0.99) ^^^^ |
| 14 | PPV23 naive | 1.91 (1.31- 2.77) | 1.23 (0.71- 2.15) |
| | PPV23 immunized | 1.32 (1.10- 1.59) * | 1.07 (0.78- 1.47) |
| 19A | PPV23 naive | 1.73 (1.33- 2.24) | 0.97 (0.65- 1.46) ^ |
| | PPV23 immunized | 1.39 (1.19- 1.63) | 0.89 (0.69- 1.16) ^^ |
| 19F | PPV23 naive | 2.49 (1.65- 3.74) | 1.74 (0.94- 3.23) |
| | PPV23 immunized | 1.90 (1.30- 2.60) | 1.06 (0.83- 1.34) |
| 23F | PPV23 naive | 1.57 (1.16- 2.14) | 1.00 (0.65- 1.53) |
| | PPV23 immunized | 1.72 (1.32- 2.23) | 1.09 (0.78- 1.51) ^ |

Geometric mean (GM), 95% confidence intervals (CI). * compares groups for each serotype for each fold change period. ^ compares day 0-28-fold change to day 0-365-fold change for each group. Fold change between day 0 and 28; PPV23 naïve n=22, PPV23 immunized n=34, between day 0 and 365; PPV23 naïve n= 17, PPV23 immunized n= 28.

Table 2a. IgM antibody concentrations in CKD patients immunized with PCV13

| Serotype | Group | Day 0<br>GM (95% CI) | Day 28<br>GM (95% CI) | Day 365<br>GM (95% CI) |
|---|---|---|---|---|
| 3 | PPV23 naive | 0.19 (0.15- 0.25) | 0.31 (0.24- 0.41) + | 0.23 (0.13- 0.38) |
| | PPV23 immunized | 0.25 (0.18- 0.33) | 0.28 (0.21- 0.37) | 0.25 (0.16- 0.36) |
| 6B | PPV23 naive | 0.64 (0.45- 0.89) | 1.30 (0.86- 1.97) + | 0.72 (0.45- 1.15) # |
| | PPV23 immunized | 0.89 (0.62- 1.26) | 1.06 (0.73- 1.54) | 0.80 (0.57- 1.11) |
| 9V | PPV23 naive | 0.44 (0.30- 0.64) | 0.66 (0.43- 1.02) + | 0.39 (0.24- 0.64) |
| | PPV23 immunized | 0.52 (0.36- 0.75) | 0.62 (0.44- 0.88) | 0.61 (0.43- 0.86) |
| 14 | PPV23 naive | 0.61 (0.43- 0.89) | 0.62 (0.42- 0.93) | 0.46 (0.28- 0.75) |
| | PPV23 immunized | 0.51 (0.38- 0.70) | 0.51 (0.35- 0.73) | 0.55 (0.38- 0.79) |
| 19A | PPV23 naive | 0.82 (0.56-1.19) | 1.26 (0.75- 2.09) | 1.10 (0.67- 1.79) |
| | PPV23 immunized | 1.05 (0.75- 1.45) | 1.25 (0.89- 1.77) | 1.31 (0.93- 1.84) |
| 19F | PPV23 naive | 0.96 (0.66- 1.39) | 1.72 (1.14- 2.61) | 1.14 (0.72- 1.81) |
| | PPV23 immunized | 1.23 (0.87- 1.73) | 1.42 (0.98- 2.06) | 1.41 (1.04- 1.91) |
| 23F | PPV23 naive | 0.19 (0.15- 0.24) | 0.24 (0.18- 0.33) | 0.18 (0.10- 0.31) |
| | PPV23 immunized | 0.28 (0.21- 0.37) | 0.24 (0.16- 0.36) | 0.21 (0.15- 0.29) |

Geometric mean (GM), 95% confidence intervals (CI). * compares groups for each serotype at each collection, + compares day 0 to day 28 and day 365 for each group and serotype, / compares day 28 to day 365, # compares days 0, 28, 365 for each serotype for each group. Day 0 and 28; PPV23 naïve n= 22, PPV23 immunized n= 34, day 365; PPV23 naïve n=17, PPV23 immunized n=29.

Table 2b. Fold change of IgM antibody concentrations in CKD patients immunized with PCV13

| Serotype | Group | Fold Change 0-28 | Fold Change 0-365 |
|---|---|---|---|
| 3 | PPV23 naive | 1.22 (1.01- 1.47) | 0.94 (0.68- 1.30) |
| | PPV23 immunized | 1.01 (0.95- 1.08) | 0.73 (0.53- 1.01) |
| 6B | PPV23 naive | 1.30 (1.05- 1.60) | 0.85 (0.64- 1.13) ^ |
| | PPV23 immunized | 1.05 (0.98- 1.12) | 0.85 (0.65- 1.12) |
| 9V | PPV23 naive | 1.60 (1.05- 2.45) | 0.91 (0.60- 1.38) ^ |
| | PPV23 immunized | 1.04 (0.96- 1.12) | 0.86 (0.64- 1.16) |
| 14 | PPV23 naive | 0.95 (0.83- 1.08) | 0.59 (0.40- 0.86) ^ |
| | PPV23 immunized | 0.98 (0.92-1.04) | 0.63 (0.46- 0.85) ^ |
| 19A | PPV23 naive | 1.21 (0.88- 1.66) | 0.98 (0.75- 1.32) |
| | PPV23 immunized | 1.07 (1.00- 1.14) | 0.97 (0.70- 1.27) |
| 19F | PPV23 naive | 1.18 (1.00- 1.39) | 0.98 (073- 1.31) |
| | PPV23 immunized | 1.08 (0.99- 1.19) | 0.97 (0.70- 1.33) |
| 23F | PPV23 naive | 1.17 (0.94- 1.45) | 0.87 (0.53- 1.43) |
| | PPV23 immunized | 0.96 (0.89- 1.05) * | 0.85 (0.60- 1.21) |

Geometric mean (GM), 95% confidence intervals (CI). * compares groups for each serotype for each fold change period. ^ compares day 0-28-fold change to day 0-365-fold change for each group. Fold change between day 0 and 28; PPV23 naïve n=22, PPV23 immunized n=34, between day 0 and 365; PPV23 naïve n= 17, PPV23 immunized n= 28.

