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Immune response to pneumococcal vaccination in chronic lymphocytic leukemia

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Abstract

Patients with chronic lymphocytic leukemia (CLL) are at increased risk of *Streptococcus pneumoniae* infections due to disease- and treatment-related immune dysfunction. Vaccine responses are often impaired. This thesis evaluates the immune response to primary immunization with pneumococcal conjugate vaccine (PCV) versus pneumococcal polysaccharide vaccine (PPSV), long-term antibody persistence and the effect of revaccination in CLL patients.

Study I was a randomized trial in treatment-naïve CLL patients comparing PCV and PPSV, demonstrating that PCV elicits an enhanced immune response.

Study II was a prospective study evaluating B-cell subsets and plasmablast dynamics before and after revaccination. It showed that repeated revaccinations with PCV in CLL patients improves early humoral response.

Study III assessed antibody persistence 5 years after primary immunization and response to revaccination, showing that CLL patients have poor long-term antibody persistence, but that revaccination with PCV enhances immunity.

Study IV examined the impact of two analytical methods, multiplex immunoassay (MIA) and enzyme immunoassay (EIA), on serotype-specific IgG measurements and demonstrated their influence on vaccine response interpretation in CLL patients.

The findings in this thesis emphasize the importance of pneumococcal conjugate vaccines in CLL patients and suggest a need for revaccination to maintain protection against severe pneumococcal disease.

Keywords: chronic lymphocytic leukemia, secondary immunodeficiency, vaccine response, pneumococcal polysaccharide vaccine, pneumococcal conjugate vaccine, pneumococcal revaccination

Inne i dig öppnar sig valv bakom valv oändligt.

Du blir aldrig färdig och det är som det skall.

Tomas Tranströmer

Ur ”Romanska bågar”

אני ואתה נשנה את העולם

אריק איינשטיין

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I. T. Svensson, M. Kättström, Y. Hammarlund, D. Roth, P-O. Andersson, M. Svensson, I. Nilsson, L. Rombo, H. Cherif, E. Kimby. **Pneumococcal conjugate vaccine triggers a better immune response than pneumococcal polysaccharide vaccine in patients with chronic lymphocytic leukemia. A randomized study by the Swedish CLL group.** *Vaccine. 2018 Jun 14;36(25):3701-3707. doi: 10.1016/j.vaccine.2018.05.012.*

II. M. Kättström, B. Uggla, E. Tina, E. Kimby, T. Norén, S. Athlin. **Improved plasmablast response after repeated pneumococcal revaccinations following primary immunization with 13-valent pneumococcal conjugate vaccine or 23-valent pneumococcal polysaccharide vaccine in patients with chronic lymphocytic leukemia.** *Vaccine. 2023 May 5;41(19):3128-3136. doi: 10.1016/j.vaccine.2023.04.016.*

III. M. Kättström, B. Uggla, C. Virta, M. Melin, N. Ekström, A. Magnuson, P-O. Andersson, Y. Hammarlund, S. Lockmer, I. Nilsson, D. Roth, M. Svensson, T. Tolf, E. Kimby, T. Norén, S. Athlin. **Revaccination with pneumococcal conjugate vaccine five years after primary immunization improves immunity in patients with chronic lymphocytic leukemia.** *Haematologica. 2025 Mar 6. doi: 10.3324/haematol.2024.286942. Epub ahead of print.*

IV. M. Kättström, C. Virta, M. Melin, N. Ekström, A. Magnuson, T. Tolf, E. Kimby, T. Norén, B. Uggla, S. Athlin. **Impact of multiplex serological testing versus enzyme immunoassay on pneumococcal vaccine response in patients with chronic lymphocytic leukemia.** *In manuscript.*

Abbreviations

APC	antigen presenting cell
BCL2	B-cell lymphoma 2
BCL2i	B-cell lymphoma 2 inhibitor
BCR	B-cell receptor
BTK	Bruton's tyrosine kinase
BTKi	Bruton's tyrosine kinase inhibitor
CLL	chronic lymphocytic leukemia
CLL-IPI Index	chronic lymphocytic leukemia International Prognostic
DAT	direct antiglobulin test
ELISA	enzyme-linked immunosorbent assay
EIA	enzyme immunoassay
FMIA	fluorescent multiplexed bead-based immunoassay
GMC	geometric mean concentration
GMT	geometric mean titer
GMR	geometric mean ratio
IG	immunoglobulin
IGHV	immunoglobulin heavy chain variable region
IPD	invasive pneumococcal disease
iwCLL	International Workshop on CLL
LLOQ	lower limit of quantification
MBL	monoclonal B-cell lymphocytosis
M-CLL	mutated CLL

MHC	major histocompatibility complex
MIA	multiplexed immunoassay
MOPA	multiplexed opsonophagocytic assay
NK	natural killer
OPA	opsonophagocytic assay
PBMC	peripheral blood mononuclear cells
PCV13	pneumococcal conjugate vaccine 13
PPSV23	pneumococcal polysaccharide vaccine 23
R/R	relapsed/refractory
SARS-CoV-2	severe acute respiratory syndrome coronavirus 2
SP	serological protection
SR	serological response
Tfh	T follicular helper
Th	T helper
Treg	T regulatory
U-CLL	unmutated CLL

Introduction

Chronic lymphocytic leukemia

CLL biology

Chronic lymphocytic leukemia (CLL) is a malignant B-lymphocyte disorder. With an incidence of 4–5 per 100,000, it is the most common leukemia in the Western world, typically diagnosed at a median age of 70 years and nearly twice as common in males (1). Most cases are asymptomatic and diagnosed as a result of lymphadenopathy or incidental finding of lymphocytosis. Due to defective apoptosis, driven by active B-cell receptors (BCRs) and complex interactions with the microenvironment that provide continuous stimulation and activation, malignant B cells accumulate in the bone marrow, lymph nodes, and spleen (2, 3). CLL B-cells are seen as increased lymphocyte counts in peripheral blood, which can be the only presentation at diagnosis. Morphological features associated with CLL are Gumprechts nuclear shadow (smudge cells) seen in peripheral blood smears (Figure 1). According to the 2018 International Workshop on CLL (iwCLL) (4), the diagnosis requires at least 5×10^9 monoclonal B-cells/L in peripheral blood, sustained for at least 3 months, with typical phenotype detected through flow cytometry. CLL cells express the B-cell marker CD19, usually with weaker expression of CD20. CD5, CD23, κ and λ are also needed to establish the diagnosis (5). Smaller clonal B-cell populations ($<5 \times 10^9$), without signs of lymphoproliferative disorders, are defined as monoclonal B-cell lymphocytosis (MBL). This can be categorized as either low-count or high-count MBL, based on whether the B-cell count is above or below 0.5×10^9/L, with greater risk of progression to CLL if the count is high (6, 7).

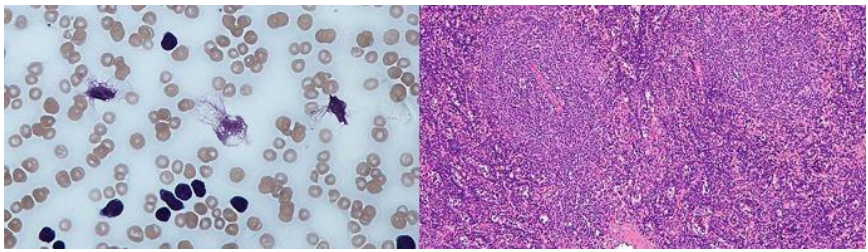

Figure 1. Morphological features of chronic lymphocytic leukemia (CLL). Left: Blood smear showing characteristic smudge cells. Right: Histological section of a lymph node involved by CLL, displaying proliferation centers (pseudofollicles). Images by Dr. Graham Beards and Nephron. Licensed under CC BY-SA 3.0. Source: Wikimedia Commons.

Many individuals are diagnosed at an early asymptomatic stage, and only approximately 15% of patients require treatment at the time of diagnosis. Around two thirds develop a need for treatment at some point during the disease course (4, 8). The heterogenous clinical course is mainly a result of different genetic alterations in the CLL cells, which have significance for the prognosis. Staging of a CLL patient traditionally involves evaluating disease burden through physical examination and laboratory data, in accordance with the classifications suggested by Rai (9) or Binet (10). Independent factors influencing prognosis and treatment response include genetic aberrations such as TP53 status, del(17p), del(11q), trisomy 12, and del(13q), as well as immunoglobulin heavy chain gene (IGHV) mutation status, clinical stage (Rai or Binet), lymphocyte doubling time, serum beta-2 microglobulin levels >3.5 mg/L, and age (11-16). The IGHV mutation status reflects the developmental origin of B-cells, with mutated IGHV (M-CLL) indicating a population derived from more mature B-cells. Unmutated IGHV (U-CLL) is characterized by the absence of somatic hypermutation in the IGHV region genes, likely originating from naïve or marginal zone-like B-cells (17).

IGHV mutation status gives prognostic information early in the course of the disease. M-CLL occurs in 50–60% of newly diagnosed patients and is associated with more favorable prognosis than U-CLL, which has faster progression and shorter remissions after

chemoimmunotherapy (18, 19). Other genetic aberrations, like mutations in the genes for NOTCH1 and SF3B1, stereotyped BCR subsets and complex karyotype, are also independent prognostic factors (20-22), but less commonly used in clinical practice.

CLL treatment

The iwCLL criteria for initiating treatment in CLL focus on identifying "active disease" (4). Treatment is recommended for patients with significant disease-related symptoms, i.e., progressive bone marrow failure, massive splenomegaly and lymphadenopathy, rapid increase of lymphocytosis, B symptoms, and refractory autoimmune complications. In the absence of these conditions, a "watch and wait" approach with regular monitoring is typically advised. One third of CLL patients never require treatment, and early-stage treatment with chemotherapy has not been shown to prolong survival (23). First-line treatment in CLL is selected based on various factors, including genetic abnormalities such as del(17p), del(11q) and TP53 mutations, as well as IGHV mutation status (U-CLL or M-CLL).

Chemoimmunotherapy has for many years been the backbone of CLL treatment, with fludarabine and cyclophosphamide used in younger, fit patients, and bendamustine preferred for elderly patients and those with comorbidities. These treatments are combined with CD20 antibodies. In recent years, so-called targeted therapies have increasingly been incorporated in CLL treatment (24). Targeted therapy, with or without CD20 antibodies, has now replaced chemoimmunotherapy for the majority of patients in Sweden (25). However, chemoimmunotherapy may still be a treatment option for patients with M-CLL who lack other high-risk features.

CD20 antibodies, such as rituximab and obinutuzumab, are essential in CLL treatment. They act by targeting B-cells for immune-mediated destruction. Rituximab, a first-generation CD20 antibody, is widely used with chemotherapy (for instance with fludarabine and cyclophosphamide or with bendamustine) and targeted therapies. Obinutuzumab, a second-generation, glycoengineered CD20 antibody, offers enhanced efficacy through stronger antibody-dependent cellular cytotoxicity and direct induction of apoptosis (26). It is

frequently combined with targeted therapies in the frontline setting, providing a fixed-duration treatment that often achieves deep remissions (27).

Bruton's tyrosine kinase inhibitors (**BTKis**) are targeted therapies for CLL and other B-cell malignancies. By inhibiting Bruton's tyrosine kinase (BTK), a key enzyme in B-cell receptor signaling, they disrupt malignant B-cell survival, proliferation and migration, inducing apoptosis. BTKis have become an established treatment option in both frontline and relapse treatment, particularly for high-risk genetic profiles, due to superior progression-free survival. In patients with del(17p) or TP53 mutations, continuous BTKi therapy is preferred for its deep and durable responses (28). However, off-target inhibition of other kinases may be a problem, especially with first-generation BTKi such as ibrutinib, as these off-target interactions are associated with adverse effects. Second-generation inhibitors such as acalabrutinib and zanubrutinib, which more selectively target BTK, have fewer off-target effects and consequently improved tolerability (29). New non-covalently binding BTKis will soon be used in clinical practice, changing the treatment landscape further.

B-cell lymphoma 2 inhibitors (**BCL2is**) are a class of targeted therapies, with venetoclax being widely used in clinical practice. These inhibitors target the B-cell lymphoma 2 (BCL-2) protein, which is overexpressed in CLL cells and plays a key role in preventing apoptosis. By inhibiting BCL-2, these drugs restore the apoptotic process, leading to the selective death of CLL cells (30). Venetoclax is often used in combination with anti-CD20 antibodies, providing a fixed-duration treatment approach (27). This time-limited therapy allows patients to achieve long-term remission without continuous treatment, reducing the risk of cumulative toxicity.

PI3K delta inhibitors are a class of targeted therapies that block the phosphoinositide 3-kinase delta (PI3Kδ) isoform, which plays a crucial role in BCR signaling. Although approved for clinical use, they are rarely given due to high risk of immune-related toxicities and increased susceptibility to infections.

Overview of the immune system

The immune defense against infection can be divided into innate immunity and adaptive immunity. Innate immunity provides an immediate, non-specific response to foreign pathogens or tissue damage. Adaptive immunity, in contrast, develops over time and generates immune memory, allowing for a more targeted and long-lasting response. Both immune systems rely on specialized cells and molecules to recognize and neutralize antigens when needed.

Innate immunity

The innate immune system, present from birth, provides an immediate, non-specific defense against infections through inflammation, phagocytosis and complement activation. It recognizes microbial structures, known as pathogen-associated molecular patterns (PAMPs), as well as danger signals from injured tissue (DAMPs) (31). Recognition occurs via pattern recognition receptors, including toll-like receptors, which activate signaling pathways leading to cytokine production (e.g., IL-1, TNF, IL-6), triggering immune responses and recruiting adaptive immune cells (32).

Key immune cells in innate immunity include neutrophils, monocytes, macrophages, dendritic cells, mast cells, and natural killer (NK) cells. Macrophages and neutrophils play a central role in phagocytosis, engulfing pathogens and killing them using toxic enzymes and oxygen radicals. Opsonization, in which pathogens are coated with antibodies immunoglobulin G (IgG) or complement factors (C3b), enhances phagocytosis and allows recognition of encapsulated bacteria, like pneumococci, which otherwise evade immune detection (33).

The complement system is a cascade of plasma proteins that supports phagocytosis, immune cell recruitment, and direct microbial lysis. It is activated through three pathways: the classical pathway, triggered by antibody-bound antigens; the lectin-binding pathway, initiated by mannose-binding lectin (MBL) binding bacterial carbohydrates; and the alternative pathway, which amplifies the response through C3b deposition on microbial surfaces (32, 33). A key function of complement is the formation of the membrane attack complex (MAC),

which lyses gram-negative bacteria and inactivates viruses. Additionally, complement fragments (C3a, C5a) enhance inflammation by attracting immune cells and increasing vascular permeability.

Natural IgM antibodies are produced without prior antigen exposure and play a role in innate-like humoral immunity. They are primarily secreted by B-1 cells (a subset of B lymphocytes), which are located in the peritoneal and pleural cavities, as well as in the spleen and bone marrow (34). Unlike adaptive IgM, which is produced after antigen exposure, natural IgM is poly-reactive, binding to conserved microbial structures. It plays a role in clearing dying cells, regulating inflammation, and activating the classical complement pathway.

Encapsulated bacteria such as *S. pneumoniae* evade immune detection by inhibiting phagocytosis. In these cases, opsonization with antibodies and complement factors is essential for effective immune clearance. Patients with antibody or complement deficiencies are at increased risk for severe pneumococcal infections, highlighting the importance of vaccination strategies targeting pneumococcal immunity.

Adaptive immunity

The adaptive immune system, unlike innate immunity, develops over time and provides specific and long-lasting protection through T and B lymphocytes. These cells possess unique antigen receptors that enable them to recognize and respond to pathogens with high specificity. A key feature of adaptive immunity is immune memory, where a portion of activated lymphocytes become memory cells, allowing for a faster and stronger response upon re-exposure to the same antigen.

