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ANTI-PHOSPHORYLCHOLINE ANTIBODIES IN CARDIOVASCULAR DISEASE

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Stockholm 2013

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to my father

ABSTRACT

Cardiovascular disease (CVD) is the leading cause of morbidity and mortality in the industrialized countries and a growing concern for the developing world. The underlying cause of almost all CVD is atherosclerosis, a process that is characterized by a low-grade inflammation in the artery walls. This inflammation is believed to be initiated by components of low density lipoproteins which, upon oxidation, display pro-inflammatory self-neo epitopes such as malonyldialdehyde (MDA) and phosphorylcholine (PC). Antibodies directed against the PC-epitope (anti-PC) have been researched for several decades but the relatively recent realization that these antibodies also recognize oxidized phospholipids has revolutionized the field and opened up a whole new avenue of investigation. Anti-PC has been shown to aid the clearance of apoptotic cells and prevent the formation of foam cells by clearing oxidized low density lipoprotein. Murine studies with PC-vaccination have shown strong beneficial effects on experimental atherosclerosis *in vivo* and human epidemiology has consistently linked anti-PC insufficiency to CVD.

All humans have detectable anti-PC IgM in serum, though the concentration varies greatly between individuals. Our group has previously shown that people with low serum/plasma levels of anti-PC IgM have increased risk of CVD and the subgroup analysis had indicated that this association was particular strong with regard to the incidence of stroke. In paper I, we tested this hypothesis in a stroke material from northern Sweden. A significant association between low plasma level of anti-PC IgM at baseline and incident stroke was seen for the whole group at anti-PC levels below the 30th percentile (OR 1.62; CI 1.11 to 2.35). Analyses of gender-specific associations indicated fairly strong associations for females, especially at the lowest 30th percentile (OR 2.65; CI 1.41 to 4.95). However, no association was noted for men.

Paper II focused on the properties of different anti-PC antibody classes/subclasses. We report that anti-PC IgM, IgA and IgG1 (but not IgG2) were negatively associated with IMT-progression, which is a surrogate marker for atherosclerosis development. Examination of binding profiles revealed that the protective isotypes (IgM, IgA and IgG1) have a different fine-specificity than the non-protective IgG2. Analysis of serum samples taken four years apart in study participants showed that anti-PC IgM titers, essentially, do not change over time. In this paper, we also demonstrate that anti-PC IgM inhibits LPC-induced cell death *in vitro* and propose that this is yet another protective mechanism.

For paper III, I had successfully designed a PC-specific probe to identify, isolate and characterize PC-reactive B cells from ten healthy human donors. We found that all ten had mounted somatically mutated antibodies towards PC utilizing a broad variety of immunoglobulin-genes. PC-reactive B cells were primarily found in the IgM^+ memory subset though significant numbers were also detected among naïve, IgG^+ and $CD27^+CD43^+$ B cells. From the isolated B cells, we derived several human monoclonal antibodies (mAbs) with proven PC specificity.

In conclusion, the future of CVD treatment lies with immunomodulation and the premise of this thesis represents one avenue of research in the quest for novel diagnostic tools and improved treatment options. My work has focused on understanding the properties and molecular ontogeny of human anti-PC antibodies. Though this thesis represents one important step in the direction of clinical application, much more research is needed before we will see use of anti-PC mAbs or PC-vaccination in the treatment of patients.

LIST OF PUBLICATIONS

I. **Fiskesund R**, Stegmayr B, Hallmans G, Vikstrom M, Weinehall L, de Faire U and Frostegard J. "Low levels of antibodies against phosphorylcholine predict development of stroke in a population-based study from northern Sweden." *Stroke. 2010 Apr; 41(4): 607-612*.

II. **Fiskesund R,** Su J, Bulatovic I, Vikström M, de Faire U, Frostegard J. IgM phosphorylcholine antibodies inhibit cell death and constitute a strong protection marker for atherosclerosis development, particularly in combination with auto-antibodies against modified LDL. *Results in Immunology. 2012. 2(0): 13-18.*

III. **Fiskesund R,** Steen J, Amara K, Murray F, Szwajda A, Liu A, Douagi I, Malmström V and Frostegård J. Naturally occurring human phosphorycholine antibodies predominantly products of affinity-matured B cells in the adult. *Manuscript to be published.*