Table 3a. IgA antibody concentrations in CKD patients immunized with PCV13

| Serotype | Group | Day 0<br>GM (95% CI) | Day 28<br>GM (95% CI) | Day 365<br>GM (95% CI) |
|---|---|---|---|---|
| 3 | PPV23 naive | 0.32 (0.18- 0.57) | 0.56 (0.33- 0.94) | 0.28 (0.18- 0.45) / |
| | PPV23 immunized | 0.42 (0.30- 0.60) | 0.65 (0.47- 0.89) | 0.31 (0.22- 0.44) // # |
| 6B | PPV23 naive | 0.13 (0.07- 0.24) | 0.52 (0.25- 1.08) ++ | 0.16 (0.09- 0.29) / ## |
| | PPV23 immunized | 0.15 (0.10- 0.23) | 0.46 (0.28- 0.75) ++ | 0.20 (0.13- 0.30) / ## |
| 9V | PPV23 naive | 0.30 (0.20- 0.45) | 0.63 (0.38- 1.06) ++ | 0.27 (0.16- 0.47) // ## |
| | PPV23 immunized | 0.23 (0.18- 0.31) | 0.48 (0.35- 0.66) +++ | 0.32 (0.22- 0.46) ## |
| 14 | PPV23 naive | 0.52 (0.31- 0.86) | 0.88 (0.51- 1.52) | 0.29 (0.17- 0.49) + /// ## |
| | PPV23 immunized | 0.37 (0.24- 0.57) | 0.62 (-.43- 0.90) | 0.37 (0.25- 0.56) |
| 19A | PPV23 naive | 0.99 (0.52- 1.87) | 1.69 (0.87- 3.27) | 1.04 (0.54- 2.01) |
| | PPV23 immunized | 0.83 (0.55- 1.26) | 1.48 (0.99- 2.21) + | 1.08 (0.71- 1.63) |
| 19F | PPV23 naive | 0.20 (0.10- 0.40) | 0.50 (0.25- 1.00) + | 0.18 (0.09- 0.34) / # |
| | PPV23 immunized | 0.17 (0.10- 0.26) | 0.52 (0.31- 0.87) +++ | 0.24 (0.14- 0.41) / ## |
| 23F | PPV23 naive | 0.15 (0.09- 0.26) | 0.32 (0.19- 0.53) + | 0.13 (0.08- 0.22) // # |
| | PPV23 immunized | 0.08 (0.06- 0.12) * | 0.19 (0.13- 0.27) * +++ | 0.12 (0.08- 0.18) ## |

Geometric mean (GM), 95% confidence intervals (CI). * compares groups for each serotype at each collection, + compares day 0 to day 28 and day 365 for each group and serotype, / compares day 28 to day 365, # compares days 0, 28, 365 for each serotype for each group. Day 0 and 28; PPV23 naïve n= 22, PPV23 immunized n= 34, day 365; PPV23 naïve n=17, PPV23 immunized n=29.

Table 3b. Fold change of IgA antibody concentrations in CKD patients immunized with PCV13.

| Serotype | Group | Fold Change 0-28 | Fold Change 0-365 |
|---|---|---|---|
| 3 | PPV23 naive | 1.45 (1.20- 1.77) | 0.73 (0.45- 1.18) ^ |
| | PPV23 immunized | 1.44 (1.23- 1.69) | 0.79 (0.56- 1.12) ^^ |
| 6B | PPV23 naive | 1.48 (0.98- 2.230 | 0.48 (0.23- 0.98) ^^^ |
| | PPV23 immunized | 1.98 (1.49- 2.64) | 0.83 (0.58- 1.20) ^^^ |
| 9V | PPV23 naive | 2.09 (1.51- 2.89) | 1.08 (0.72-1.62) ^ |
| | PPV23 immunized | 1.91 (1.48- 2.45) | 1.29 (1.01- 1.64) ^ |
| 14 | PPV23 naive | 1.30 (0.86- 1.96) | 0.47 (0.28- 0.78) ^^^^ |
| | PPV23 immunized | 1.29 (1.13- 1.47) | 0.88 (0.71- 1.09) * ^^^ |
| 19A | PPV23 naive | 1.83 (1.37- 2.44) | 0.83 (0.46- 1.50) ^^ |
| | PPV23 immunized | 1.65 (1.38- 1.98) | 1.0 (0.65- 1.52) ^ |
| 19F | PPV23 naive | 2.52 (1.76- 3.60) | 0.80 (0.43- 1.47) ^^^ |
| | PPV23 immunized | 2.56 (1.91- 3.43) | 1.38 (1.01- 1.88) ^^ |
| 23F | PPV23 naive | 1.69 (1.25- 2.30) | 0.59 (0.36- 0.95) ^^^ |
| | PPV23 immunized | 1.92 (1.49- 2.49) | 0.96 (0.67- 1.39) ^^ |

Geometric mean (GM), 95% confidence intervals (CI). * compares groups for each serotype for each fold change period. ^ compares day 0-28-fold change to day 0-365-fold change for each group. Fold change between day 0 and 28; PPV23 naïve n=22, PPV23 immunized n=34, between day 0 and 365; PPV23 naïve n= 17, PPV23 immunized n= 28.