T cells mature in the thymus and are activated when antigens are presented by major histocompatibility complex (MHC) molecules. CD4+ T helper (Th) cells coordinate immune responses by releasing cytokines, activating macrophages, B cells, and cytotoxic CD8+ T cells. CD8+ cytotoxic T cells kill virus-infected and malignant cells by inducing apoptosis through perforins and granzymes. T-cell subsets such as Th1, Th2, and Th17 specialize in different immune responses,

while regulatory T cells (Tregs) prevent excessive immune activation and autoimmunity (32).

B-cell activation occurs when the B-cell receptor (BCR) binds to its specific antigen. Membrane-bound immunoglobulins (BCRs) and soluble antibodies share the same basic Y-shaped structure, composed of two heavy chains and two light chains (Figure 2). The variable regions at the tips of the Y provide antigen specificity, while the constant region of the heavy chain determines the antibody class and its effector functions (35). BCRs possess a transmembrane domain that anchors them to the B-cell surface, whereas soluble antibodies are secreted and circulate freely in the bloodstream.

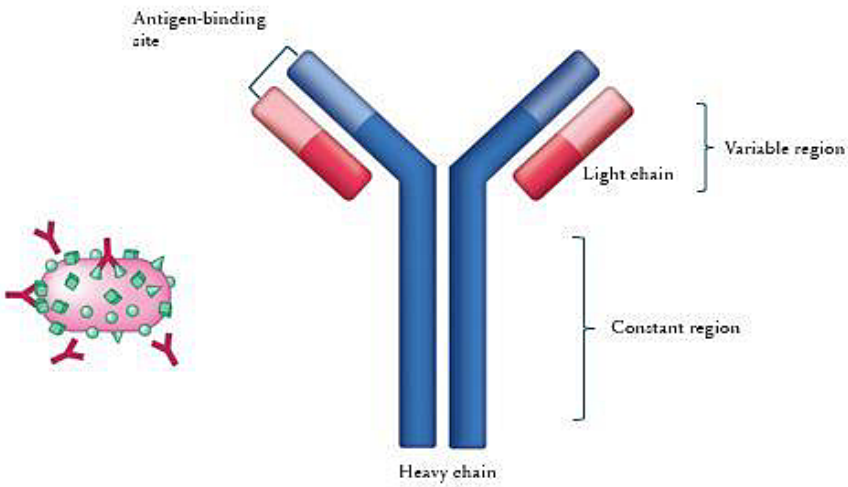

Figure 2. Basic structure of an immunoglobulin molecule, composed of two heavy chains and two light chains forming a Y-shaped configuration.

The BCR is associated with the signaling molecules Igα (CD79a) and Igβ (CD79b), which transduce activation signals upon antigen binding (32). Naïve B-cells primarily express IgM and IgD, but after class

switching, they can produce IgG (for opsonization and complement activation), IgA (for mucosal defense), or IgE (for allergic and antiparasitic responses). IgD regulates the BCR signaling and is involved in the transition from naïve to antigen-experienced B-cells (32). IgM is the first immunoglobulin produced in response to infection and is particularly important in defense against pneumococci as it enhances opsonization and complement activation (31).

In T-cell-dependent responses, an antigen presenting cell (APC), that has digested an antigen, is displaying an antigen fragment via MHC class II molecules which activates the T cell receptor (TCR). T cells secrete cytokines with further regulates the immune response. This interaction promotes B-cell proliferation, differentiation, and the germinal center reaction, a process for antibody maturation and memory formation (Figure 3)(36). The germinal center, located in secondary lymphoid organs (e.g., lymph nodes and spleen), consists of a dark zone where B-cells (centroblasts) undergo somatic hypermutation, introducing genetic changes to enhance antigen affinity, and a light zone, where B-cells (now centrocytes) interact with follicular dendritic cells. Only those with high-affinity BCRs survive, while lower-affinity clones undergo apoptosis. This ensures the selection of highly effective antibody-producing B-cells. In the germinal center, class-switch recombination allows B-cells to switch antibody classes while maintaining antigen specificity. This process is regulated by cytokines from Tfh cells (37). In contrast, T-cell-independent responses occur when repetitive antigens, such as bacterial polysaccharides, directly activate B-cells, leading to rapid IgM production but no memory formation.

Many B-cells enter the germinal center reaction, and some differentiate into plasmablasts, which serve as an early source of antibodies but have a short lifespan (Figure 3). Plasmablasts are short-lived, rapidly proliferating cells that secrete antibodies without undergoing extensive affinity maturation (38). They arise quickly after antigen exposure and produce IgM or, in some cases, IgG. Plasma cells, in contrast, are terminally differentiated B-cells. They arise from the germinal center reaction but migrate to the bone marrow and secrete high-affinity antibodies continuously, contributing to long-term immunity.

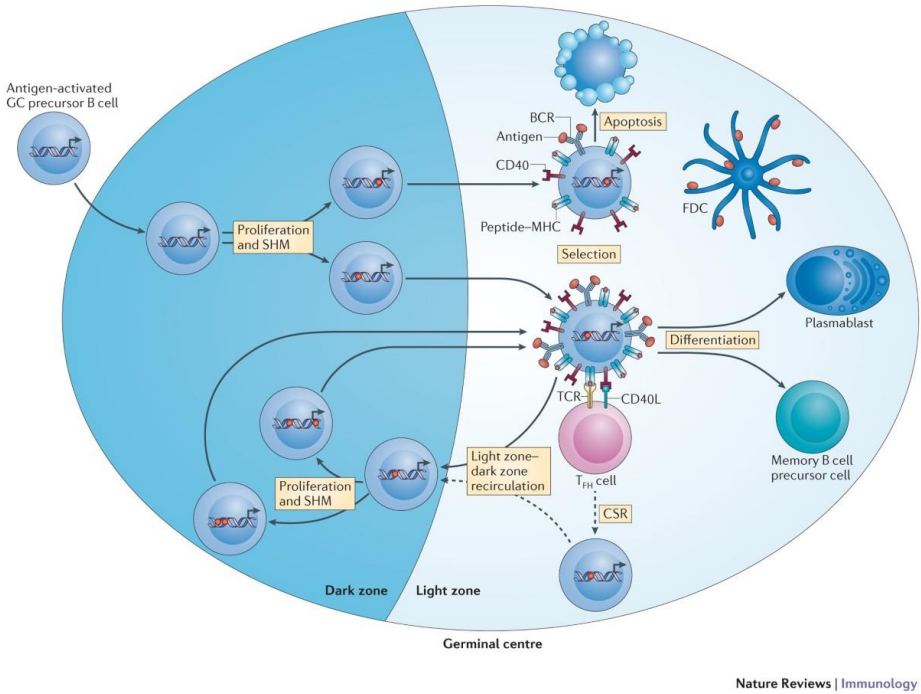

Figure 3. Dynamics of B cells in the germinal center reaction. Reproduced from De Silva et al., Nature Reviews Immunology, open access under a Creative Commons license (36).

Immune dysfunction in CLL

Most CLL patients are asymptomatic at diagnosis, but some present with recurrent infections already early in the disease course. All CLL patients are considered to have some degree of immunodeficiency. The immune dysfunction includes both the effect of the leukemic cells on the immune system, leading to humoral and cellular dysfunction, and the impact of CLL-specific treatment.

Disease-related immune dysfunction

Immune dysfunction is seen from the early stages of the disease and usually worsens with disease progression (39-42). Patients with MBL already exhibit immune defects and infectious complications are common (6, 43-46). This state of secondary immunodeficiency caused by the lymphoproliferative disease involves both qualitative and quantitative defects of the adaptive and innate immune system, which result in abnormal cellular and humoral-mediated responses (Figure 4).

The CLL tumor microenvironment refers to the dynamic and complex network of immune cells, stromal elements, signaling molecules and extracellular matrix components that surround CLL cells. This microenvironment promotes leukemic cell survival and proliferation while simultaneously impairing both innate and adaptive immune responses (47). For example, interactions between CLL cells and nurse-like cells or Tregs promote the secretion of IL-10 and other immunosuppressive cytokines, leading to both immunosuppression and tumor proliferation (48).

Hypogammaglobulinemia is observed in approximately 25% of newly diagnosed CLL patients and typically worsens over time, ultimately affecting up to 85% of all CLL patients (49-51). Lower immunoglobulin levels correlate with an increased risk of infections, with IgG subclasses, particularly IgG3 and IgG4, being the most affected (52). This likely contributes to increased susceptibility to bacterial infections, as these subclasses play a key role in opsonization and complement activation. Hypogammaglobulinemia reflects decreased survival and function of plasma cells and is also a consequence of the reduced number of normal, non-CLL B-cells, along with dysregulation of both

regulatory T- and B-cells. Additionally, an inadequate B-cell response to interleukin 2 (IL-2) impairs differentiation and antibody production, further compromising immune function (41, 47).

Alterations in the innate immune system, seen in most patients at diagnosis, cause defects in neutrophils and NK cells and impaired complement system with reduced levels of complement proteins (40). The complement system is primarily a first-line defense mechanism within innate immunity and almost 40% of CLL patients present with decreased levels of some complement proteins and activity, in particular C3b, which has an impact on protection against bacterial infections (40). Additionally, defects in activation, binding, and expression of cell surface complement receptors 1 and 2 is seen, which affects immune regulation, complement system activation, and particularly B-cell responses and clearance of immune complexes (40, 47). Monocytes and NK cells seem to be increased in number in CLL but usually have several functional defects, e.g. in cytotoxic activity (53). Dendritic cells have impaired maturation with reduced ability for IL-12 release, leading to ineffective T-cell stimulation and response (54). Neutrophils show impaired bactericidal activity and reduced C5A-induced chemotaxis (55).

T-cells in CLL show defects in activation, proliferation, and cytotoxic function (56-58). In early disease stages, T-cell numbers are often elevated but the cells are functionally impaired. As the disease progresses, T-cells accumulate further and exhibit increasing signs of exhaustion. CLL cells actively drive this immune dysfunction through the expression of cytotoxic surface molecules and extracellular mediators, which suppress T-cell proliferation and function. Upregulation of inhibitory receptors, such as PD-1, CTLA-4, TIM-3 and LAG-3, contributes to impaired cytotoxicity and diminished T-cell activation (39, 47, 51, 59). Additionally, T-cells in CLL patients have defective immunological synapse formation, failing to form proper immune synapses with APCs. T-cell subpopulation imbalances are also observed, including an altered CD4+/CD8+ ratio, with an increased proportion of circulating CD8+ cells (with reduced cytotoxic capacity) (58). CLL cells also exert direct inhibitory effects on CD4+ Th, further contributing to immune dysfunction.

Tregs are elevated in CLL and exert immunosuppressive effects on CD4+, CD8+, and NK cells. Studies suggest that CLL progression is associated with Treg expansion and Th17 downregulation, whereas increased Th17 cell numbers in peripheral blood correlate with a more favorable prognosis (60-64), highlighting the relevance of these subpopulations. Furthermore, the Treg/Th17 imbalance has been implicated in the development of autoimmune cytopenia and is suggested as a potential prognostic marker in CLL (60).

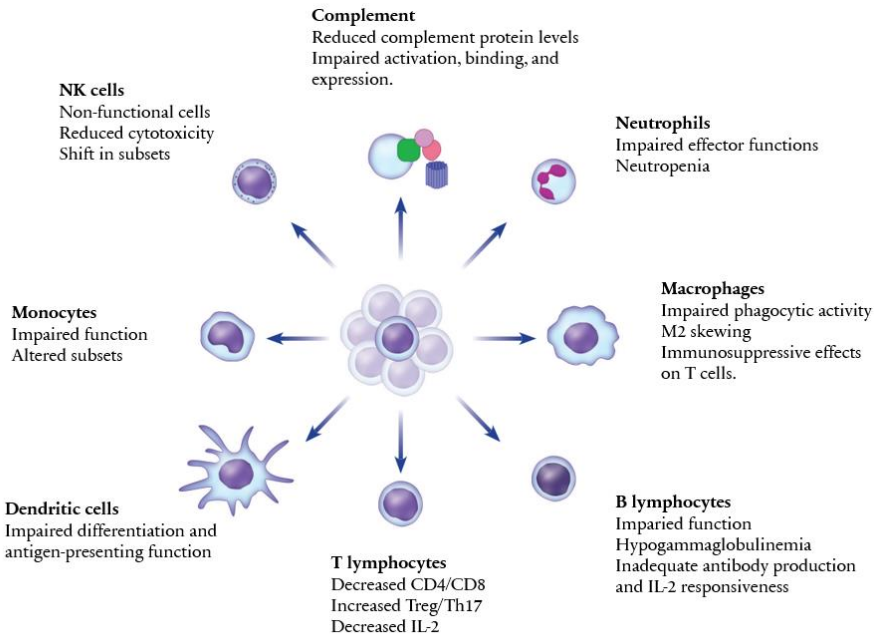

Figure 4. Disease-related immune dysfunction in chronic lymphocytic leukemia.

Treatment-related immune dysfunction

The immunosuppressive effects of various treatment modalities such as chemotherapy or new targeted therapies, with or without CD20 antibodies, can further increase infectious complications (51, 65). However, a partial restoration of immune function is also observed during or after treatment, as a consequence of reduced tumor burden.

Chemotherapy with alkylating agents (mainly cyclophosphamide, bendamustine or chlorambucil) are associated with myelosuppression and an increased risk of bacterial infections. Purine analogs (fludarabin) interfere with DNA synthesis, leading to a depletion of CD4+ T-cells and decreasing B-cells and monocytes, also increasing the risk of opportunistic infections with viral and mycotic agents (66).

When CD20 antibodies were introduced as a standard of therapy, a significant survival benefit for CLL patients was seen in combination with chemotherapy (67). However, anti-CD20 antibodies bind to CD20 on B-cells, inducing apoptosis and prolonged B-cell depletion, which can persist for up to 24 months and contribute to long-lasting immunosuppression (68). Also, obinutuzumab has been shown to induce depletion of NK cells and reduction of both CD4+ and CD8+ T-cells (69).

BTK is crucial for BCR signaling, supporting malignant B-cell survival. Although BTKis effectively disrupt tumor growth, they also impair normal B-cell function and affect various hematopoietic cells, including macrophages, granulocytes, and mast cells. BTK is involved in multiple immune pathways, such as Toll-like receptor, chemokine receptor and Fc receptor signaling pathways, contributing to broader immune modulation (70). BTKis also exert off-target effects, particularly on IL-2–inducible T-cell kinase (ITK), which plays a key role in T-cell activation through T-cell receptor signaling. ITK inhibition weakens Th2 responses and reduces cytokine production, which is more pronounced with first-generation BTKi and may explain the higher risk of opportunistic infections seen (71). Second-generation BTKis are more selective and exhibit reduced ITK inhibition (72). Both first- and second-generation BTKis impair monocyte and macrophage function, reducing phagocytosis and inflammatory cytokine

secretion, which also contributes to susceptibility to invasive fungal infections (73-75).

Despite these immunosuppressive effects, BTKis can also enhance T-cell function by increasing the T-cell repertoire diversity and reconstitution, which is mainly seen after long-term treatment (76-78). When CLL cells are depleted, their immunosuppressive effects on other immune cells decrease, potentially restoring the immune balance. Research has also shown that BTKis can reduce T-cell exhaustion, improve activation, repair immune synapse formation, and shift the immune profile toward a Th1 response, enhancing anti-tumor immunity (72, 77, 79). During treatment, T-cell counts initially rise, possibly due to treatment-induced lymphocytosis, but then gradually decline (76).

Early reports on the immunomodulatory effects of first-generation BTKis suggested an initial increase in immunoglobulin levels in patients treated with ibrutinib, though a decline was observed over time (72, 80). Also, the absolute number of non-CLL B-cells remained low during BTKi treatment as compared with in healthy individuals. These negative effects of BTKis on normal B cell function may also contribute to a weaker vaccine response.