RELATED PUBLICATIONS

Ajeganova S, **Fiskesund** R, de Faire U, Hafström I, Frostegård J. Effect of biological therapy on levels of atheroprotective antibodies against phosphorylcholine and apolipoproteins in rheumatoid arthritis - a one year study. *Clin Exp Rheumatol. 2011 Nov-Dec;29(6):942-50.*

Liu A, Ming JY, **Fiskesund R**, Ninio E, Karabina SA, Bergmark C, Frostegård AG, Frostegård J. Induction of dendritic cell-mediated T cell activation by modified but not native LDL in humans and inhibition by Annexin A5: heat shock proteins as possible T-cell antigens. *Manuscript to be published*

LIST OF ABBREVATIONS

PC	Phosphorylcholine
Anti-PC	Antibody against phosphorylcholine
CVD	Cardiovascular Disease
Ig	Immunoglobulin
LDL	Low density lipoprotein
oxLDL	Oxidized low density lipoprotein
TCR	T cell receptor
BCR	B cell receptor
AID	Activation induced deaminase
MDA	Malonyldialdehyde
PTC	Phosphatidylcholine
PAF	Platelet activating factor
LPC	Lysophosphatidylcholine
ScR	Scavenger receptor
APC	Antigen presenting cell
DC	Dendritic cell
NK cell	Natural Killer cell
NKT cell	Natural Killer T cell
SMC	Smooth muscle cell
PAMP	Pathogen associated molecular pattern
PRR	Pattern recognition receptor
TLR	Toll-like receptor
LPS	Lipopolysacharide
CRP	C-reactive protein
mAb	monoclonal antibody
ELISA	Enzyme linked immunosorbent assay
IMT	Intima-media thicknness
FACS	Flowcytometry activated cell sorting
CDR	Complementarity determining region
Lp-PLA2	Lipoprotein associated phospholipase A2

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1. INTRODUCTION

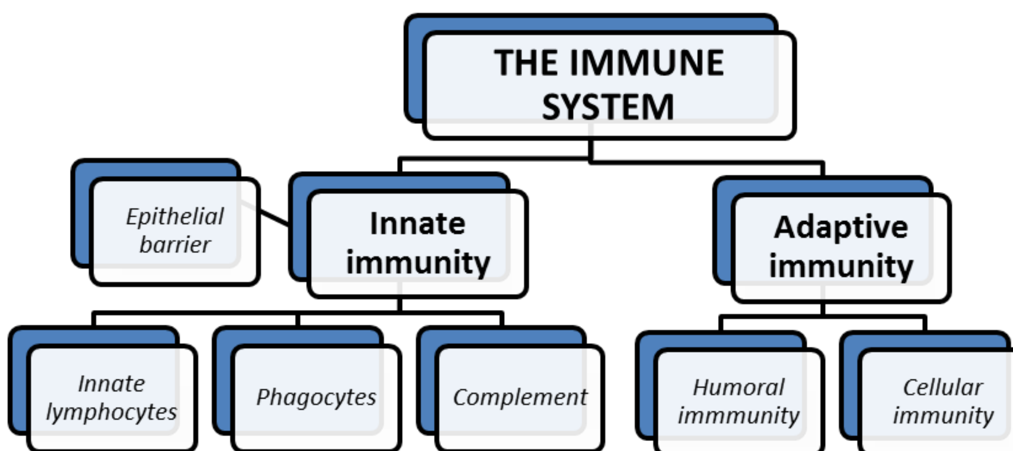
Cardiovascular disease (CVD) is the leading cause of morbidity and mortality the industrialized countries and a growing concern for the developing world. During the past century, research has made enormous strides in understanding this disease from a clinical perspective. At present, medical doctors have a multitude of proven drugs at their disposal and a good understanding of some major risk factors for developing CVD (smoking, obesity, hypertension, diabetes etc). However, the molecular understanding of atherosclerosis, which is the underlying cause behind almost all CVD is still patchy and a lot of research is currently devoted to studying this process for the purpose of identifying new risk factors/markers and finding novel targets for a new generation of drugs.

The scientific work in this thesis represents one avenue of research in the quest for better diagnostic tools and treatment options for patients with CVD. The contents revolve around the proposed protective role of phosphorylcholine antibodies (anti-PC) in CVD, their properties and their origin. This introduction will facilitate reader understanding of the experimental and epidemiological studies performed by providing a brief overview of the immune system, atherosclerosis and anti-PC.