Table 4a. Correlation between proportions of B cells vs. fold change in IgG antibodies.

| PPV23 naïve participants | | | | | |
|---|---|---|---|---|---|
| Fold Change | | Cells (day 0) (r, p value) | | Cells (day 7) (r, p value) | |
| | | CD27+IgM- | CD5- | CD27+IgM- | CD5- |
| | | n= 13 | n= 13 | n= 22 | n= 14 |
| 0-28 | 3 | -0.21, p > 0.05 | **-0.64, p < 0.05** | -0.15, p > 0.05 | **-0.76, p < 0.01** |
| | 6B | -0.13, p > 0.05 | -0.50, p > 0.05 | 0.25, p > 0.05 | -0.44, p > 0.05 |
| | 9V | 0.055, p > 0.05 | -0.43, p > 0.05 | -0.14, p > 0.05 | -0.49, p > 0.05 |
| | 14 | 0.036, p > 0.05 | -0.035, p > 0.05 | 0.25, p > 0.05 | 0.14, p > 0.05 |
| | 19A | 19A: 0.042, p > 0.05 | -0.055, p > 0.05 | 0.19, p > 0.05 | -0.26, p > 0.05 |
| | 19F | 19F: 0.48, p > 0.05 | -0.12, p > 0.05 | 0.28, p > 0.05 | -0.33, p > 0.05 |
| | 23F | 23F: -0.37, p > 0.05 | -0.45, p > 0.05 | 0.0010, p > 0.05 | -0.52, p > 0.05 |
| | | n= 10 | n= 10 | n= 17 | n= 11 |
| 0-365 | 3 | -0.017, p > 0.05 | -0.43, p > 0.05 | -0.24, p > 0.05 | -0.41, p > 0.05 |
| | 6B | -0.024, p > 0.05 | -0.28, p > 0.05 | -0.073, p > 0.05 | -0.37, p > 0.05 |
| | 9V | 0.45, p > 0.05 | -0.33, p > 0.05 | 0.032, p > 0.05 | **-0.65, p < 0.05** |
| | 14 | -0.15, p > 0.05 | -0.47, p > 0.05 | **0.60, p < 0.05** | 0.045, p > 0.05 |
| | 19A | 0.57, p > 0.05 | -0.37, p > 0.05 | 0.15, p > 0.05 | -0.44, p > 0.05 |
| | 19F | 0.38, p > 0.05 | -0.63, p > 0.05 | 0.19, p > 0.05 | -0.55, p > 0.05 |
| | 23F | -0.017, p > 0.05 | **-0.65, p < 0.05** | 0.16, p > 0.05 | -0.45, p > 0.05 |
| PPV23 immunized participants | | | | | |
| Fold Change | | Cells (day 0) (r, p value) | | Cells (day 7) (r, p value) | |
| | | CD27+IgM- | CD5- | CD27+IgM- | CD5- |
| | | n= 18 | n= 18 | n= 33 | n= 22 |
| 0-28 | 3 | 0.15, p > 0.05 | -0.20, p > 0.05 | -0.064, p > 0.05 | **-0.55, p < 0.05** |
| | 6B | 0.18, p > 0.05 | 0.38, p > 0.05 | 0.15, p > 0.05 | 0.37, p > 0.05 |
| | 9V | **0.59, p < 0.05** | -0.024, p > 0.05 | 0.34, p > 0.05 | 0.26, p > 0.05 |
| | 14 | 0.32, p > 0.05 | -0.21, p > 0.05 | 0.24, p > 0.05 | 0.29, p > 0.05 |
| | 19A | 0.04, p > 0.05 | 0.068, p > 0.05 | 0.10, p > 0.05 | 0.36, p > 0.05 |
| | 19F | 0.021, p > 0.05 | 0.28, p > 0.05 | -0.0031, p > 0.05 | 0.15, p > 0.05 |
| | 23F | 0.18, p > 0.05 | -0.20, p > 0.05 | 0.24, p > 0.05 | 0.057, p > 0.05 |
| | | n= 15 | n= 15 | n= 27 | n= 18 |
| 0-365 | 3 | -0.12, p > 0.05 | 0.21, p > 0.05 | -0.025, p > 0.05 | -0.0018, p > 0.05 |
| | 6B | -0.0091, p > 0.05 | -0.078, p > 0.05 | 0.40, p > 0.05 | -0.21, p > 0.05 |
| | 9V | -0.029, p > 0.05 | 0.35, p > 0.05 | 0.079, p > 0.05 | 0.11, p > 0.05 |
| | 14 | -0.25, p > 0.05 | 0.56, p > 0.05 | 0.16, p > 0.05 | 0.21, p > 0.05 |
| | 19A | -0.015, p > 0.05 | -0.31, p > 0.05 | -0.17, p > 0.05 | -0.21, p > 0.05 |
| | 19F | 0.082, p > 0.05 | 0.62, p > 0.05 | -0.054, p > 0.05 | -0.072, p > 0.05 |
| | 23F | 0.055, p > 0.05 | **0.68, p < 0.05** | -0.085, p > 0.05 | 0.35, p > 0.05 |

Table 4b. Correlation between absolute numbers of B cells vs. fold change in IgG antibodies.