The BCL2i venetoclax is used as monotherapy or in combination with CD20 antibodies. In CLL patients treated with venetoclax, 32–58% were found to develop severe (common toxicity criteria grade 3–4) neutropenia in a range of studies, especially early during treatment (81-83). The mechanism for venetoclax-induced neutropenia seems to be the increased BCL2 expression in neutrophil precursors, triggering apoptosis (83). The effects of venetoclax on non-leukemic cells in lymph nodes and peripheral blood have also been described, showing immune recovery after the elimination of leukemic cells (84).

Combination therapy with BCL2is and BTKis is emerging as a time-limited treatment option and analysis of immune cell subsets from treatment studies shows restoration of immune function, as observed after single-agent BTKi, but with faster kinetics (85, 86).

Infectious complications in CLL

Due to the previously described immunodeficiencies, CLL patients have increased susceptibility to bacterial, viral, and fungal infections and remain a major cause of morbidity and mortality in this population.

Infection risk, types of infection and prevention

It is estimated that around 80% of CLL patients encounter infections during the disease course (65, 87) and that the mortality rate due to infections ranges from 30–60% (88, 89). Although the risk of infection increases with disease progression and CLL-specific treatment, one study demonstrated that infections are common even before therapy is initiated, with 31% of patients experiencing at least one infection prior to treatment (90). Risk factors of infectious episode included older age, male sex, advanced disease stage (Binet), elevated β2-microglobulin, and unmutated IGHV. Immunoglobulin deficiencies, particularly low IgA levels, were strongly linked to infection risk. Additionally, CLL patients who experience infections seem to have lower overall survival (91, 92). Interestingly, increased risk of infections is already seen in MBL patients (93).

Bacterial infections originating from skin or mucosal surfaces are a major cause of infections in CLL. Respiratory tract infections caused by *Streptococcus pneumoniae* (pneumococci) are frequent, while other common pathogens include *Staphylococcus aureus, Haemophilus influenzae*, *Escherichia coli, Klebsiella pneumoniae*, and *Pseudomonas aeruginosa* (87, 94, 95). An increased risk of invasive pneumococcal disease (IPD) has been reported, with a 29- to 36-fold higher incidence compared to the general population (96-100).

Viral infections, including influenza, herpes simplex virus, varicella-zoster virus and cytomegalovirus, are also prevalent, especially in relapsed/refractory (R/R) CLL. Opportunistic infections, including fungal infections, are also a concern in RR/ CLL and prophylactic therapy varies depending on treatment regimen and additional risk factors (101). Furthermore, the COVID-19 pandemic underscored

that CLL patients were a particularly high-risk group for developing severe disease with increased mortality (66, 102, 103).

The increased infection risk is multifactorial and, as mentioned previously, relates to immune dysregulation due to advanced-stage disease or treatment, but also to age and comorbidities (40, 65, 87, 104, 105). Fludarabine based regimen with their strong T-cell suppressive effect increases the risk for opportunistic infections (106). Infections do however still remain a concern in patients with CLL receiving targeted agents (65, 107-109). For patients receiving first-line therapy with BTKis, infection rates range between 11.4% and 27.4%, coming close to 30% in relapsed/refractory patients. A slightly increased rate of invasive fungal infections (but still <3%) during BTKi treatment has been reported (71, 73, 74). Fixed-duration venetoclax-based treatments show variable rates, with maximum values around 20% (107).

Preventive measures, such as immunoglobulin replacement and vaccination, can reduce the risk of infections in patients with hematological malignancies, but prophylactic antibiotics do not (110). Preventive measures vary across guidelines depending on population and treatment regimen (101). High-quality studies on CLL patients regarding immunoglobulin replacement are lacking. CLL patients exhibit a reduced response to most studied vaccines, with the impairment becoming more pronounced in advanced disease stages, during active treatment, or within 6–12 months following CD20 antibody therapy (68, 111-115). Immunoglobulin replacement therapy may be considered in patients with low IgG levels in combination with severe bacterial infections, recurrent antibiotic-requiring respiratory infections or poor resolution despite adequate antibiotic treatment (116). Reduced immunoglobulin levels are commonly observed following CD20 antibody therapy, and in such cases, substitution may also be considered if the deficiency is deemed clinically significant (117). However, no preventive measures seem to reduce all-cause mortality (110).

Pneumococcal infections

There are over 100 serotypes of *S. pneumoniae*, but only a few frequently cause IPD. The distribution of the serotypes varies by age group, geographic region, and vaccination status (118). Still, pneumococcal disease is the leading source of infection-caused mortality in the world (119). The cell walls of pneumococci contain a thick peptidoglycan layer (characteristic of Gram-positive bacteria), teichoic acids, cell wall polysaccharides and surface proteins, all of which contribute to bacterial structure, immune modulation and virulence. In addition, pneumococci are surrounded by a polysaccharide capsule, considered their major virulence factor, which protects the bacteria from phagocytosis and enhances their ability to evade host immune responses (120). Surface-associated virulence factors such as pneumolysin also contribute to tissue damage and inflammation. The serotypes are numbered and grouped based on similarities in polysaccharide structures (121). Normally, pneumococci are opsonized by antibodies and complement, facilitating phagocytosis and removal by splenic macrophages (33). Patients with impaired splenic function are therefore particularly vulnerable to pneumococcal infections, as the spleen plays a crucial role in clearing *S. pneumoniae* from the bloodstream.

Historically, before the introduction of routine pneumococcal vaccination (in the pre-pneumococcal conjugate vaccine era), the most dominant serotypes causing IPD were 1, 3, 4, 6B, 9V, 14, 18C, 19F, and 23F. These serotypes were specifically targeted by the first 7-valent pneumococcal conjugate vaccine (PCV7), which significantly reduced their prevalence (118). With the introduction of PCV13, the incidence of vaccine-covered serotypes declined; however, non-vaccine serotypes began to emerge, a process called serotype replacement. Despite PCV13 coverage, serotypes 1, 3, 7F, and 19A remained significant contributors to IPD. In a retrospective study, non-PCV13 serotypes caused the majority of IPD cases in Southwest Sweden in patients with predisposing factors. However, serotype 3, included in PCV13, was prevalent and often caused severe disease (122). Newly emerging serotypes post-PCV13, including 10A, 15A, 22F, 23B, and 33F, are becoming more prevalent. To address these shifts in serotype

distribution, PCV15 and PCV20 were developed, incorporating additional serotypes such as 8, 10A, 11A, 15B, 22F, and 33F to provide broader protection in response to evolving epidemiological trends (123).

In Sweden, the incidence of IPD was 13.7/100,000 in 2023, with an increased risk in those aged above 65 years. Of all typed isolates from 2023, only 2.6 percent belonged to serotypes included in PCV10, compared with 57 percent in 2009 (124). In 2023, 54 percent of the typed isolates were serotypes covered by PCV15, 71 percent were covered by PCV20, and 74 percent by the 23-valent pneumococcal polysaccharide vaccine (PPSV23).

Pneumococcal vaccines

In an article published in The Lancet 1914, Wright et al. described the first studies on inoculation of humans using killed pneumococci, with substantial reduction in cases of pneumonia and deaths among miners in South Africa (125). In subsequent years, studies led to identification of capsular polysaccharides and an understanding of how different strains varied in disease and immunogenicity (126). Two types of vaccines are today used for immunization against pneumococcal infections: conjugate and polysaccharide vaccines. The capsular polysaccharides included in them are selected based on their prevalence in IPD. New vaccine-strategies are also being explored such as protein-based vaccines (targeting pneumococcal proteins and thereby able to induce immune response to all serotypes) and whole-cell pneumococcal vaccines (123). The Multiple Antigen-Presenting System is a technology combining polysaccharides with protein-based vaccines. For instance, a 24-valent ASP3772 pneumococcal conjugate candidate vaccine is being tested in phase I studies (127).

Pneumococcal polysaccharide vaccine, PPSV

A polysaccharide vaccine covering 23 serotypes was introduced in 1983. PPSV23, still marketed and used today, contains purified polysaccharides from serotypes 1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20, 22F, 23F, and 33F and is administered as an intramuscular injection. The polysaccharides induce a B-

cell-dependent (and T-cell-independent) immune reaction, first stimulating the release of IgM (Figure 3) (128). It induces only serotype-specific antibodies and no memory cells, which leads to limited duration of vaccine-induced immune response. Polysaccharide vaccines are not recommended in children under 2 years of age due to their immature immune systems with decreased ability to respond to B-cell stimulus. Studies have also shown decreased response in the elderly and a need for revaccination after approximately 5 years (129). The studies on PPSV show effects against IPD, but there are no studies confirming decreased risk of non-invasive disease. In Sweden, PPSV23 is recommended for adults ≥ 65 years without other risk factors for severe pneumococcal disease. Revaccination with PPSV23 is recommended after 5 years (130). Single-dose use of PPSV23 is no longer part of primary immunization in the Center for Disease Control guidelines (131).

Pneumococcal conjugate vaccine, PCV

Pneumococcal conjugate vaccines (PCVs) include a protein, CRM197 (a non-toxic variant of diphtheria toxin), conjugated to pneumococcal capsular polysaccharides. This induces a T-cell-dependent immunological memory that elicits an improved response in groups with less effective adaptive immune responses (128). For many years, PCV containing 13 serotypes (PCV13) has been used, and this vaccine was also used in the studies included in this thesis. It covers serotypes 1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F, and 23F.

After vaccination, APCs internalize the conjugate vaccine, process the protein carrier, and present it on MHC class II molecules to Th cells. This induces cytokine secretion (IL-2, IL-4, IL-21) promoting B-cell activation and differentiation (128). This interaction drives class switching and affinity maturation, leading to the production of high-affinity IgG antibodies. Memory B-cells are generated, providing long-term immune protection, allowing a rapid antibody response upon subsequent exposure (Figure 3, 5).

PCVs were first approved for infants and are globally recommended as a routine childhood immunization. PCV also induces a mucosal immunity thought to inhibit carriage of serotypes in vaccinated

children, leading to herd protection in the unvaccinated and elderly (128, 132). PCV13 was approved for adult use in 2011 and was incorporated into vaccine recommendations for high-risk groups in Sweden in 2016. However, PCV20 has now replaced PCV13 in most current guidelines.

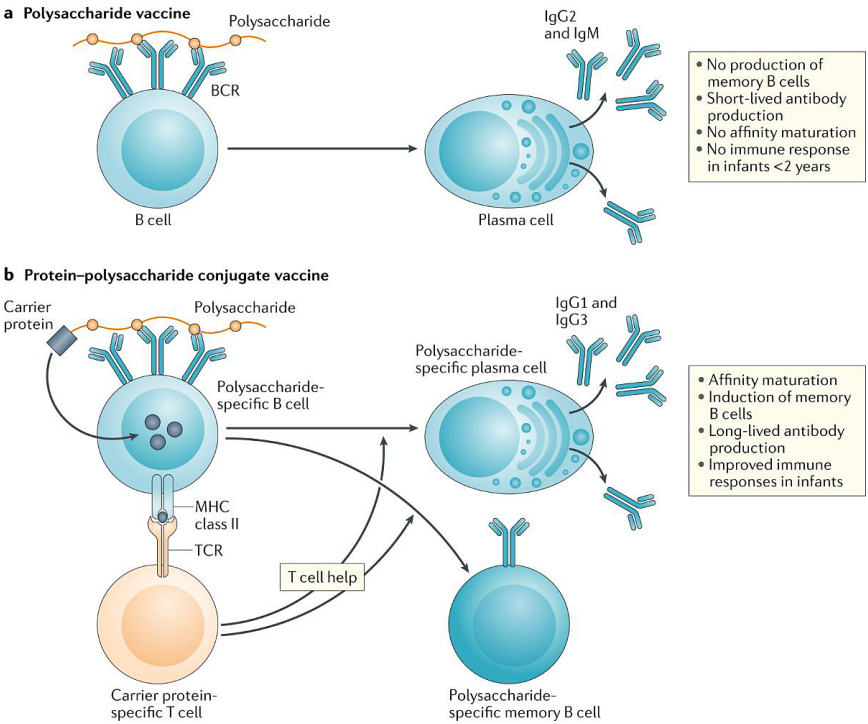

Figure 5. Immune response to pneumococcal polysaccharide and conjugate vaccines. Reproduced from Pollard et al., Nature Reviews Immunology (2021), open access under a Creative Commons license (128).

Aspects on revaccination with pneumococcal vaccines

Revaccination with pneumococcal vaccines is an area of debate. It is suggested that revaccination with PPSV23 within 5–10 years after primary immunization enhances protection in risk groups (131). But hyporesponsiveness, i.e., a weakened or diminished immune response to subsequent doses of a vaccine after an initial priming dose, is an area of concern. Hyporesponsiveness can occur after repeated administration of pneumococcal vaccines, especially if PPSV23 is given multiple times over a short time or administered before PCV (133). The exact mechanisms remain unclear but repeated exposure to antigenic stimulation may deplete the memory B-cell pool by driving pre-existing memory B-cells toward terminal differentiation into antibody-secreting cells, leading to attenuated responses upon re-exposure to the same antigen (133, 134). The clinical implications of hyporeponsivness are unknown, however, some research suggests that revaccination strategies may carry a risk, although this risk appears to be lower with the use of PCV (134, 135).

Revaccination with a conjugate vaccine in healthy elderly adults has been proven to be efficient and safe (136). Repeated doses of pneumococcal conjugate vaccines as part of primary immunization is recommended for infants (137) and has also been shown to improve protection in patients with hematological malignancies after allogenic stem cell transplantation, where it is a recommended strategy (138-140). No studies on repeated vaccinations with pneumococcal conjugate vaccines in CLL patients, either as part of primary immunization or as periodic booster vaccinations, have been conducted.

Assessment of antibody response to pneumococcal antigens

Enzyme immunoassays

Enzyme immunoassay (EIA), also known as enzyme-linked immunosorbent assay (ELISA), is a commonly used method for measuring serotype-specific IgG antibody responses following pneumococcal vaccination and is standardized and well-validated (141, 142). It is the primary method recommended by the World Health Organization (WHO) to evaluate immune response to pneumococcal vaccination (143). The assay quantifies antibodies directed against the capsular polysaccharides of *S. pneumoniae*.

The process begins with microtiter plates coated with purified pneumococcal polysaccharides representing various serotypes, ensuring that only specific antibodies bind. When measuring pneumococcal antibodies by EIA, cross-reactivity can lead to overestimation of serotype-specific responses due to non-specific antibodies binding to similar structures or cell wall polysaccharides. To improve specificity, serotype 22F polysaccharide is used as an adsorbent to remove these cross-reactive antibodies before analysis, ensuring that only truly serotype-specific antibodies are measured. After addition of patient serum, serotype-specific antibodies attach to the antigens and multiple washing steps remove unbound components. An enzyme-labeled secondary antibody is then added, to bind human IgG. Subsequent addition of a substrate solution triggers a colorimetric reaction, with the color intensity correlating with the antibody concentration (Figure 6). The results are quantified by measuring the optical density (expressed in µg/mL using a spectrophotometer, calibrated against WHO reference standards (144)). However, EIA is impractical for evaluations in vaccine studies since a separate assay needs to be performed for each serotype.

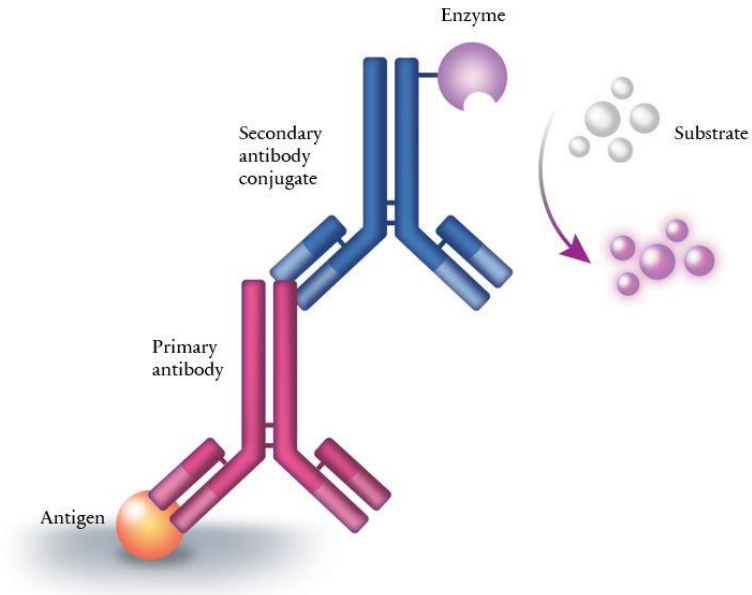

Figure 6. Principle of enzyme immunoassay.