1.1 THE IMMUNE SYSTEM

The main purpose of the immune system is to defend the organism against invading pathogens such as bacteria, fungi, parasites and viruses. However, there are also several other vital purposes. One such auxiliary purpose is the surveillance of endogenous processes in order to locate and eliminate tumor cells. Another proposed function of the immune system is the clearance of apoptotic cells and oxidatively damaged molecules. Figure 1 provides a schematic overview of the immune system, showing its major branches.

Figure 1. The organization of the immune system.



1.1.1 Innate immunity

All multicellular organisms possess a pre-programmed system for eliminating tumor cells, invading microbes and cleaning up damaged endogenous molecules/cells. The receptors and effector molecules of innate immunity are *germline encoded* and this system represents the organisms' first line of defense. These are the components of the innate immune system:

1. An epithelial barrier covers the entire interface between the body and the outside world. Besides the obvious physical barrier function, epithelial cells are also capable of producing antimicrobial peptides and enzymes among many things. This layer is also home to many dendritic cells (discussed below) and the mast cells, which are important in fighting parasites and mediating allergic reactions.

2. The complement system is a collection of circulating membrane-associated proteins, many of which possess proteolytic activity. Complement does not attack endogenous cells because these exhibit regulatory proteins on their surface. Bacteria lack these regulatory proteins and after complement associates with a microbial surface, a cascade is activated which results in the formation of pores and subsequently bacterial lysis and death. This microbial surface association may be spontaneous but it can be enhanced by the presence of antibodies on the microbe.

3. Phagocytes can be subdivided into neutrophils, dendritic cells and monocytes/macrophages. They ingest microbes and clean tissues by removing cell debris and dead cells. Engulfed pathogens are subjected to killing by reactive oxygen species (ROS) and degradation by enzymes. *Neutrophils* are the most abundant class of immune cells in circulation. They are quickly mobilized to the site of infection, where they engulf microbes and die after a few hours. *Monocytes* are less abundant in circulation but once they enter extravascular tissue, they differentiate into *macrophages* which live for long periods of time. As a general rule, monocytes/macrophages are involved in chronic inflammatory conditions, e.g. atherosclerosis and rheumatoid arthritis whereas neutrophils are associated with acute inflammation.

   Dendritic cells are the precursors professional antigen presenting cells (APCs) that connect the innate with the adaptive immune system. These cells share genealogical lineage with monocytes and reside in extravascular tissue, where their main purpose is to detect foreign molecules by engulfing particles around them and after degradation, initiate powerful adaptive immune responses by presenting these non-self peptides to lymphocytes of the adaptive immune system.

4. Innate lymphocytes can be broadly divided into NK cells, NKT cells, γδ T cells and B1 cells. *Natural Killer cells* or NK cells have been extensively studied, since their discovery at Karolinska

Institutet many years ago. They primarily respond to intracellular microbes by killing infected host cells but are also believed to be important in removing tumor cells. *Natural Killer T (NKT) cells* and *γδ T cells* are less well understood but are believed to recognize non-peptide antigens, such as polysaccharides and phospholipids.

B1 cells are perhaps the most enigmatic among the innate lymphocytes. Theoretically speaking, these are B cells that possess a limited set of gene rearrangements for their antigen receptor and spontaneously secrete "natural" IgM antibodies without external antigen stimulation. Furthermore, B1 cells are supposed to be primarily located in the peritoneal cavity and respond to lipid and polysaccharide antigens. In textbooks as well as many research papers, phosphorylcholine (PC) is often portrayed as a prototypic B1 antigen and antibodies against PC (anti-PC) are assumed to be the product of B1 cells.

One major problem in this research field is that nobody has convincingly shown that B1 cells exist in humans. All research on B1 cells has been done in mice and the data has simply been extrapolated to humans. While mice unequivocally have a population of $CD5^{+}$ B cells that fulfill the above-mentioned description; in humans CD5 does not distinguish innate B1 cells from regular B cells (B2 cells). In 2011, Griffin *et al* published a paper in the prestigious Journal of Experimental Medicine, where they claim that human B1 cells are $CD20^{+}CD27^{+}CD43^{+}$ and $CD70^{-}$ *(Griffin, Holodick et al. 2011)*. However, their data could not be reproduced by renowned fellow scientists in letters to the aforementioned journal *(Descatoire, Weill et al. 2011)*. Our group has not been able to fully reproduce their data either, as will be discussed later. In conclusion, the presence of B1 cells in humans is an interesting but highly controversial subject. This is also an issue that is intimately linked with this thesis since anti-PC antibodies are supposedly products of B1 cells.