| PPV23 naïve participants | | | | | |
|---|---|---|---|---|---|
| Fold Change | | Cells (day 0) | | Cells (day 7) | |
| | | CD27+IgM- | CD5- | CD27+IgM- | CD5- |
| | | n= 13 | n= 13 | n= 13 | n= 13 |
| 0-28 | 3 | -0.34, p > 0.05 | -0.57, p > 0.05 | -0.39, p > 0.05 | **-0.68, p < 0.05** |
| | 6B | -0.53, p > 0.05 | -0.56, p > 0.05 | -0.30, p > 0.05 | -0.35, p > 0.05 |
| | 9V | -0.24, p > 0.05 | -0.61, p > 0.05 | -0.045, p > 0.05 | -0.0061, p > 0.05 |
| | 14 | 0.035, p > 0.05 | 0.0091, p > 0.05 | -0.25, p > 0.05 | -0.23, p > 0.05 |
| | 19A | 0.073, p > 0.05 | 0.079, p > 0.05 | -0.18, p > 0.05 | -0.39, p > 0.05 |
| | 19F | 0.18, p > 0.05 | -0.28, p > 0.05 | -0.079, p > 0.05 | -0.26, p > 0.05 |
| | 23F | -0.49, p > 0.05 | -0.030, p > 0.05 | -0.28, p > 0.05 | **-0.65, p < 0.05** |
| | | n= 10 | n= 10 | n= 10 | n= 10 |
| 0-365 | 3 | 0.26, p > 0.05 | 0.26, p > 0.05 | 0.33, p > 0.05 | -0.68, p > 0.05 |
| | 6B | 0.23, p > 0.05 | 0.23, p > 0.05 | 0.15, p > 0.05 | -0.67, p > 0.05 |
| | 9V | 0.20, p > 0.05 | 0.20, p > 0.05 | 0.10, p > 0.05 | -0.43, p > 0.05 |
| | 14 | 0.36, p > 0.05 | 0.36, p > 0.05 | 0.54, p > 0.05 | -0.25, p > 0.05 |
| | 19A | 0.38, p > 0.05 | 0.38, p > 0.05 | 0.27, p > 0.05 | -0.69, p > 0.05 |
| | 19F | 0.38, p > 0.05 | 0.38, p > 0.05 | 0.18, p > 0.05 | **-0.82, p < 0.05** |
| | 23F | 0.30, p > 0.05 | 0.30, p > 0.05 | 0.53, p > 0.05 | -0.47, p > 0.05 |
| PPV23 immunized participants | | | | | |
| Fold Change | | Cells (day 0) | | Cells (day 7) | |
| | | CD27+IgM- | CD5- | CD27+IgM- | CD5- |
| | | n= 18 | n= 18 | n= 18 | n= 18 |
| 0-28 | 3 | 0.37, p > 0.05 | -0.97, p > 0.05 | **0.62, p < 0.01** | -0.0049, p > 0.05 |
| | 6B | 0.49, p > 0.05 | 0.82, p > 0.05 | **0.62, p < 0.05** | 0.34, p > 0.05 |
| | 9V | 0.088, p > 0.05 | **-0.56, p < 0.05** | 0.22, p > 0.05 | -0.29, p > 0.05 |
| | 14 | -0.039, p > 0.05 | **-0.84, p < 0.0001** | -0.018, p > 0.05 | -0.47, p > 0.05 |
| | 19A | -0.16, p > 0.05 | -0.46, p > 0.05 | 0.12, p > 0.05 | 0.12, p > 0.05 |
| | 19F | 0.29, p > 0.05 | -0.094, p > 0.05 | **0.58, p < 0.05** | 0.30, p > 0.05 |
| | 23F | 0.23, p > 0.05 | -0.32, p > 0.05 | 0.46, p > 0.05 | 0.093, p > 0.05 |
| | | n= 15 | n= 15 | n= 15 | n= 15 |
| 0-365 | 3 | 0.34, p > 0.05 | 0.16, p > 0.05 | -0.0022, p > 0.05 | -0.14, p > 0.05 |
| | 6B | 0.045, p > 0.05 | -0.40, p > 0.05 | 0.084, p > 0.05 | 0.13, p > 0.05 |
| | 9V | -0.033, p > 0.05 | 0.23, p > 0.05 | -0.0071, p > 0.05 | -0.018, p > 0.05 |
| | 14 | -0.31, p > 0.05 | -0.15, p > 0.05 | -0.049, p > 0.05 | 0.011, p > 0.05 |
| | 19A | **-0.56, p < 0.05** | -0.27, p > 0.05 | -0.37, p > 0.05 | -0.018, p > 0.05 |
| | 19F | -0.30, p > 0.05 | -0.13, p > 0.05 | 0.049, p > 0.05 | 0.52, p > 0.05 |
| | 23F | 0.099, p > 0.05 | 0.20, p > 0.05 | -0.061, p > 0.05 | 0.29, p > 0.05 |

**Supplementary Material**

Supplementary table 1a. Number and proportion of participants that had a post-immunization IgG concentration of ≥ 1 μg/ mL and antibody fold change increase of ≥ two-fold.

| Serotype | Group | Day 28 | Day 365 |
|---|---|---|---|
| 3 | Naïve | 5/ 22 (22.7%) | 2/17 (11.8%) |
| | PPV23 | 5/34 (14.7%) | 2/28 (7.1%) |
| 6B | Naïve | 14/ 22 (63.6%) | 10/17 (58.8%) |
| | PPV23 | 19/34 (55.9%) | 10/28 (35.7%) |
| 9V | Naïve | 15/ 22 (68.2%) | 4/17 (23.5%) |
| | PPV23 | 16/34 (47.1%) | 5/28 (17.9%) |
| 14 | Naïve | 14/ 22 (63.6%) | 8/17 (47.1%) |
| | PPV23 | 12/34 (35.3%) | 9/28 (32.1%) |
| 19A | Naïve | 14/ 22 (63.6%) | 6/17 (35.3%) |
| | PPV23 | 14/34 (41.2%) | 7/28 (25.0%) |
| 19F | Naïve | 16/ 22 (72.7) | 9/17 (52.9%) |
| | PPV23 | 18/34 (52.9%) | 10/28 (35.7%) |
| 23F | Naïve | 11/ 22 (50.0%) | 3/17 (17.6%) |
| | PPV23 | 20/34 (58.8%) | 11/28 (39.3%) |