Multiplex immunoassays

Multiplex immunoassay (MIA), also known as fluorescent multiplexed bead-based immunoassay (FMIA), is an advanced laboratory technique that enables the simultaneous quantification of antibodies against multiple pneumococcal serotypes. This makes it more efficient and better suited for large-scale data analysis and it is increasingly being implemented in vaccine studies (145). The principle of MIA involves coupling polysaccharides from different pneumococcal serotypes to uniquely fluorescent microspheres (beads). When patient serum is added, serotype-specific IgG antibodies bind to their corresponding bead-bound antigens. A fluorescent-labeled secondary antibody targeting human IgG is then introduced. The results are read using a flow-based detection system, where lasers detect the specific bead fluorescence and quantify bound antibodies based on the intensity of the fluorescent signal. The results are typically expressed in micrograms per milliliter (µg/mL) using calibration curves standardized

against WHO reference serum with assigned antibody concentrations for specific pneumococcal serotypes (144, 146, 147).

One of the key advantages of MIA is the ability to measure IgG responses to 23 or more pneumococcal serotypes simultaneously, which is useful when evaluating responses after pneumococcal vaccination. Further, MIA requires smaller sample volumes than EIA and is increasingly used in laboratories due to being less laborious and more flexible. However, the method is not standardized, although evaluations of cross-laboratory results in Europe indicate high correlation (146).

Opsonophagocytic assay

Opsonophagocytic assay (OPA) and multiplexed opsonophagocytic assay (MOPA) are functional assays used to measure the ability of antibodies to mediate bacterial clearance following pneumococcal vaccination (148). These analyses complement the measurement of IgG concentrations, as antibody titers not always correlate with functional activity or protection against pneumococcal infections.

Both assay types assess the functional capacity of antibodies by evaluating their ability to promote opsonization and phagocytosis, key processes in the defense against *S. pneumoniae* (148). MOPA, unlike OPA, allows for simultaneous measurement of functional antibody responses against multiple pneumococcal serotypes in a single assay. Patient serum is incubated with live pneumococcal bacteria, complement, and phagocytic cells. If functional antibodies are present, they will opsonize the bacteria, marking them for ingestion and destruction by phagocytes.

The assay measures bacterial killing by determining the reduction in viable bacterial colonies compared with in control samples. The OPA/MOPA titer is defined as the highest serum dilution that still achieves 50% bacterial killing compared to a control without serum (149). Higher titers indicate a greater presence of functional antibodies in the serum. Titers are expressed as the reciprocal of the serum dilution, meaning that a titer of 1:64 indicated that the serum can be diluted 64 times while still maintaining 50% bacterial killing. The

lowest detectable titer is typically 1:8, and if no effect is observed at this dilution, the sample is considered negative (149). MOPA results can be presented as the geometric means of titers (GMTs), as percentage of subjects with detectable titers, as EIA:OPA ratio (e.g., μg/mL antibody needed for 50% killing) or as correlations between MOPA titers and antibody concentrations.

In MOPA, a mixture of pneumococcal strains, each carrying a unique antibiotic resistance marker, is used. After incubation, the remaining live bacteria are grown on selective media containing antibiotics, allowing to determine which bacterial serotypes were effectively killed by the antibodies. The use of antibiotic resistance markers enables precise identification and quantification of the immune response to each pneumococcal serotype.

These assays require specialized laboratory expertise due to their complexity and are costly and time consuming. Interlaboratory comparison has shown acceptable agreement (150, 151). The assay provides essential data on the effectiveness of antibodies in mediating protection, a complement to analyzing IgG concentrations where the titers do not always correspond to the functionality of the antibodies and the protection against pneumococcal infections.

Cut-offs and correlate of protection

Although cut-off levels for serological protection (SP) and serological response (SR) are commonly applied in pneumococcal studies, they should not be interpreted as definitive protective thresholds or as indicators of adequate vaccine response at the individual level. The definitions vary between studies and serve only as surrogate markers of protection. They are influenced by factors such as the study population, vaccine type, and the specific objectives of the measurement, whether to assess response to vaccines or to evaluate overall immune competence.

SP, typically used in clinical vaccine studies, is commonly defined as the proportion of patients achieving serotype-specific IgG concentrations ≥0.35 μg/mL in 50-70% of the measured serotypes, indicating an antibody level considered sufficient to provide clinical protection

against invasive disease. However, the 0.35 µg/mL threshold, established by the WHO more than 20 years ago from pooled data of three PCV7 trials in children under 2 years, correlates with population-level protection against IPD in children (143, 152). It does not account for mucosal immunity or non-invasive pneumococcal diseases such as pneumonia and otitis media (153). Also, standard cut-off levels have not been validated as protective in adults or immunocompromised individuals, for whom higher thresholds, such as 1.0 µg/mL or 1.3 µg/mL, have been proposed (154). Protective levels are also defined by the proportion of tested serotypes, typically 50–70%, that exceed antibody concentrations of 0.35 µg/mL or 1.3 µg/mL, although the number of serotypes assessed varies between studies. For children under 6 years, 50% is considered sufficient, while 70% or more is recommended for older children and adults.

Alternative cut-off values aimed at more accurately defining immune protection have been proposed, though they require further validation (155, 156). Serotype-specific thresholds, rather than a single uniform cut-off value, have been suggested to allow more accurate predictions of protective antibody levels. However, the relationship between IgG concentrations following short and long-term protection remains poorly understood and requires further investigation.

Serological response (SR) following pneumococcal vaccination is typically defined as a ≥2-fold or ≥4-fold increase in IgG levels for 50-70% of the measured serotypes compared to pre-vaccination levels and refers to the change in antibody concentration in response to vaccination. Using a 4-fold increase has been suggested in adults and immunocompromised patients (154), but use of a 2-fold increase is more commonly seen in vaccine studies.

Using geometric mean concentrations (GMCs) and geometric mean ratios (GMRs) to evaluate pre- and post-vaccination titers for each serotype is also a widely used method for assessing immune response. The WHO recommends analyzing vaccine efficacy approximately 4 weeks post-immunization (143). Some studies extend this to 4–8 weeks.

There are limited data on standardized cut-off levels for OPA titers and their correlation with protective antibody levels, though some data suggest that an IgG concentration of ≥0.2 µg/mL corresponds to an OPA titer of ≥1:8 for certain serotypes (148). A titer of 1:64 has been suggested as a threshold for adults but is based on limited data (149). Studies have also shown that some serotypes may have a strong OPA response even at lower IgG levels, whereas others may require higher IgG concentrations to achieve effective opsonization. IgM, also being part of opsonophagocytic activity may play a role in discrepancies between IgG levels and OPA activity (149).

Newer assays, such as MIA, are emerging, potentially requiring bridging studies to align with the WHO's EIA reference method (145). Inter-laboratory variability must also be considered and cut-off levels might need to be adjusted for individual serotypes to ensure accurate evaluation of vaccine response (146, 157, 158). The influence of methodological differences and interlaboratory variability on the interpretation of results in relation to predefined response criteria has been previously emphasized, along with the risk that these factors may affect the evaluation of vaccine responses in clinical studies (158, 159).

Defining optimal serological thresholds that approximate protective cut-offs in clinical vaccine studies remains challenging, largely due to the difficulty of conducting trials with clinical endpoints. Variation in serotype-specific immunogenicity, differences in measurement methods and the limited number of studies in certain populations highlight the need for ongoing efforts to establish consensus in defining vaccine responses.

Pneumococcal vaccination in CLL

Vaccination recommendations

High-risk groups are defined as patient populations at higher risk of developing severe illness or complications from *S. pneumoniae* infection. Guidelines in Sweden and in the Western world have for many years recommended both PCV13 and PPSV23 to high-risk groups, including patients with hematological malignancies (130, 160, 161). These recommendations, with PCV13 followed by PPSV23, are based

on the theory of a so-called prime-boost strategy in vaccine-naïve individuals, to enhance an already activated immune system with PPSV23 and broaden the protection against additional serotypes (162). Recently, PCVs with additional serotypes has been introduced (PCV15, PCV20). Vaccination recommendations have now largely shifted to single-dose PCV20, decreasing the need to add additional serotype protection with PPSV23 (25, 130, 163, 164). In CLL patients, pneumococcal vaccination is recommended as early as possible after diagnosis, partly based on results of Study I in this thesis (25, 115, 164). There are no current recommendations on revaccination with pneumococcal conjugate vaccines in CLL patients but revaccination after 5 years with PPSV23 may be considered according to Swedish guidelines (25, 130).

Current knowledge on antibody response to pneumococcal vaccination in CLL

The impaired immune response to pneumococcal vaccination in CLL patients was first described more than 20 years ago. Pneumococcal vaccine studies conducted in CLL patients, including type of vaccine and method for antibody detection, are summarized in Table 1. Vaccine-induced immunological response to PPSV23 is seen in less than 25% in previous studies (165-168). When pneumococcal conjugate vaccines became available, Sinisalo et al. showed that almost 40% achieved an immune response after PCV7 vaccination, with a superior response if vaccinated early in the disease course. However, the response was still impaired compared with in controls (169). In a follow-up study of the same cohort 5 years after PCV7 immunization, performed by Lindström et al., the median antibody concentrations had declined by 50–75%, depending on serotype. Still, more than half of the CLL patients showed remaining protective levels for 4/7 serotypes, suggesting that a conjugate vaccine can provide long-term immunity (170). However, revaccination with PPSV23 did not improve immunity further (171). Mauro et al. showed that treatment-naïve patients had a response rate of 36% after PCV13 vaccination, whereas patients previously treated with chemoimmunotherapy or with BTKis showed a very low (0–8%) immune response (172). The impaired response in BTKi treated patients confirm a previous report on lack of

response in 4 ibrutinib treated patients receiving PCV13 (173). Pasiarski et al. described early humoral response, evaluated as plasmablast increase, and SR after PCV13 vaccination in CLL patients and controls. SR was detected in 58.3% of CLL patients, which also correlated with plasmablast response (174). Haggenburg et al. evaluated the recommended sequential vaccination strategy, starting with PCV13 and adding PPSV23 after 8 weeks, and found no additional immunological benefit from PPSV23, confirming the findings previously reported by Lindström et al. (175). Overall, the responses presented were very low, with an overall SR of 10.5% (results highly influenced by treatment status and tumor burden). However, more stringent cut-off criteria were used compared with in the other studies and MIA was used for antibody detection. Two of the vaccine studies measuring antibody response in this thesis (Study I and Study III) have been included at the bottom of Table 1 for comparison with previous studies (167, 176).

Table 1. Summary of studies evaluating pneumococcal vaccine responses in patients with chronic lymphocytic leukemia, highlighting variability in methodology across studies.

Study	Vaccine	Nr. patients	Nr. serotypes	Definition of protection and response	Method	Effect
Hartkamp et al. 2001 (165)	PPSV23	25	3	*1	EIA	SP 22%
Sinisalo et al. 2001 (166)	PPSV23	31	6	*2	EIA	SR 0%
Safdar et al. 2008 (168)	PPSV23	32	6	*3	EIA	SR 10%
Sinisalo et al. 2007 (169)	PCV13	52	7	*4	EIA	SP 49-92% SR 20-47%
Lindström et al. 2018 (170)	PCV7 (5 y. follow-up)	24	7	*4	EIA	SP 29-71%
Lindström et al. 2019 (171)	PCV13/PPSV23 (5 y. between vaccinations)	20	9	*4	EIA	SR 10-15% SP 30-75%
Pasiarski et al. 2014 (174)	PCV13	24	23	*5	EIA	SR 58%
Mauro et al. 2021 (172)	PCV13	112	med. PC IgG	*6	EIA	SP 30% SR 8%
Haggenburg et al 2023 (175)	PCV13/PPSV23 (8 weeks between vaccinations)	143	9	*7	MIA	SR 11% SP 11%
Svensson et al. 2018 (167)	PPSV23 vs PCV13	126	12	*8	EIA OPA	SR PPSV23 22% PCV13 40%

Kättström et al. 2025 (176)	A: PPSV23/PCV13/PCV13 vs B: PCV13/PCV13/PPSV23	74	12	*9	MIA	SR A: 24%/30% B: 12%/30% SP A:52%/56% B: 27%/49%

*1 SP: if 2/3 serotypes reached 20% of serotype IgG concentration reference levels in hyperimmune plasma pool, *2 GMT rise and SR: sum of serotype-specific antibody levels, changes pre- and post-immunization.*3 SR: 2-fold >0.5 µg/mL *4 SP: >0.35 µg/mL of serotype-specific IgG concentration, SR: 2-fold ≥ 0.35 µg/mL *5 SR: 2-fold increase *6 SP: ≥ 40 mg/L median PC IgG levels, SR: 2-fold increase baseline PC-IgG concentration *7 SR: 4-fold increase in ≥70% of serotypes, SP ≥1.3 µg/mL for ≥70% of serotypes, *8 SR: OPA geometric mean titers for every serotype and response defined as a post-vaccination OPA titer ≥ LLOQ in ≥8 /12 serotypes. EIA GMCs for each serotype and change, as a geometric mean ratio, pre- and post-vaccination. *9 SP: ≥ 0.35µg/mL for ≥70% of serotypes, 2-fold increase ≥ 0.35 µg/mL in ≥70% of serotypes. GMC and GMR for each serotype.

Aims of the thesis

General

The aims of this thesis were to compare immune responses after pneumococcal polysaccharide vaccine (PPSV23) versus pneumococcal conjugate vaccine (PCV13), to evaluate antibody persistence and to explore whether revaccination with pneumococcal conjugate vaccines improves immunity in patients with chronic lymphocytic leukemia.

Aims of the individual studies

Study I: To compare the immune response, measured by OPA and ELISA, following vaccination with PPSV23 or PCV13 in treatment-naïve CLL patients.

Study II: To characterize the normal composition of the peripheral blood B-cell repertoire in CLL patients and to explore the dynamics of plasmablast response following revaccination with either repeated PCV13 or PCV13 followed by PPSV23.

Study III: To evaluate antibody persistence five years after primary immunization with PPSV23 or PCV13 and to assess the antibody response to revaccination with either repeated PCV13 or PCV13 followed by PPSV23.

Study IV: To investigate whether the choice of analytical method, MIA or EIA, for measuring serotype-specific IgG concentrations following pneumococcal vaccination influences the assessment of vaccine response in CLL patients.

Materials and Methods

Study I

A randomized, multi-center trial was conducted between 2013 and 2016, enrolling 128 untreated CLL patients from eight hematology clinics in Sweden (Figure 7). Patients were stratified based on Rai stage and serum IgG levels before being randomly assigned to receive either PPSV23 (Pneumovax®23) or PCV13 (Prevenar13®). Key exclusion criteria included planned initiation of CLL-specific treatment within 1 month, other malignancies, prior pneumococcal vaccination within the preceding 5 years, history of severe allergic reactions to vaccines, neutropenia ($<0.5 \times 10^9$/L), a positive direct antiglobulin test (DAT), ongoing infection or previously known hemolysis.

The primary objective of the study was to compare immune responses using OPA at 4 weeks post-vaccination. Secondary outcomes included the evaluation of serotype-specific IgG concentrations, measured by ELISA, at 4 weeks and 6 months, as well as OPA titers at 6 months to assess the durability of the immune response. Post-vaccination analyses were conducted for 12 pneumococcal serotypes (1, 3, 4, 5, 6B, 7F, 9V, 14, 18C, 19F, 19A, and 23F), shared between PCV13 and PPSV23.