Supplementary table 1b. Number and proportion of participants that had a post-immunization IgM concentration of ≥ 1 μg/ mL and antibody fold change increase of ≥ two-fold.

| Serotype | Group | Day 28 | Day 365 |
|---|---|---|---|
| 3 | Naïve | 3/22 (13.6%) | 1/17 (5.9%) |
| | PPV23 | 3/34 (8.8%) | 3/28 (10.7%) |
| 6B | Naïve | 8/22 (36.4%) | 2/17 (11.8%) |
| | PPV23 | 3/34 (8.8%) | 5/28 (17.9%) |
| 9V | Naïve | 9/22 (40.9%) | 6/17 (35.3%) |
| | PPV23 | 2/34 (5.9%) | 5/28 (17.9%) |
| 14 | Naïve | 3/22 (13.6%) | 2/17 (11.8%) |
| | PPV23 | 1/34 (2.9%) | 4/28 (14.3%) |
| 19A | Naïve | 6/22 (27.3%) | 4/17 (23.5%) |
| | PPV23 | 4/34 (11.8%) | 11/28 (39.3%) |
| 19F | Naïve | 8/22 (36.4%) | 3/17 (17.6%) |
| | PPV23 | 6/34 (17.6%) | 8/28 (28.6%) |
| 23F | Naïve | 3/22 (13.6%) | 1/17 (5.9%) |
| | PPV23 | 3/34 (8.8%) | 4/28 (14.3%) |

Supplementary table 1c. Number and proportion of participants that had a post-immunization IgA concentration of ≥ 1 μg/ mL and antibody fold change increase of ≥ two-fold.

| Serotype | Group | Day 28 | Day 365 |
|---|---|---|---|
| 3 | Naïve | 6/22 (27.3%) | 1/17 (5.9%) |
| | PPV23 | 12/34 (35.3%) | 5/28 (17.9%) |
| 6B | Naïve | 7/22 (31.8%) | 3/17 (17.6%) |
| | PPV23 | 15/34 (38.2%) | 4/28 (14.3%) |
| 9V | Naïve | 13/22 (59.1%) | 4/17 (23.5%) |
| | PPV23 | 13/34 (38.2%) | 4/28 (14.3%) |
| 14 | Naïve | 13/22 (59.1%) | 2/17 (11.8%) |
| | PPV23 | 8/34 (23.5%) | 4/28 (14.3%) |
| 19A | Naïve | 13/22 (59.1%) | 5/17 (29.4%) |
| | PPV23 | 14/34 (41.2%) | 7/28 (25.0%) |
| 19F | Naïve | 9/22 (40.9%) | 2/17 (11.8%) |
| | PPV23 | 17/34 (50.0%) | 7/28 (25.0%) |
| 23F | Naïve | 7/22 (31.8%) | 0/17 (0%) |
| | PPV23 | 7/34 (20.6%) | 1/28 (3.6%) |

Supplementary table 2a. Comparison of Mitra *et al*. (2016) results to our study

| Comparison | Our PPV23 naïve CKD patients | Our PPV23 immunized CKD patients | Mitra *et al*. (2016) CKD patients |
|---|---|---|---|
| The lowest response day 28, 2 months Mitra *et a*l. (2016) | ST 3 (22.7%) | ST 3 (14.7%) | ST 3 (47%) |
| Highest response day 28, 2 months Mitra *et al*. (2016) | ST 19F (72.7%) | ST 23F (58.8%) | ST 6B (~ 94%) |
| A minimum of 50% of that responded to a ST | STs 6B, 9V, 14, 19A, 19F, and 23F | STs 6B,19F, and 23F | STs 6B,14,19A,19F, and 23F |
| Concentrations day 365 that were significantly lower than day 28, 2 months Mitra *et al*. (2016) | STs 3 and 19A | STs 3, 9V and 19A | STs 3, 6B, 9V, 19A, 19F and 23F |

IgG antibody concentration of at least 1 µg/ mL and a ≥ two-fold antibody concentration increase was defined as a response to PCV13

Supplementary table 2b. Comparison of Vandecasteele *et al.* (2018) results to our study.

| | Our Study | | Vandecasteele et al. (2018) | | |
|---|---|---|---|---|---|
| Comparison | PPV23 naïve CKD patients | PPV23 immunized CKD patients | PPV23 naïve CKD patients | PPV23 immunized CKD patients < 4 years | PPV23 immunized CKD patients > 4 years |
| Day 28 that were significantly higher than pre-immunization | STs 6B, 9V, 14, and 19F | STs 6B, 9V, 19F and 23F | STs 3, 6B, 9V, 14, 19A, 19F, 23F | STs 6B, 9V, 14, 19A, 19F, and 23F | STs 6B, 9V, 14, 19A, 19F, and 23F |
| Day 365 that were significantly higher than pre-immunization | ST 19F | STs 6B and 23F (ST 3 was significantly lower) | STs 6B, 9V, 14, 19A, 19F, 23F | STs 6B, 9V, 14, 19A, 19F, and 23F | STs 6B, 9V, 14, 19A and 19F |
| PPV23 naïve vs. PPV23 immunized pre-immunization | PPV23 immunized patients had significantly higher pre-immunization concentrations for STs 14 and 19A compared to PPV23 naïve patients. | | No significant differences | | |
| PPV23 naïve vs. PPV23 day 28 | PPV23 naïve had significantly lower ST 19A concentrations | | PPV23 naïve patients had significantly higher titres for STs 3, 6B, 9V, 14, 19A, 19F, and 23F | | |
| PPV23 naïve vs. PPV23 day 365 | PPV23 naïve had significantly lower ST 23F concentrations | | PPV23 naïve patients had significantly higher titres for STs 6B, 9V, 14, 19A, and 23F | | |