Validated OPA assays and ELISA were conducted at Pfizer's Vaccine Research Clinical Testing Laboratory in the United States according to previous published studies (177, 178). OPA titers were defined as the reciprocal serum dilution required to achieve 50% complement-mediated bacterial killing. A lower limit of quantification (LLOQ) was defined for each serotype and results presented as GMTs. Serotype-specific IgG was measured using ELISA and GMCs was compared across serotypes. Additionally, the study compared the proportion of patients in each of the two vaccination groups who achieved a positive immune response, defined as a post-vaccination OPA titer ≥ LLOQ in at least 8 of the 12 serotypes shared by PCV13 and PPSV23. The predefined LLOQ response criteria for each serotype were as follows: serotype 1, 1:18; serotype 3, 1:12; serotype 4, 1:21; serotype 5, 1:29; serotype 6A, 1:37; serotype 6B, 1:43; serotype 7F, 1:113; serotype

9V, 1:141; serotype l4, 1:35; serotype 18C, 1:31; serotype 19A, 1:18; serotype 19F, 1:48; and serotype 23F, 1:13.

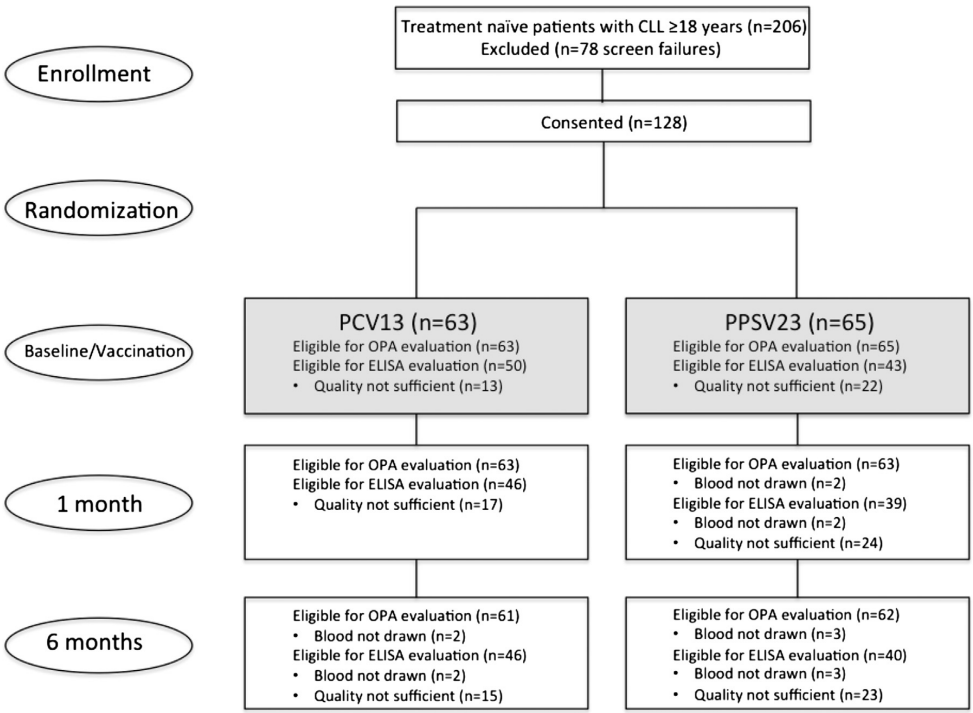

Figure 7. Study design and study population in Study I.

Study II

This prospective study included CLL patients from the Hematology Unit at Örebro University Hospital, Sweden, who had previously received PCV13 or PPSV23 as part of a randomized vaccination trial (Study I) conducted between 2013 and 2016. Between October 2019 and February 2020, 14 CLL patients were enrolled in a revaccination study. Following informed consent, a physical examination was performed, followed by blood chemistry analysis, including measurements of total IgG, IgA, and IgM levels, as well as IgG subclasses. Main exclusion criteria included ongoing treatment with high-dose corticosteroids (≥20 mg prednisolone) or other immunosuppressive drugs not part of active CLL therapy, history of allergic reactions to vaccines, positive DAT, current or previous autoimmune hemolytic anemia or immune thrombocytopenic purpura. Vaccination was temporarily postponed in cases of febrile illness, recent antibiotic therapy (within 72 hours), inactivated vaccine administration (within 14 days), or live vaccine administration (within 28 days). None of the patients had received pneumococcal revaccination since their primary immunization. The revaccination strategy was determined by prior immunization (Figure 8). CLL patients who had initially received PCV13 were given a single dose of PCV13, followed by PPSV23 after 8 weeks. Those who had originally received PPSV23 were revaccinated with two doses of PCV13, administered 8 weeks apart. A control group of immunocompetent individuals (n = 31) who had previously received PCV13 or PPSV23 as part of routine vaccination 3-6 years earlier was included. These individuals were revaccinated with a single dose of PCV13, without a second revaccination.

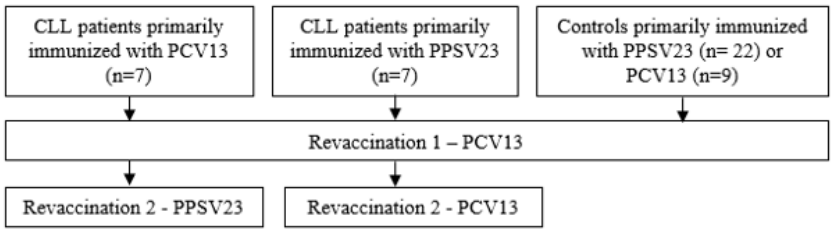

Figure 8. Revaccination strategy of CLL patients and controls.

The primary objective of the study was to investigate the early humoral response to pneumococcal revaccination in previously immunized CLL patients by assessing plasmablast proportions before and seven days after revaccination, using immunocompetent individuals as controls. A further objective was to characterize the baseline composition of the B-cell repertoire in peripheral blood among CLL patients and immunocompetent controls and to evaluate its dynamic changes following pneumococcal revaccination.

B cells were analyzed using flow cytometry following standard staining and sample preparation protocols. Data acquisition was performed on a Gallios Flow Cytometer, and analysis was conducted using Kaluza software (v2.1, Beckman Coulter). To assess B-cell subsets and the plasmablast response before and after revaccination, whole blood was collected in heparinized tubes. Flow cytometry was performed at baseline and 7 days post-revaccination to analyze B-cell populations. Total B cells were identified by gating on $CD19^{+}/CD45^{+}$ cells, and the following subsets were characterized: naïve B cells ($CD27^{-}/IgD^{+}$), switched memory B cells ($CD27^{+}$), non-switched memory B cells ($CD27^{+}/IgD^{+}$), double-negative memory B cells ($CD27^{-}/IgD^{-}$), and plasmablasts ($CD38^{++}/CD27^{++}/IgD^{-}/IgM^{+}$).

Study III

In this prospective study, 74 CLL patients previously enrolled in the randomized multicenter vaccination study (Study I), in which they had received either PCV13 or PPSV23, were recruited from eight hematology clinics in Sweden between October 2019 and February

2020. Additionally, 31 immunocompetent controls who had received PCV13 or PPSV23 between 2013 and 2017 were included. Participants were included at a median of 5 years after their primary immunization and were stratified into two revaccination groups based on their initial vaccination with either PCV13 or PPSV23 (Figure 9).

The primary outcomes of the study were to evaluate the proportion of CLL patients reaching the criteria of SP five years after primary immunization with PCV13 or PPSV23, as well as SR at eight weeks following PCV13 revaccination. Secondary outcomes included assessing the impact of a second revaccination with either PCV13 or PPSV23 on SR. Additional objectives involved evaluating SP rates post-revaccination at every time point, analyzing serotype-specific responses through GMCs and GMRs, investigating the influence of hypogammaglobulinemia and CLL treatment on revaccination outcomes, and monitoring the incidence of invasive pneumococcal disease (IPD) since the initial vaccination study (2013–2016), along with the prevalence of nasopharyngeal pneumococcal carriage.

SR was defined as a ≥2-fold increase in serotype-specific IgG to ≥0.35 µg/mL, and SP was defined as a post-revaccination IgG concentration of ≥0.35 µg/mL. Both criteria had to be fulfilled for at least 70% (9-12 out of 12) of the shared serotypes. A higher cut-off of ≥1.3 µg/mL, previously suggested as a protective threshold for immunocompromised adults, was also evaluated (154). Serotype-specific vaccine responses were further analyzed by calculating GMCs and GMRs for each serotype.

A bead-based fluorescent MIA was used to quantify serum IgG (µg/mL) levels against the 12 pneumococcal serotypes common to PCV13 and PPSV23 (1, 3, 4, 5, 6B, 7F, 9V, 14, 18C, 19A, 19F, and 23F). The serological assay was performed and validated by the Finnish Institute for Health and Welfare (THL), Helsinki, Finland as described previously, but some modifications (179).

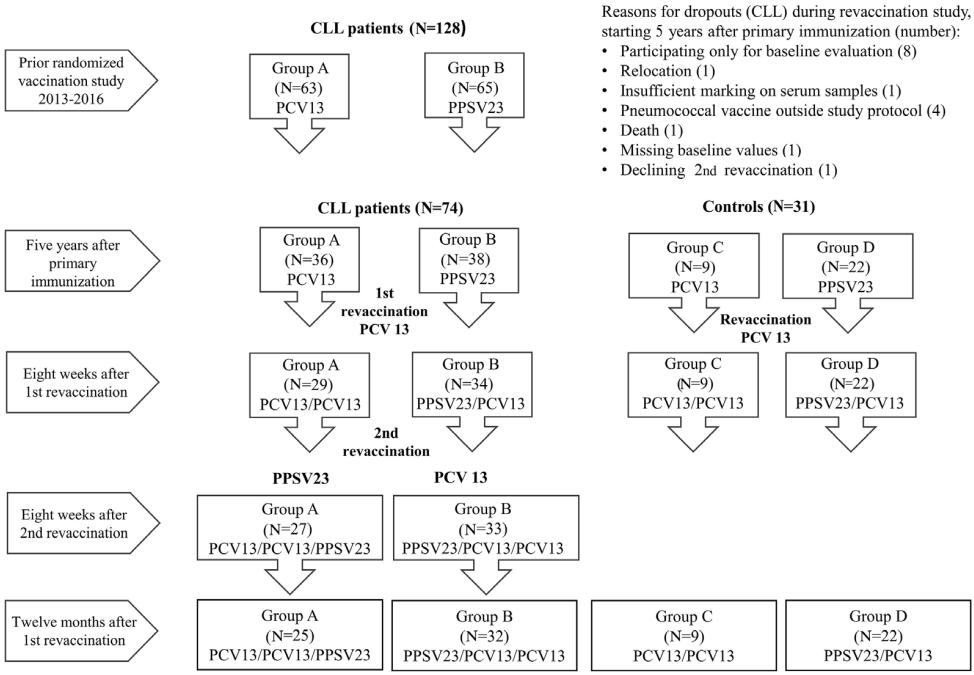

Figure 9. Study design, study population and revaccination strategy among CLL patients and controls.

Study IV

This study re-analyzed serum samples collected from Study I, the multicenter randomized vaccination trial that enrolled treatment-naïve CLL patients to compare immune responses to PPSV23 and PCV13. In Study I, serotype-specific IgG concentrations were measured by Pfizer using EIA.

The primary objective of Study IV was to explore whether reanalyzing serum samples by MIA would influence the assessment of responses to vaccination in CLL patients, as compared to the original results, based on EIA.

Blood samples for immunogenicity assessment were collected at three timepoints in Study I: immediately before vaccination, 4 weeks post-immunization and after 6 months. Serum samples were initially stored at minimum -20 °C at local sites. Upon study completion, all samples were transferred to Pfizer's Vaccine Research High-Throughput Clinical Testing Laboratory in Pearl River, NY, USA, for analysis. There, serum samples were evaluated using standardized EIA as described previously (177), and was performed for each of the 12 pneumococcal serotypes common to PCV13 and PPSV23 (serotypes 1, 3, 4, 5, 6B, 7F, 9V, 14, 18C, 19A, 19F, and 23F).

After laboratory testing was finalized, the remaining serum samples were returned in 2018 and stored at Karolinska Institute, Sweden. In 2022, the samples were moved to the biobank at Örebro University, Sweden, before being shipped to THL in Helsinki, Finland, where MIA was used to quantify serum IgG concentrations (µg/mL) against the same 12 pneumococcal capsular polysaccharides. This serological assay was conducted and validated by THL and accredited by the Finnish Accreditation Service in compliance with SFS-EN ISO/IEC 17025 standards. Sufficient serum for reanalysis at THL was available from 56 patients (146 sampling events), including 27 who had received PCV13 and 29 who had received PPSV23.

Additionally, MIA analysis was performed and compared to WHO-assigned values for pneumococcal quality control serum samples using the WHO EIA for serotypes 1, 3, 4, 5, 6A, 6B, 7F, 9V, and 14 (12 samples from the WHO Pneumococcal QC Serum Panel, NIBSC code 12/278). For values below the detection threshold, both methods applied half the detection limit.

Statistical analysis

Study I: Since no previous clinical trials had evaluated pneumococcal vaccine responses in CLL patients using OPA geometric mean titers (GMTs) as a primary outcome, the initial sample size was estimated based on an expected immune response rate of 15% in PPSV23 recipients and 35% in PCV13 recipients—a difference of 20%. To detect this difference with 80% power ($\beta = 0.20$) and a significance level of 5% ($\alpha = 0.05$), a total of 145 evaluable patients was required. Allowing

for a 5% dropout rate, 154 patients (77 per group) were planned for inclusion. Due to slow recruitment, a revised power calculation was conducted after 120 patients had been enrolled. Based on new data from non-immunocompromised individuals, which suggested a larger difference between groups, it was concluded that a minimum of 120 patients would be sufficient to meet the primary study objective.

Statistical analyses of immunogenicity were conducted using linear mixed models to calculate geometric mean ratios (GMRs), incorporating vaccination group, time points (e.g. 1 and 6 months) and their interaction. The data were log-transformed, and GMRs were presented as exponentiated estimates. Responder rates were evaluated using logistic regression models, with vaccination group and baseline antibody levels as predictors. All results were reported with 95% confidence intervals and p-values. Missing or indeterminate values were excluded from the analyses without imputation.

Study II: Normality of the data was assessed using the Shapiro–Wilk test. For inter-group comparisons, the Mann–Whitney U test was applied to non-normally distributed variables, while normally distributed variables were analyzed using the independent t-test. Categorical variables were compared using the chi-square test. For paired samples, the Wilcoxon matched-pairs signed-rank test was used. Expansion of a B-cell subset was defined as an increase in its percentage from baseline to post-revaccination. A p-value < 0.05 was considered statistically significant. All statistical analyses were performed using SPSS version 25
Study III: Baseline characteristics were compared using the Mann–Whitney U test for continuous variables and either the chi-square or Fisher's exact test for categorical variables. SR and SP rates were compared using random intercept mixed Poisson regression models, with results presented as relative risk ratios (RR) and 95% confidence intervals (CI). Fixed effects included group, time (five years after primary immunization, eight weeks after first and second revaccination, and 12 months after first revaccination), and their interaction (group × time). Missing data were assumed to be missing at random, and pretreatment status (PCV13 or PPSV23) was included as an adjustment when comparing CLL patients with controls.

In cases where the mixed model did not converge due to sparse data, Exact McNemar's or Fisher's exact test was used. Serotype-specific GMCs of IgG were analyzed using a random intercept linear mixed model and reported as GMRs with 95% CIs. A p-value < 0.05 was considered statistically significant. All analyses were performed using SPSS version 29 and Stata release 17.

Study IV: The correlations between IgG concentrations obtained from MIA and EIA (for serotypes 1, 3, 4, 5, 6B, 7F, 9V, 14, 18C, 19A, 19F, and 23F), as well as between MIA and WHO EIA, were evaluated both graphically and by calculating concordance correlation coefficients (CCCs) with 95% confidence intervals (CIs). CCCs were derived from log-transformed data for each serotype and interpreted according to predefined thresholds: <0.50 (poor), 0.50–0.75 (moderate), 0.75–0.90 (good), and >0.90 (excellent)(180).To compare serotype-specific IgG geometric mean concentrations (GMCs) between MIA vs. EIA and MIA vs. WHO EIA, linear regression was used, generating geometric mean ratios (GMRs) with 95% CIs as measures of association. These analyses were conducted at individual time points and across all time points combined, with robust standard errors adjusted for patient clustering in the latter analysis.