Supplementary table 3a. Antibody concentrations of adults in response to PCV13

| Study | Location | Time post-PCV13 immunization | IgG concentrations (μg/ mL) | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | 3 | 6B | 9V | 14 | 19A | 19F | 23F |
| Severe CKD pneumococcal vaccine naïve adults mean age = 59 years (this study) | Canada | Pre- immunization | 0.64 | 0.92 | 1.50 | 2.81 | 4.60 | 1.37 | 0.66 |
| | | 28 days post-immunization | 0.89 | 2.13 | 3.43 | 8.03 | 6.77 | 4.47 | 1.20 |
| | | 365 days post-immunization | 0.37 | 1.73 | 1.55 | 5.29 | 5.98 | 3.05 | 0.93 |
| Severe CKD adults immunized with PPV23 ≥ 1-year pre-PCV13 mean age = 60 years (this study) | Canada | Pre- immunization | 0.97 | 1.07 | 1.64 | 5.05 | 8.45 | 2.44 | 1.05 |
| | | 28 days post-immunization | 1.04 | 2.34 | 2.70 | 8.25 | 12.13 | 5.30 | 2.34 |
| | | 365 days post-immunization | 0.52 | 2.47 | 1.71 | 7.88 | 8.79 | 4.27 | 1.83 |
| Severe CKD adults received a pneumococcal vaccine > 5 years ago mean age = 63 years (1) | USA | Pre- immunization | 0.47 | 0.57 | 1.11 | 2.09 | 2.18 | 1.36 | 0.62 |
| | | 2-months post-immunization | 1.88 | 8.35 | 3.47 | 13.84 | 9.86 | 9.99 | 6.12 |
| | | 12-months post-immunization | 0.79 | 3.08 | 1.36 | 5.95 | 3.11 | 4.56 | 2.24 |
| Healthy pneumococcal vaccine naïve adults mean age = 30 (2) | USA | 1-month post-immunization | 2.21 | 24.47 | 5.13 | 12.89 | 17.25 | 14.43 | 17.45 |
| Healthy pneumococcal vaccine naïve adults mean age = 71 years (3) | South Africa | 1-month post-immunization | 2.5 | 14.6 | 11.9 | 18.7 | 27.9 | 12.0 | 10.8 |
| Healthy pneumococcal vaccine naïve adults (group 1a) mean age = 55 years (4) * | USA | 1-month post-immunization | 1.15 | 7.58 | 4.96 | 20.77 | 26.80 | 6.13 | 7.17 |
| Healthy pneumococcal vaccine naïve adults (group 2a) mean age = 55 years (4) * | USA | 1-month post-immunization | 1.46 | 10.09 | 6.97 | 14.05 | 18.84 | 7.13 | 8.54 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Healthy pneumococcal vaccine naïve adults (group 1b) mean age = 72 (5) * | Germany, Netherlands, Belgium and Hungary | 1-month post-immunization | 1.08 | 6.24 | 4.97 | 8.95 | 11.93 | 4.78 | 5.82 |
| Healthy pneumococcal vaccine naïve adults (group 2b) mean age = 72 (5) * | Germany, Netherlands, Belgium and Hungary | 1-month post-immunization | 1.15 | 6.43 | 6.21 | 12.44 | 17.10 | 7.39 | 6.11 |
| Pneumococcal vaccine naïve adults mean age = 73 years (6) ^ | Netherlands | Pre- immunization | 0.44 | 2.18 | 1.21 | 1.97 | 3.45 | 1.32 | 1.43 |
| | | 1-month post-immunization | 1.35 | 9.30 | 7.57 | 11.99 | 18.07 | 7.23 | 7.36 |
| | | 12-months post-immunization | 0.7 | 5.35 | 3.91 | 7.66 | 8.62 | 3.67 | 3.97 |
| Adults received PPV23 ≥ 1-year pre-PCV13 mean age = 73 years (7) / | USA | Pre- immunization | 0.19 | 0.67 | 1.04 | 4.1 | 3.29 | 1.45 | 0.68 |
| | | 1-month post-immunization | 0.46 | 3.7 | 2.72 | 6.45 | 9.41 | 4.35 | 0.6 |
| Pneumococcal vaccine naïve adults 18- 49 years group (8) + | USA | 1-month post-immunization | 1.77 | 16.78 | 6.36 | 20.05 | 18.93 | 10.92 | 14.10 |
| Pneumococcal vaccine naïve adults 60- 64 years (8) + | USA | 1-month post-immunization | 1.65 | 7.87 | 6.32 | 7.09 | 12.30 | 4.57 | 6.98 |
| Adults with asplenia received PPV23 ≥ 0.5 years pre-PCV13 median age= 61years (9) & | Sweden | Pre- immunization | 0.24 | 1.85 | 2.06 | 3.44 | 4.45 | 2.99 | 2.07 |
| | | 4- 6 weeks post-immunization | 0.47 | 1.33 | 2.11 | 3.99 | 7.02 | 3.79 | 6.06 |
| HIV-positive adults received 2 doses of PCV13 mean age = 50 (10) | Italy | Pre- immunization | 0.74 | 1.38 | 0.56 | 5.75 | 3.8 | 4.57 | 1.78 |
| | | 1-year post-immunization | 0.79 | 3.09 | 1.35 | 12.3 | 6.03 | 6.76 | 3.24 |
| HIV positive pneumococcal vaccine naïve adults mean age= 41 years (11) | South Africa + Romania | Pre- immunization | 0.48 | 3.13 | 1.56 | 2.45 | 4.78 | 1.33 | 1.61 |
| | | 1-month post-immunization | 0.77 | 7.29 | 5.16 | 16.54 | 13.03 | 4.88 | 5.30 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| HIV positive adults received at least 1 dose of PPV23 ≥ 6 months prior to PCV13 Mean age = 47 years (12) | USA | Pre- immunization | 0.2 | 1.17 | 0.92 | 2.40 | 2.69 | 0.72 | 0.81 |
| | | 1-month post-immunization | 0.49 | 3.06 | 2.63 | 6.29 | 6.53 | 2.71 | 2.70 |
| Chronic lymphocytic leukemia adults, received a pneumococcal vaccine > 5 years ago, median age = 70 years (13) | Sweden | Pre- immunization | 0.2 | 0.7 | 0.5 | 0.9 | 2.0 | 0.7 | 0.7 |
| | | 1-month post-immunization | 0.3 | 1.3 | 1.4 | 1.8 | 3.5 | 1.5 | 1.4 |
| | | 6-months post-immunization | 0.2 | 1.2 | 1.1 | 1.9 | 3.3 | 1.3 | 1.5 |