The proportions of patients with IgG concentrations ≥0.35 µg/mL for individual serotypes, as well as those achieving SP, defined as IgG concentrations ≥0.35 µg/mL for at least 70% (9-12/12) of the shared serotypes, were compared across methods using McNemar's exact test. A p-value <0.05 was considered statistically significant. Statistical analyses were conducted using SPSS version 29 and STATA release 17.

Ethical considerations

The studies have been performed in accordance with the ethical principles of the Declaration of Helsinki and good clinical practice. All patients and controls gave written informed consent to participate. The studies have been approved by the Swedish Ethical Review Authority and the Swedish Medical Products Agency. The studies were also registered in EUDRA-CT and at www.clinicaltrials.gov. Study I was monitored by the Karolinska Trial Alliance and Studies II and III

were monitored by the Clinical Epidemiology and Biostatistics Unit, Region Örebro.

Study I: Swedish Ethical Review Authority: 2009/1731-31, 2009/1731-31/1, 2012/293-32, 2013/1000-32, 2014/2222-32). EudraCTnr: 2009-012642-22. Swedish Medical Products Agency: 151:2009/64686, Biobank: RBC 2013-368

Study II: Swedish Ethical Review Authority: 2018/483, 2019-02172, 2020-00982. EudraCTnr: 2018-003377-97. Swedish Medical Products Agency: 5.1-2018-86887, Biobank: RBC 19250 2 2018/483

Study III: Swedish Ethical Review Authority: 2018/483, 2019-02172, 2020-00982. EudraCTnr: 2018-003377-97. Swedish Medical Products Agency: 5.1-2018-86887, Biobank: RBC 19250 2 2018/483

Study IV: Swedish Ethical Review Authority: 2009/1731-31, 2009/1731-31/1, 2012/293-32, 2013/1000-32, 2014/2222-32)2022-00145-02, 2022-00145-02-234575, EudraCTnr: 2009-012642-22. Swedish Medical Products Agency: 151:2009/64686, Biobank: 2013-368

Results

Study I

Of the 128 patients enrolled, 126 completed the one-month follow-up and were evaluable for the primary endpoint, while 123 completed the six-month follow-up. The median age at vaccination was 69 years, and the majority of patients were in Rai stage 0–I. Hypogammaglobulinemia were seen in 27% prior to vaccination.

One month after vaccination, PCV13 elicited higher OPA GMTs than PPSV23 for 10 of the 12 shared serotypes. A significantly greater proportion of PCV13 recipients showed a positive immune response compared to those receiving PPSV23: 25 of 63 (40%) in the PCV13 group versus 14 of 63 (22%) in the PPSV23 group ($p = 0.034$) (Figure 10). At six months, PCV13 continued to demonstrate higher OPA GMTs for five serotypes, with a higher overall response rate than PPSV23: 21 of 63 (33%) vs. 11 of 63 (17%) ($p = 0.041$). In ELISA analyses, PCV13 also resulted in higher serotype-specific IgG concentrations (GMCs) for 7 serotypes at one month and 6 at six months, although sample availability limited testing in some cases.

Subgroup analyses revealed that patients with hypogammaglobulinemia had significantly lower OPA GMTs across most serotypes. No vaccine responses were observed in patients with IgG levels below 4.9 g/L. Additionally, patients with a shorter disease duration (<31 months) showed stronger vaccine responses than those with longer disease duration. The impact of Rai stage could not be meaningfully evaluated due to the small number of high-risk patients.

The difference in immune response between the two vaccines declined over time and was generally lower after six months than after one month.

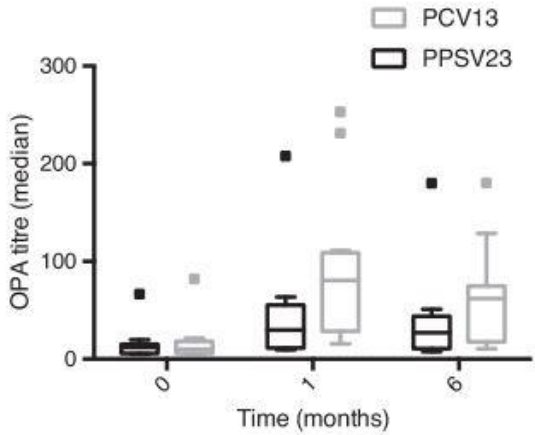

Figure 10. Immunogenicity data measured as OPA GMTs at baseline, 1 month, and 6 months after vaccination.

Study II

All participants had previously received pneumococcal vaccination, with a majority (71%) of controls having received PPSV23. The mean time from primary immunization to revaccination was similar between CLL patients and controls (63 vs. 65 months, $p = 0.65$). Median total Ig levels and all IgG subclasses were significantly lower in CLL patients compared to controls.

At baseline, plasmablasts were undetectable in CLL patients but present in all controls ($p < 0.001$) (Figure 11 and 12). Seven days after the first revaccination with PCV13, plasmablast proportions increased significantly in controls ($p < 0.001$), but not in CLL patients ($p = 0.13$). Plasmablasts were detectable in four of the 14 CLL patients, three of whom had received PCV13 and one PPSV23 as primary immunization. In controls, no difference in response was observed between those primarily immunized with PCV13 versus PPSV23 ($p = 0.33$). Seven days after the second revaccination, eight of 14 CLL patients showed increased plasmablast proportions compared to baseline ($p < 0.01$). Among patients who showed plasmablast expansion only after the second revaccination ($n = 4$), all had received PPSV23 as

primary immunization and PCV13 at both revaccinations ($p = 0.04$). A second revaccination with PPSV23, after PCV13 had been given as both primary immunization and first revaccination, did not result in further increase in plasmablast proportions. Plasmablasts were not detected after any revaccination in patients with ongoing BTK inhibitor treatment or recent rituximab exposure. Among patients with hypogammaglobulinemia, three out of four with total IgG below the reference interval did not respond to either revaccination, while one responded only after the second PCV13 dose.

Regarding B-cell subsets, CLL patients had significantly lower or absent naïve B cells at baseline compared to controls ($p < 0.001$). Due to interference from the leukemic clone, switched and non-switched memory B cells were not clearly distinguishable in CLL patients and were presented together. No statistically significant expansion of total memory B cells was seen in CLL patients after first ($p = 0.06$) or second ($p = 0.24$) revaccination. In contrast, controls showed a significant expansion of switched memory B cells ($p < 0.001$), but not non-switched memory cells ($p = 0.16$), and a significant reduction in naïve B cells ($p < 0.001$). Double-negative memory B cells were lower at baseline in CLL patients ($p = 0.01$) and did not increase significantly after either revaccination ($p = 0.07$ and 0.14), while a significant expansion was observed in controls ($p = 0.01$).

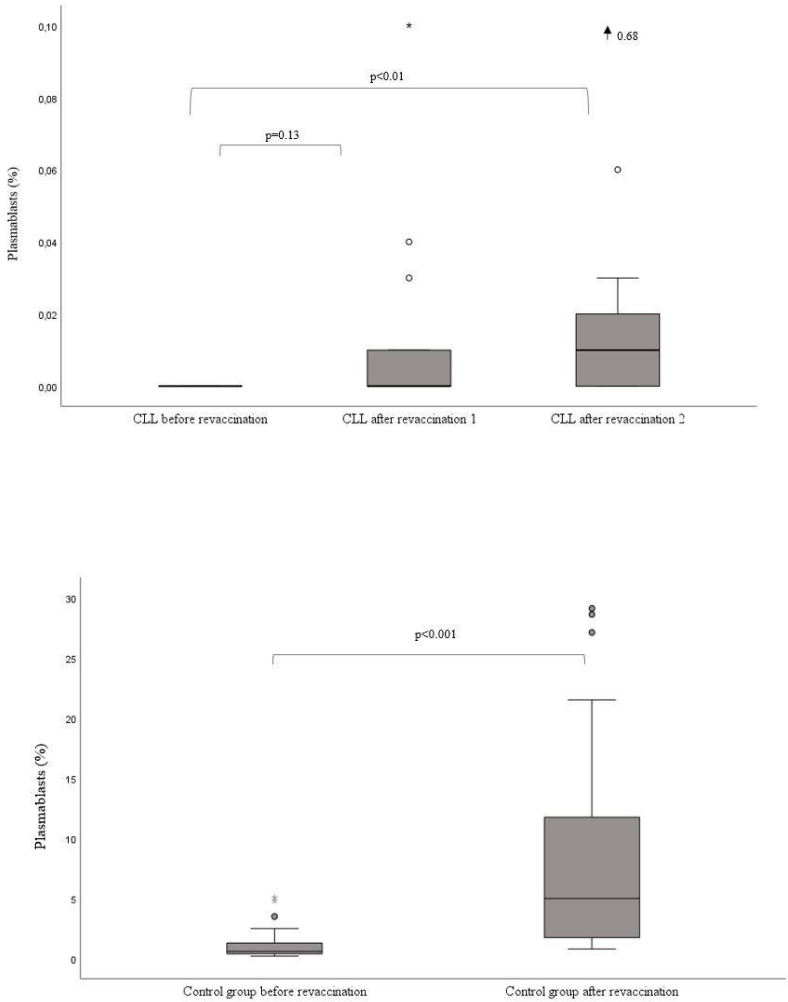

Figure 11. Plasmablast proportions in CLL patients (top) and controls (bottom) before and after pneumococcal revaccination

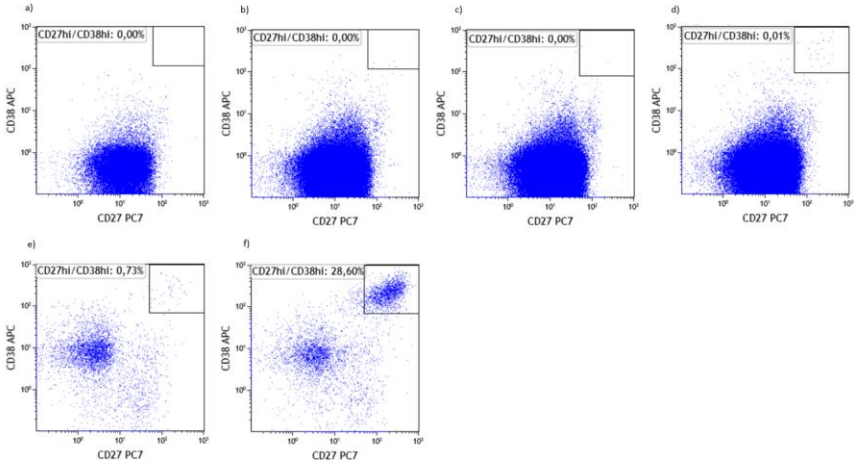

Figure 12. Flow cytometric analysis of plasmablast proportions before and after pneumococcal revaccination. Panels a-d show changes in a CLL patient primary immunized with PPSV23 and before and after each of two revaccinations with PCV13. Panels e-f show response from a healthy control before and after revaccination with PCV13.

Study III

At inclusion, 25% of CLL patients had hypogammaglobulinemia. The majority (82%) were treatment-naïve, 7% were in remission off treatment, and 9% were on BTK inhibitors or had received anti-CD20 therapy within the past 12 months. Five years after primary immunization with PCV13 or PPSV23, the proportion of CLL patients maintaining SP at ≥0.35 µg/mL did not differ significantly between groups (14% vs. 5%, respectively; p=0.23), though CLL patients had significantly lower SP rates than controls (10% vs. 32%; p=0.006). None of the CLL patients, but two controls previously immunized with PCV13, reached SP at ≥1.3 µg/mL. Serotype-specific IgG GMCs did not differ significantly between PCV13 and PPSV23 groups but were higher for 8/12 serotypes in controls compared with CLL patients.

Following PCV13 revaccination, SR was observed in 24% of group A (PCV13/PCV13) compared to 12% of group B (PPSV23/PCV13) (p=0.25) (Figure 13), whereas CLL patients overall had significantly lower SR rates than controls (18% vs. 42%; p=0.04). SP rates (≥0.35

μg/mL) increased significantly in all groups after revaccination (Figure 13 and 14). When the ≥1.3 μg/mL cut-off was applied, SP improved in group A and controls but not in group B. GMCs increased significantly after revaccination in both CLL patients and controls, with GMCs in 4/12 serotypes being higher in group A than in group B.

After a second revaccination, 30% of group B patients achieved SR, up from 12% ($p = 0.017$), and SP increased from 27% to 49% ($p < 0.01$) (Figure 13). No significant change was observed at the higher SP cut-off. In group A, second revaccination with PPSV23 did not lead to further increases in SR or SP. GMCs increased in 8 of 12 serotypes in group B, but no further increases were seen in group A. GMCs increased significantly in 8/12 serotypes after a second PCV13 revaccination but not after PPSV23, reducing the difference between groups seen after the first revaccination.

SP remained significantly lower in CLL patients compared to controls (40% vs. 71%, $p = 0.002$) after 12 months (Figure 14). SR rates declined significantly in group B (from 30% to 13%, $p = 0.021$), but not in group A. GMCs decreased over 12 months in both groups but remained higher than pre-revaccination levels. Decline was seen in 11 of 12 serotypes in group A and 6 of 12 in group B. GMCs remained higher in controls for 10 of 12 serotypes, and the antibody decline was less pronounced than in the CLL group.

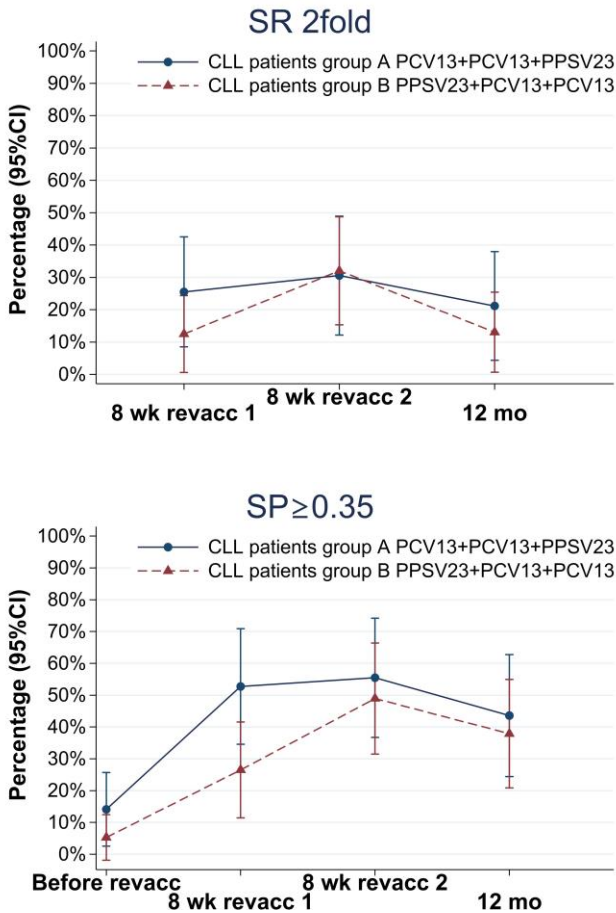

Figure 13. Serological response (SR) after revaccination in CLL patients, group A and B, defined as 2-fold increase of IgG levels ≥ 0.35 ug/ml in at least nine (70%) of the 12 serotypes compared to baseline (top) and serological protection (SP) defined as ≥0.35 ug/ml in at least nine (70 %) of the 12 serotypes (botton).