* group 1a- PCV13 administered with trivalent inactivated influenza vaccine (TIV) followed by placebo, group 2a- placebo with TIV followed by PCV13, group 1b- PCV13 with TIV followed by placebo, group 2b- placebo with TIV followed by PCV13

^ includes healthy adults, adults with heart disease, diabetes mellitus, lung disease and asthma

/ pre-existing conditions were not listed

+ adults that are stable but have chronic conditions e.g., cardiovascular, pulmonary, renal, or liver diseases including alcoholic liver disease and alcoholism, and diabetes mellitus

& received between 1-3 doses of PPV23 prior to PCV13 immunization, mean number of years before PCV13 immunization was 4.6 years

Supplementary table 3b. Studies that have reported responses to PCV13

| Study | Definition of response | Response post-immunization | Proportion of participants that responded (%) | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | 3 | 6B | 9V | 14 | 19A | 19F | 23F |
| Severe CKD pneumococcal vaccine naïve adults mean age = 59 years (this study) | post-immunization IgG concentration of at least 1 µg/ mL and a ≥ two-fold antibody concentration increase | Day 28 | 22.7 | 63.6 | 68.2 | 63.6 | 63.6 | 72.7 | 50.0 |
| | | Day 365 | 11.8 | 58.8 | 23.5 | 47.1 | 35.3 | 52.9 | 17.6 |
| Severe CKD adults immunized with PPV23 ≥ 1-year pre-PCV13 mean age = 60 years (this study) | post-immunization IgG concentration of at least 1 µg/ mL and a ≥ two-fold antibody concentration increase | Day 28 | 14.7 | 55.9 | 47.1 | 35.3 | 41.2 | 52.9 | 17.6 |
| | | Day 365 | 7.1 | 35.7 | 17.9 | 32.1 | 25.0 | 35.7 | 39.3 |
| Severe CKD adults (could have received a pneumococcal vaccine > 5 years ago) mean age = 63 years (1) | post-immunization IgG concentration of at least 1 µg/ mL and a ≥ two-fold antibody concentration increase | 2 months | ~ 47 | 94.1 | ~ 47 | ~ 82 | 58.8 | ~76 | ~71 |
| | | 1 year | ~ 23 | ~ 65 | ~ 29 | ~ 53 | 47 | ~59 | ~59 |
| Pneumococcal vaccine naïve adults mean age = 73 years (6) ^ | 4-fold increase in IgG concentrations post-immunizations | 1 month | 32.7 | 43.1 | 59.5 | 55.2 | 50.9 | 53.4 | 51.9 |
| Adults received PPV23 ≥ 1-year pre-PCV13 mean age = 73 years (7) / | 4-fold increase in IgG concentrations post-immunizations | 1 month | 17.1 | 46.2 | 23.9 | 9.4 | 35.0 | 33.3 | 45.3 |
| Adults with asplenia received PPV23 ≥ 0.5 years pre-PCV13 median age= 61years (9) & | IgG level ≥ 0.35 µg/ml | 4- 6 weeks post-immunization | ~ 55 | ~ 77 | ~ 90 | ~ 83 | 100 | ~ 91 | ~ 95 |
| | IgG level ≥ 1 µg/ml | | ~ 27 | ~ 75 | ~ 85 | ~ 77 | ~ 95 | ~ 90 | ~ 90 |
| | IgG level ≥ 5 µg/ml | | ~ 5 | ~ 23 | ~ 23 | ~ 55 | ~ 65 | ~ 50 | ~ 70 |
| HIV-positive adults received 2 doses of PCV13 mean age = 50 (10) | IgG level ≥ 0.35 µg/ml | 5 years post-immunization | 88 | 100 | 88 | 98 | 100 | 100 | 100 |
| | IgG level ≥ 1 µg/ml | | 44 | 88 | 33 | 98 | 95 | 98 | 90 |

* group 1a- PCV13 administered with trivalent inactivated influenza vaccine (TIV) followed by placebo, group 2a- placebo with TIV followed by PCV13, group 1b- PCV13 with TIV followed by placebo, group 2b- placebo with TIV followed by PCV13
^ includes healthy adults, adults with heart disease, diabetes mellitus, lung disease and asthma
/ pre-existing conditions were not listed
+ adults that are stable but have chronic conditions e.g., cardiovascular, pulmonary, renal, or liver diseases including alcoholic liver disease and alcoholism, and diabetes mellitus
& received between 1-3 doses of PPV23 prior to PCV13 immunization, mean number of years before PCV13 immunization was 4.6 years