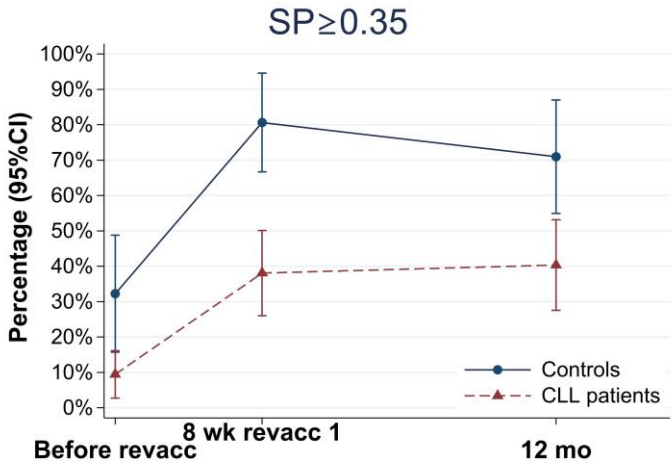

Figure 14. Serological protection (SP), defined as ≥0.35 ug/ml in at least nine (70 %) of the 12 serotypes, in CLL patients and controls five years after primary immunization and after revaccination,

Study IV

After excluding samples with insufficient quantity or indeterminate results, 127 samples from 46 patients (42 at baseline, 42 at 4 weeks, and 43 at 6 months post-vaccination) remained for comparative analysis between the two methods. MIA provided complete data for all 13 serotypes measured, while missing or incomplete data were more common in the EIA dataset due to quantity-not-sufficient (QNS) or indeterminate results.

Across all time points, IgG concentrations measured by MIA were consistently lower than those obtained via EIA (Table 2). The degree of correlation between MIA and EIA varied by serotype. The lowest correlation was observed for serotype 5 (CCC = 0.14), while the highest was seen for serotype 19F (CCC = 0.86). Based on predefined concordance thresholds, 2 of 12 serotypes showed poor correlation, 5 moderate, and 5 good correlation between methods. These differences impacted the classification of patients reaching the IgG threshold ≥0.35 µg/mL. For every serotype, fewer patients reached this threshold when measured by MIA than by EIA. For instance, for

serotype 5, 23% reached ≥0.35 µg/mL by MIA compared to 91% by EIA; for serotype 19F, 62% vs. 81%, respectively.

Using the SP definition with IgG ≥0.35 µg/mL in at least 70% of the 12 shared vaccine serotypes, the proportion of CLL patients classified as protected was significantly lower with MIA than with EIA at all time points: 5% vs. 56% at baseline, 42% vs. 79% at 4 weeks, and 26% vs. 74% at 6 months (all $p < 0.001$) (Table 3).

Table 2. Comparison of serotype specific IgG geometric mean concentrations (GMC; µg/ml) and geometric mean ratios (GMR) with 95% confidence intervals (95% CI) in CLL patients measured by MIA and EIA at all time points combined

			MIA	EIA	MIA vs. EIA	
	no. of patients	no. of samples	GMC (µg/ml)	GMC (µg/ml)	GMR[a] (95% CI)	P[a]
All timepoints						
st1	45	114	0.13	0.32	0.40 (0.30 - 0.54)	<.001
st3	45	117	0.19	0.34	0.56 (0.43 - 0.71)	<.001
st4	46	123	0.08	0.19	0.42 (0.32 - 0.54)	<.001
st5	46	126	0.09	1.83	0.05 (0.03 – 0.08)	<.001
st6B	43	110	0.21	1.31	0.16 (0.11 – 0.22)	<.001
st7F	46	123	0.55	1.16	0.48 (0.34 – 0.67)	<.001
st9V	46	124	0.33	1.45	0.22 (0.16 – 0.32)	<.001
st14	46	127	1.14	2.12	0.54 (0.40 – 0.72)	<.001
st18C	46	126	0.96	1.66	0.58 (0.44 – 0.76)	<.001
st19A	46	127	0.80	3.61	0.22 (0.15 – 0.33)	<.001
st19F	44	114	0.77	1.57	0.49 (0.40 – 0.59)	<.001
st23F	45	120	0.42	1.74	0.24 (0.15 – 0.39)	<.001

Table 3. Comparing proportions of patients with serological protection (SP) defined as ≥0.35 ug/ml in at least nine (70 %) of the 12 serotypes, analyzed with MIA and EIA.

			MIA ≥70% (9 of 12) serotypes ≥0.35 µg/ml	EIA ≥70% (9 of 12) serotypes ≥0.35 µg/ml	
	no. of patients	no. of samples	n (%)	n (%)	P[a]
All	44	116	28 (24.1)	81 (69.8)	<.001
Baseline	39	39	2 (5.1)	22 (56.4)	<.001
4 weeks after vaccination	42	42	16 (42.1)	30 (79.0)	<.001
6 months after vaccination	43	43	10 (25.6)	29 (74.4)	<.001

General discussion

The immunodeficiency observed in patients with CLL not only predisposes them to infectious complications but also significantly impairs their ability to mount effective immune responses to vaccinations. Given that pneumococcal infections represent one of the most common and severe infectious threats to CLL patients, preventive strategies are of high importance. Vaccination is an established approach to reduce the risk of severe pneumococcal disease; however, multiple studies have demonstrated that CLL patients have an impaired response to various vaccines, with the SARS-CoV-2 vaccine being the most extensively investigated. Only a few studies have previously evaluated pneumococcal vaccines in CLL and vaccine strategies need to be improved. The studies presented in this thesis contribute to the understanding of the immune response to pneumococcal vaccination in CLL and may contribute to optimization of vaccination recommendations and improvement of protection against infections.

Efficacy of pneumococcal vaccines in CLL

Study I, conducted between 2013 and 2016, is the first and only randomized pneumococcal vaccine study in CLL patients. The findings demonstrated enhanced immunogenicity of conjugate vaccines compared with polysaccharide vaccines, as measured using both ELISA and OPA, supporting the use of conjugate vaccines in this patient group. These findings also supported the vaccine recommendations that were updated 2016, including conjugate vaccine as part of primary immunization in CLL patients. Since then, the recommendation has been to administer PCV13 followed by PPSV23, 8 weeks later, a strategy supported by studies in healthy adults (181). However, only one study has previously evaluated this sequential primary immunization strategy in CLL, finding no additional benefit from adding PPSV23 (175). Similarly, in a study where PPSV23 was administered 5 years after PCV13, no improvement in immunity was found (171). The results of Study III confirm the lack of benefit from polysaccharide vaccines in CLL patients, raising questions about the role of PPSV23 in vaccination strategies for this group. This is further supported by the findings of Study II, which showed that plasmablast

responses improved only following administration of conjugate vaccines. However, with the recent availability of PCV20, the recommendation has shifted to a single dose of PCV20 for CLL patients (130, 163), decreasing the need for broadening serotype protection with PPSV23. Results of Study I confirm, in a larger study group compared with previous smaller studies (165, 166), that pneumococcal vaccination early in the disease course is beneficial for CLL patients, a principle that is now also incorporated into several vaccination recommendations and guidelines (8, 25, 115, 161). Further, results from both Study I and Study III confirm that hypogammaglobulinemia is a negative prognostic factor for vaccine response, defining a patient subgroup that requires special attention regarding infection susceptibility and improved vaccination strategies.

Evaluating clinical outcomes to pneumococcal vaccination such as pneumonia or IPD is challenging due to the need for large study populations. In the CAPiTA study (with >80,000 participants) PCV13 reduced vaccine-type IPD by 75% and pneumococcal pneumonia by 45.6% (182). In CLL patients, a retrospective study by Draliuk (n>600) showed that PCV13 vaccination prior to treatment was associated with a 45% reduction in hospital admissions for pneumonia or sepsis (183). Mauro et al. assessed both serological and clinical outcomes in CLL, but the study was limited by a small cohort and short follow-up (172). In our studies, infections were not primary endpoints due to the limited study cohort; however, CRF data reported only one case of culture-confirmed pneumococcal pneumonia approximately four years after PPSV23 vaccination in Study I and no cases of IPD during the whole study period.

Long-term immunity and revaccination strategies

Low serotype specific IgG concentrations were observed 5 years after primary immunization in Study III, with only 14% of PCV13-vaccinated patients and 5% of PPSV23-vaccinated patients achieving SP (10% overall in CLL patients). Interestingly, even among immunocompetent controls, only 33% reached SP. Similarly, Lindström et al. (170) reported a decline in antibody levels 5 years after PCV7 in both CLL patients and controls. While these findings may support

revaccination at a five-year interval, the decline in GMCs observed as early as 6 months in Study I and 12 months in Study III raises the possibility that earlier revaccination could be beneficial, although further studies are needed to confirm this. In a previous study of patients who underwent allogeneic stem cell transplantation, protective antibody levels – albeit with some serotype variability – were observed in 40% even a decade after primary immunization with repeated doses of pneumococcal conjugate vaccines (140). This suggests that repeated use of conjugate vaccines as a primary immunization strategy may offer advantages for both short- and long-term immune responses. Further investigation is warranted, particularly in subgroups of CLL patients who are expected to have a weaker vaccine response, such as those with hypogammaglobulinemia or ongoing/recent treatment.

When designing the revaccination studies (Studies II and III), the hypothesis was that revaccination with a conjugate vaccine would improve immunity in CLL. At the time the study was conducted, PPSV23 was still part of the recommended primary pneumococcal vaccination regimen, and half of the study participants had received either PCV13 or PPSV23 as their primary immunization. To ensure that all study participants received PPSV23, which covers a broader range of serotypes, a revaccination strategy was designed that would answer a) if PCV improved immunity after previous PCV or PPSV and if the effects differed depending on type of primary immunization, b) if adding PPSV to PCV improved immunity and c) if adding one more PCV after 8 weeks was safe and could further enhance the response, given the participants' underlying immune dysfunction. Our research hypothesis was supported by studies on healthy adults demonstrating that initial vaccination with PCV13 facilitates recall anti-pneumococcal responses upon subsequent vaccination, whether with a conjugate or polysaccharide vaccine (181).

We were unable to statistically confirm that priming with PCV13 enhanced the response to revaccination with another dose of conjugate vaccine after 5 years, but both the plasmablast response and serotype-specific IgG concentrations were higher in patients who had received PCV13 as their primary immunization. It is possible that this

hypothesis could be validated with a larger sample size, allowing for a more robust statistical analysis.

The results also confirmed however our hypothesis that revaccination with PCV13 after 5 years, regardless of type of primary immunization, improved immunity, but also that administration of two consecutive doses of PCV13 was beneficial.

Although repeated doses of pneumococcal conjugate vaccines have been shown to be safe in other patient groups and in studies on healthy adults (181, 184-186), it remains essential to evaluate vaccination strategies in clinical studies to confirm safety, before modifying recommendations. Additionally, hyporesponsiveness is a factor to consider when assessing pneumococcal vaccination and avoiding the use of PPSV23 could potentially reduce the risk of hyporesponsiveness (133, 134, 181). However, even though this was not a specific aim of the study and is mostly seen after PPSV, we did not observe any indication of this effect after revaccination with conjugate vaccines. AE and SAE were monitored for up to 8 weeks post-immunization, and all reported events in CLL patients and controls were grade I-II, consistent with expected reactions and supporting the overall safety of this vaccination strategy.

Nasopharyngeal samples were collected regularly throughout the study to assess the proportion of patients carrying *S. pneumoniae* and to determine whether carriage status changed following vaccination. The rationale for this investigation was based on the theoretical premise that conjugate vaccines may also induce mucosal protection. However, only two positive samples were detected (from two different patients, one from each study arm) preventing us from drawing any definitive conclusions on this matter. Additionally, no cases of IPD were reported among patients after the start of the first study and only one cultured-verified pneumococcal pneumonia, reflecting the fact that the study group was too small to reliably assess clinical outcomes.

Interestingly, the control group demonstrated surprisingly lower long-term antibody concentrations and vaccine response than expected, particularly when PPSV23 was administered as the primary

vaccine. Although our study was not specifically designed to evaluate vaccine responses in healthy elderly individuals, we observed that they responded well to revaccination with PCV13, especially if primarily immunized with a conjugate vaccine. This finding warrants further investigation, as it may provide insight into the effects of immunosenescence in elderly individuals without diagnosed immunocompromising conditions and also highlights the potential need for revaccination in this population.

Immune cell responses to pneumococcal vaccines

An additional aim in this vaccination project is to gain a deeper understanding of the immune cell response to vaccines, why peripheral blood mononuclear cells (PBMCs) have been collected both pre- and post-immunization from a substantial number of study participants following PPSV23, PCV13, and COVID-19 vaccinations, for further study purposes.

The first step in this investigation was presented in Study II, where B-cell subsets and plasmablast responses were analyzed for potential indicators of the ability to respond to repeated pneumococcal revaccinations and as markers of early immune activation (187). A key finding was the significant differences in B-cell subsets and plasmablast levels before and after immunization between CLL patients and controls, highlighting that disruption of the normal B-cell compartment could be one factor contributing to the impaired ability to mount an effective vaccine response. The plasmblast dynamics before and after pneumococcal immunization confirm the findings of Pasiarski et al. (174), demonstrating that CLL patients have lower plasmablast levels compared with controls but that an increase is seen after vaccination with a conjugate vaccine. Moreover, their study supported an association between plasmablast expansion and antibody response, a finding also observed in other immunocompromised patient groups (188). Although defining serotype-specific IgG concentrations was not part of our study design, the plasmablast response observed in Study II aligns with the findings of Study III, both suggesting an enhanced immune response following PCV13. This supports the feasibility of using plasmablast expansion as a method for evaluating early vaccine responses.

In Study II, we also demonstrated that naïve B-cells were either undetectable or present at very low levels in CLL patients at baseline, whereas, in controls, the majority of circulating B-cells were naïve. The suppression of precursor B-cells, which are essential for antigen response, may contribute to and predict impaired immune responses following vaccination (189). Few studies have previously characterized the normal B-cell repertoire across different stages of CLL. However, low proportions of circulating pre-germinal center B-cells have been reported in both CLL patients and individuals with MBL (43, 46) and was confirmed in Study II. This finding suggests that the reduced numbers of certain B-cell subsets result from decreased production, rather than solely from a lower proportional representation due to clonal lymphocytosis in the blood. An interesting study on the normal B lymphocyte repertoire in peripheral blood of CLL patients treated with either BTKi or FCR concluded that BTKi treatment preserves antigen-experienced B cells, while naïve B cells are reduced (190). In contrast, following FCR, a large number of naïve B cells reappear (as seen in one of the patients in Study II) whereas antigen-experienced B cells appear to be depleted.

Additionally, the dynamics of B-cell subsets following revaccination in the control group showed an expansion of switched memory B-cells, exhausted B-cells and plasmablasts, along with a reduction in naïve B-cells. However, these expected vaccine-induced changes could not be assessed in all subsets in the CLL cohort due to disruption by the leukemic clone, which prevented verification of changes in the switched and non-switched B-cell populations.

Immune response in treated CLL patients

Although Studies II and III included limited numbers of patients undergoing treatment, the lack of response in those receiving active or recent therapy was consistent across all results, with neither antibody nor plasmablast response in case of ongoing BTKi or recent treatment with anti-CD20 antibodies. This is consistent with previous studies on various vaccines, which have demonstrated impaired immune responses associated with CLL-specific treatments (68, 111, 112, 173, 191-199). As BTKis are recommended as continuous treatment until

progression, ongoing studies are exploring temporary discontinuation of BTKi before vaccination, which appears to be a promising approach (200-202). Additionally, studies on SARS-CoV-2 vaccination indicate that CLL patients who have undergone time-limited treatments and are in a treatment-free interval or disease remission have superior vaccine responses (195, 196). Repeated booster doses with Covid-19 vaccines in BTKi treated patients have demonstrated increased seroconversion rates, even in CLL patients who did not respond to the initial doses (195, 196, 203). Additionally, studies indicate the presence of an antigen-specific T-cell response, even in patients who fail to achieve seroconversion, which likely is important in the protection of severe disease (195, 204).

While little is known about these immune mechanisms in the context of pneumococcal vaccination, they underscore the need for further investigation—both into vaccination strategies and into T-cell responses, which remain largely unexplored. The timing of vaccination, especially early in the disease course and prior to treatment initiation, remains crucial for optimizing immune protection.

Challenges in evaluating vaccine responses

Throughout the performance of the studies in this thesis, significant differences in evaluating pneumococcal vaccine responses in CLL patients were encountered. Variability in response definitions, the number of serotypes analyzed and antibody measurement methods across studies has led to significant heterogeneity in the evaluation of pneumococcal vaccine responses in CLL patients (Table 1). The widely used cut-off values established by the WHO were originally designed to assess protection against IPD in vaccine studies for children (143), rather than clinical outcomes in immunocompromised adults. Orange et al. (2012) attempted to establish a consensus on cut-off values for immunocompromised patient groups (154) but these criteria have not been updated despite significant advancements in pneumococcal vaccination research. Furthermore, the methodologies used to evaluate antibody concentrations are shifting from EIA to MIA, and in some cases OPA, adding another layer of variability.

Although functional assays are considered the best way to correlate immune responses with protection, they remain costly, resource-intensive and require specialized research laboratories capable of applying complex methodologies. Most studies confirm that OPA correlates with EIA, whereas others report conflicting results (148, 167, 205-207), however, OPA remains the method by which antibody functionality is assessed and may better reflect protective immunity.

Differences in evaluation methods and cut-off thresholds are rarely addressed in studies, making cross-study comparisons challenging – a critical issue given the limited data available on CLL patients. Even within our own research, different evaluation methods and predefined response criteria were applied. In Study I, OPA GMTs (including predefined response cut-offs) and ELISA GMCs and GMRs were presented. In Study III, pneumococcal antibodies were evaluated using MIA, presenting GMCs and GMRs, but also adding predefined response criteria for SP and SR.

Validation studies support MIA as a replacement for EIA, with high inter-laboratory agreement (146, 208). However, most laboratories reported lower IgG concentrations for the majority of serotypes when using MIA, particularly within the ≤1 µg/mL range. This may be due to the requirement for higher-avidity antibodies to bind antigens in the MIA assay, as well as increased nonspecific binding in EIA. This may also impact the conclusions drawn when evaluating serotype-specific IgG concentrations and responses in immunosuppressed patients, such as those with CLL, who typically exhibit lower IgG levels and impaired antibody responses. Tan et al. compared MIA to EIA in a clinical vaccine study, finding a linear correlation across all 13 serotypes after PCV13 immunization. However, EIA consistently measured higher IgG levels at the lower assay range and the authors suggested to lower cut-offs for certain serotypes to align MIA with EIA-based responder rates. In Study IV, we explored the impact of using different methods on response criteria from serum samples initially analyzed in Study I and re-analyzed in study IV. Findings suggest that the selected method can impact how vaccine responses are interpreted based on commonly used response criteria, ultimately influencing the conclusions of a clinical vaccine study. This observation has also been

investigated and discussed in previous studies (158, 159, 209). This underscores the need for standardization of laboratory methodologies and possibly adjusted cut-off levels, particularly in studies involving immunocompromised populations like CLL patients. Although efforts have been made to align laboratory methods at a general level (146, 210), their clinical application in pneumococcal vaccine studies and consensus on cut-offs to evaluate response and presumed protection has not yet been sufficiently addressed.

Clinical and future aspects

While this thesis has addressed several key questions, it has also highlighted new areas that warrant further investigation. Our findings on low protective antibody levels five years after primary immunization and the benefits of revaccination after five years with pneumococcal conjugate vaccines in CLL patients should initiate discussions on improving vaccination guidelines for this patient group.

Optimizing vaccination guidelines and identifying high-risk subgroups

The result of this thesis underscores the need to refine vaccination strategies by addressing several critical aspects. These include the optimal timing of vaccination and revaccination (i.e. vaccine planning during the disease course), the identification of high-risk patient groups prone to infections and reduced vaccine responses, such as those with high disease burden, hypogammaglobulinemia or undergoing active treatment. This also includes a possible need for personalized vaccination strategies, tailored to different levels of immunodeficiency. Future studies should investigate whether certain CLL subpopulations would benefit from enhanced primary immunization strategies (e.g., repeated conjugate vaccines) in addition to later revaccinations to ensure sustained protection and if patients with more indolent disease could follow the same vaccination strategy as other risk groups. However, implementing differentiated vaccination strategies in CLL subgroups in clinical practice would require improved risk stratification tools. An ongoing study is using a machine learning model to predict the risk of infection or treatment within two years of

CLL diagnosis (211). Additionally, in another publication, risk scores for predicting infection risks incorporating factors such as age, IGHV mutation status, Binet stage, and IgG levels have been proposed to stratify patients at diagnosis (212). Such approaches may be crucial for identifying individuals who could benefit from tailored vaccination strategies, ultimately enhancing infection prevention in CLL patients.

Additionally, large-scale, industry-sponsored treatment trials in CLL should incorporate secondary studies with a more comprehensive approach, focusing on immune function and restoration. Evaluating how different treatment regimens minimize immunosuppressive effects and improve immune reconstitution could help refine treatment and vaccine strategies. This integration would also aid in identifying optimal vaccination timepoints, particularly in relation to time-limited therapies.

With an increasing number of patients receiving continuous BTKi treatment, results from current studies investigating BTKi interruption to improve vaccine response are highly needed. If consistently positive outcomes are observed, this strategy may also be incorporated into vaccination guidelines, including the option to pause BTKi therapy at the time of vaccination in patients with stable remission. However, the optimal duration of such a pause remains unclear and will hopefully be addressed in upcoming study results.

The importance of vaccination early in the disease course should continue to be reinforced in clinical guidelines and actively implemented in outpatient clinics. Standardized vaccination information should be provided to patients at the time of diagnosis to improve adherence to vaccination recommendations, both among patients and healthcare providers.

The results of this thesis support current recommendations for primary immunization with PCV as early as possible after diagnosis and suggest revaccination at a minimum interval of five years. Furthermore, based on our findings, the use of polysaccharide vaccines in CLL patients is not supported. Further research is warranted to determine the optimal timing for revaccination, which may need to occur earlier than five years, considering the decline in serotype-specific IgG

levels observed over time following both primary immunization and revaccination. Our research group is currently investigating MOPA responses in this study population to gain deeper insights into immune responses in the context of revaccination - data that may contribute to future vaccination guidelines for patients with CLL.

Future research directions

Future studies assessing multiple aspects of pre- and post-vaccination immunity may further improve our understanding of immune response dynamics and contribute to refining response criteria and vaccination strategies. As vaccine research progresses, it is important to recognize that applying a single, uniform cut-off value across all pneumococcal serotypes may not fully capture the complexity of the immune response. Emerging evidence suggests that serotype-specific thresholds may need to be revised to more accurately reflect protective immunity. Addressing this issue in future research is important to improve our understanding of serotype-specific vaccine effectiveness, both in the short and long term, and to refine the correlates of protection.

Future clinical vaccine studies in CLL should be designed to investigate the effects of repeated PCV administration as a primary immunization strategy, compared to the current single-dose regimen, in terms of both short- and long-term protection. Studies on dosing schedules may also be extended to other vaccines recommended for patients with CLL, such as herpes zoster and respiratory syncytial (RS) virus vaccines. Investigations should also aim to identify optimal approaches for subgroups at particularly high risk of poor vaccine response. This also includes exploring strategies for stratifying these patients based on known risk factors and evaluating criteria or tools applicable in clinical practice.

An important area for future research is T-cell immunity and its role in pneumococcal vaccine responses, an aspect that remains largely unexplored, both in patients with CLL and in the broader context of pneumococcal vaccination. Evaluating CD4+/CD8+ T-cells including Th1, Th2, Treg, Tfh, Th17 and NK cells through phenotypic assessment using flow cytometry, along with assessing antigen-specific

responses following primary immunization and revaccination, would provide valuable insights into the immune mechanisms underlying vaccine responses in CLL patients and immunocompetent controls.

There is particular interest in further investigating Th17 cells, both in relation to disease pathogenesis and their potential role in the immune response to pneumococcal vaccination. In CLL, Th17 cells have been suggested to play a significant role in disease progression, with higherTh17 levels potentially exerting a protective effect (60, 63, 213). Th17 cells also appear to be involved in protection against pneumococcal infection and in vaccine responses (214, 215). Reduced Th17 levels have been observed in methotrexate-treated patients with rheumatic diseases, correlating with impaired antibody and plasmablast responses to pneumococcal vaccination (188). This subject warrants further investigation in the context of CLL. Key research questions include whether pneumococcal vaccination induces Th17, IL-17A, and Th17 memory responses in CLL patients and controls, and whether these responses correlate with increased IgG production and opsonophagocytic activity - or if baseline Th17 levels can even predict vaccine responsiveness.

Future research efforts in our group, already underway, include analysis of the extensive PBMC, serum and plasma samples collected throughout the study period (2013–2022), covering both primary immunizations and revaccinations with pneumococcal and COVID-19 vaccines in CLL patients and controls. This will yield a unique dataset capturing immune cell responses to polysaccharide vaccines, pneumococcal conjugate vaccines, vector-based vaccines and mRNA vaccines within the same study population. The cohort offers a valuable opportunity to assess B- and T-cell populations and to correlate these findings with patient characteristics, antibody titers, immune cell function (e.g., cytokine levels), vaccine type and vaccination strategies. Gaining a deeper understanding of these relationships may provide important insights into the immunological mechanisms underlying pneumococcal vaccine responses in CLL patients, potentially identifying predictors of vaccine efficacy and contributing to future strategies aimed at optimizing mucosal, humoral and cellular immunity.

Limitations

In Study I, one limitation was that approximately one third of participants did not have sufficient serum volume for ELISA analysis. Nevertheless, the available samples were adequate to evaluate the primary endpoint with OPA. Additionally, for at least 3 serotypes more than 30% had OPA titers above the LLOQ, potentially reflecting prior infections or vaccinations administered more than five years before inclusion. However, this information was not further specified in the CRFs at inclusion, which limits further interpretation. Despite this, significant increases in both GMTs and GMCs were observed, suggesting that these factors did not substantially affect the study's conclusions. Statistical adjustments were also made to account for differences in pre-vaccination antibody levels in the different groups. It should also be noted that the company responsible for performing the laboratory analyses also sponsored the study, which may introduce a potential source of bias despite efforts to ensure methodological quality.

In Study II, the sample size was limited due to recruitment being restricted to a single center and the requirement for PBMC sampling seven days post-immunization. Despite this, significant changes in plasmablast responses were observed before and after revaccination with conjugate vaccines. A further limitation was that plasmablasts and other B-cell subsets were assessed as proportions of the total B-cell population rather than as absolute cell counts, which may limit direct comparability between individuals or groups with differing B-cell numbers. Additionally, plasmablast kinetics were not correlated with pneumococcal antibody responses or T-cell responses, which could have provided a more comprehensive understanding of the immunological mechanisms underlying vaccine responsiveness.

Although Study III represents the largest study to date on pneumococcal revaccination in CLL patients, one limitation is the relatively small number of patients in each study arm. This may have prevented some findings from reaching statistical significance, although clear trends were observed. Moreover, measurements of serotype-specific circulating antibody concentrations do not necessarily reflect antibody functionality, studies of which would require OPA analysis.

Furthermore, a limitation is that the current vaccine recommendations are based on PCV20, whereas this study used PCV13. However, the findings remain relevant for the other pneumococcal conjugate vaccines, as they share similar immunological principles and mechanisms of action. Additionally, as the majority of patients in this study were treatment-naïve, conclusions cannot be drawn regarding the impact of vaccination in the few CLL patients undergoing specific treatment regimens.

A major limitation of Study IV was that it was not specifically designed for direct method comparison. Although this analysis does not allow for definitive conclusions regarding methodological differences, it highlights the impact of method selection on the interpretation of vaccine responses. In addition, the sample size of the study is also small, meaning that individual samples can have a significant impact on the observed differences in concentrations measured by the EIA and MIA methods, which may affect generalizability of the results. Since the data consists solely of patient samples, it is likely that both the quantity and quality of antibodies in the subjects are deficient compared to healthy individuals. This may result in a higher number of samples containing low-avidity antibodies in the dataset and such samples may fall below the detection limit with the MIA method but yield positive results with the EIA method. Another limitation was therefore that the evaluation was not conducted also in a control group of immunocompetent individuals, making it unclear whether the observed differences are attributable to methodological variations or potentially the quality of antibodies in some CLL patients. Additionally, MIA analyses were conducted several years after EIA, requiring long-term storage and transport of serum samples, which could potentially affect sample quality. However, serum antibodies are generally considered stable, provided that freeze-thaw cycles are minimized and established storage protocols are followed. As far as we know, these conditions were adhered to in this study.

Conclusions

- PCV13 induces a stronger immune response than PPSV23 in untreated CLL patients for most shared serotypes, although responses are impaired in patients with longer disease duration and hypogammaglobulinemia. These findings support administering PCV as early as possible after CLL diagnosis.

- Suppression of precursor B-cells and an impaired plasmablast response to pneumococcal revaccination were observed in CLL patients. Repeated revaccinations with PCV enhanced the plasmablast response, suggesting its potential as an early indicator of vaccine efficacy.

- Impaired antibody persistence was observed in CLL patients five years after immunization, but revaccination with PCV improved immunity. These findings highlight a potential need for revaccination in CLL and may support updates to vaccination guidelines for this patient population.

- Two consecutive doses of pneumococcal conjugate vaccines enhanced antibody responses further and should be evaluated systematically in subgroups with impaired immunity, such as patients with hypogammaglobulinemia or those undergoing active treatment.

- The choice of analytical method for antibody measurement may influence the interpretation of vaccine response according to established criteria. There is a need to harmonize response criteria and to evaluate the impact of assay selection in order to improve consistency in results and conclusions across pneumococcal vaccine studies in immunocompromised patients.

Svensk sammanfattning (Summary in Swedish)

Patienter med blodsjukdomen kronisk lymfatisk leukemi (KLL) har en ökad risk för infektioner orsakade av *Streptococcus pneumoniae* (pneumokocker), en av de vanligaste orsakerna till lunginflammation. Vaccination mot pneumokocker kan förebygga allvarliga infektioner, men vaccinationssvaret är ofta nedsatt i denna patientgrupp eftersom både sjukdomen och dess behandlingar påverkar immunförsvaret negativt. Kunskapen om vilket vaccin och vilken vaccinationsstrategi som ger bäst immunsvar hos patienter med KLL är begränsad. För närvarande används två typer av pneumokockvaccin, konjugerat vaccin (PCV) och polysackaridvaccin (PPSV), vilka stimulerar immunförsvaret på olika sätt. Syftet med denna avhandling är att undersöka vilket pneumokockvaccin som ger bäst skydd hos KLL-patienter och om immunsvaret kan förbättras genom revaccination med PCV.

I **studie I** jämfördes PCV och PPSV hos KLL-patienter som ännu inte hade fått behandling för sin sjukdom. Vaccinationssvaret utvärderades efter 4 veckor och 6 månader, och resultaten visade att PCV gav ett bättre immunsvar än PPSV hos dessa patienter.

I **studie II och III** undersöktes kvarvarande immunsvar efter 5 år samt effekten av revaccination. Patienterna fick antingen två doser PCV med 8 veckors mellanrum eller en dos PCV följt av en dos PPSV. Immunsvaret utvärderades genom att mäta ökningen av antikroppar samt plasmablaster (antikroppsproducerande celler) före och efter vaccination. Resultaten visade ett lågt kvarvarande immunsvar efter första vaccinationen men att PCV förbättrade svaret. Detta stödjer att patienter med KLL kan ha nytta av revaccination efter fem år.

I **studie IV** undersöktes om valet av analysmetod vid antikroppsmätning påverkade tolkningen av immunsvar enligt etablerade kriterier för vaccinationssvar. Vi fann att valet av analysmetod hade betydelse för tolkningen, vilket understryker vikten av konsensus kring metodval och standardisering av hur immunsvaret ska utvärderas hos KLL-patienter efter pneumokockvaccination.

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