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## Chapter 5: Conclusions

The goal of this dissertation was to address two questions, the first was: are there differences in naturally acquired humoral immunity against Hia and pneumococcus in healthy adults and adults with CKD, or First Nations (FN) and non-First Nations (non-FN) adults of the same health status? Due to the immune dysfunction in adults with CKD, it is hypothesized that humoral immunity against Hia and pneumococcus could be decreased in CKD patients compared to healthy adults. The increased incidence of invasive disease in the FN population of northwestern Ontario, also suggests that humoral immunity in FN adults could be decreased compared to non-FN adults. It was found that patients with CKD had a significantly higher proportion of B cells compared to healthy adults (this group consists of primarily non-FN with a couple FN adults) which is thought to be due to the chronic inflammation caused by uremia as well as comorbidities such as diabetes mellitus. Non-FN CKD had an increased proportion of IgM memory and double negative B cells. This was thought to be due to the higher mean age of the non-FN CKD patients because it has been previously found that amounts of these B cells increase with age. When Hia specific capsular polysaccharide IgG and IgM concentrations were compared between CKD groups, the FN patients had significantly higher concentrations of Hia capsular polysaccharide IgM which could be an indication of recent exposure to Hia, or part of the natural IgM antibody repertoire. Both healthy and CKD FN groups had higher naturally acquired pneumococcal 6B and 14 IgG concentrations compared to healthy and CKD non-FN groups. The higher concentrations in the FN groups is thought to be due to antibodies that are cross-reactive with other pathogens or environmental antigens (i.e. *Pseudomonas aeruginosa* and *Klebsiella pneumoniae*). It is also important to consider that issues associated with low socioeconomic status within the FN communities such as inadequate accesses to clean water

(decreased hand washing) and overcrowding in households increases carriage of pneumococcus and Hia. This contributes to the increased rates of invasive disease caused by pneumococcus and Hia in the FN population. The collective data suggest that the increased prevalence of invasive disease caused by pneumococcus and Hia within the FN population is not due to decreased numbers of B cells or concentrations of naturally acquired antibodies. The increased prevalence of diseases such as CKD causing immune dysfunction in a higher proportion of the FN population increases their risk for invasive disease.

The second question was: are adults with CKD able to respond to PCV13 immunization and does previous immunization with PPV23 affect their ability to respond to subsequent immunization with PCV13? It was hypothesized that although patients with CKD are immunocompromised, they have shown the ability to respond to the conjugate Hib vaccine, therefore it is expected that they will also be able to respond to PCV13. Other studies have shown that previous immunization with PPV23 has a negative effect on pneumococcal specific humoral immune response to subsequent immunization with PCV13. It is hypothesized that patients with CKD that have previously been immunized with PPV23 will also have decreased humoral immune responses to immunization with PCV13. Humoral immune responses to immunization with one dose of PCV13 was evaluated in two groups of adults with CKD, one group was pneumococcal vaccine naïve and the other received PPV23 ≥ 1 year ago. The results demonstrated that patients with CKD were able to respond to PCV13, however, a higher proportion of PPV23 naïve patients were able to meet the response criteria (a postimmunization IgG antibody concentration of at least 1 μg/ mL and a ≥ two-fold antibody concentration increase). The data suggest that immunization with PPV23 may result in long-term changes in B-cell subpopulations such as increased prevalence of CD5- B cells and decreased prevalence of

class switched memory B cells in the peripheral blood. The significant decrease in the total class switched memory B cells in PPV23 immunized CKD patients in response to subsequent immunization with PCV13, suggests that previous PPV23 immunization may reduce PCV13 immunogenicity. Previous studies have found an association between the CD5- B cells and production of IgG antibodies in response to PPV23. Therefore, we attempted to determine if there were any differences in CD5-expressing B cells between CKD groups. There was an increased prevalence of CD5- B cells and decreased prevalence of class switched memory B cells in PPV23 immunized patients. When correlation analyses were performed, there was a negative correlation of CD5- B cells and the fold change in IgG concentrations. A positive correlation of class switched memory B cells and the fold change in IgG concentrations was found. This suggests that previous immunization with PPV23 favored the production of CD5- B cells and is associated with decreased antibody responses to subsequent immunization with PCV13 in patients with CKD.

These findings emphasize the need for further studies to optimize a pneumococcal immunization schedule for adults with CKD. Taking into consideration that immunization with PPV23 is recommended for adults with CKD, immunization with additional doses of PCV13 when following PPV23 to elicit a stronger humoral immune response should also be investigated. Currently the National Advisory Committee on Immunization recommends for adults ≥ 65 years old receive PCV13 followed by PPV23, perhaps this schedule could also be assessed for adults with CKD. Analysis of antibody secreting cells for serotypes other than 6B or 14 should also be quantified since these serotypes have been found to be less immunogenic than other serotypes. Quantification of pneumococcal specific peripheral blood memory B cells using flow cytometry rather than enzyme-linked immunospot assay (ELISPOT) may also provide more

information on the effects of previous PPV23 immunization in response to PCV13. All of the studies emphasize the need for establishment of protective antibody titres for adults. This information could determine adults that need to receive penumococcal immunization before the age of 65 years old and provide a better means of interpretation of clinical data since many studies use different definitions of a response to immunization. Determination of protective antibody concentrations should also be determined for adults with CKD specifically as they may need higher concentrations to protect them from invasive disease caused by pneumococcus and Hia. Additional studies on carriage as well as risk factors that contribute to invasive diseases caused by pneumococcus and Hia may also provide valuable insight to better understand the increased burden of invasive disease in the FN population.