The components of innate immunity recognize evolutionarily conserved structures that are shared by various classes of pathogens but absent from healthy endogenous cells. These structures represent “danger signals” or pathogen associated molecular patterns (PAMPs), which the immune system has evolved to identify. Examples of such foreign PAMPs are bacterial lipopolysaccharide (LPS), double stranded RNA and unmethylated CpG nucleotides, which are common in bacterial DNA but absent in mammalian DNA. These structures are detected by pattern recognition receptors (PRR), which are germline encoded and are capable of recognizing approximately 10^3 different patterns. Examples of PRRs are Toll like receptors (TLRs), scavenger receptors (ScRs).

1.1.2 Adaptive immunity

Whenever pathogens manage to breach the strong defense mechanisms that constitute the innate immunity, the hope of the organism lies with its adaptive immunity. Unlike the very rapid innate responses (immediate to a few hours), the more powerful adaptive immune system is slow to mobilize (days to weeks). This system consists of lymphocytes and their products; it has a very high degree of specificity and can recognize and respond to literally billions of different antigens.

The adaptive immunity is divided into *cellular immunity* mediated by cytotoxic T cells expressing T cell receptors (TCR) and *humoral immunity,* which consists of B cells producing antibodies. Central to both systems are the dendritic cells (DCs) briefly mentioned in the previous section on innate immunity. Tissue residing DCs are constantly sampling/monitoring the extra cellular fluid around them. During a localized infection, the microbes will inevitably trip the pattern recognition receptors (PRRs) of the DCs, thus triggering production of cytokines such as tumor necrosis factor alpha (TNF-α) and the migration of DCs to the local lymph node. With them, the DCs bring microbial antigens that they have acquired either through pinocytosis of soluble antigens or phagocytosis of entire microbes. During the journey to the lymph node, the DCs mature into professional antigen presenting cells.

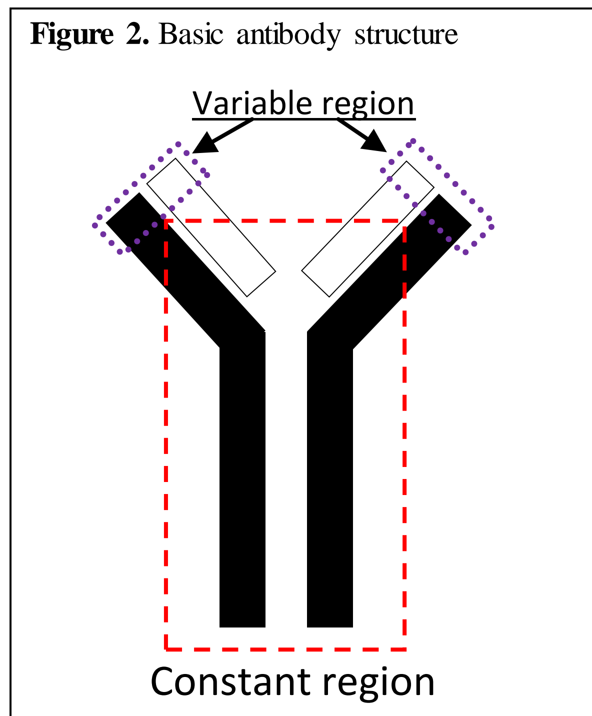
The DCs break down everything they engulf and present the fragments on their surface using antigen presenting molecules called MHC class II. A foreign peptide presented on the MHC:peptide complex will be recognized by T lymphocytes specific for that particular epitope, at the lymph node, and result in the activation of these T cells. Another group of important antigen presenting molecules on DCs is the CD1 family which is believed to present lipid antigens.

1.1.2.1 Humoral immunity

Antibodies are the effector molecules of the humoral immunity. The basic antibody unit consists of four polypeptide chains, two identical long heavy

chains (H) and two identical short light chains (L) which are combined to form a Y-like structure. The light chains may be either of the Kappa (κ) or the Lambda (λ) type.

Figure 2. Basic antibody structure



This basic unit has one constant (C) region which is shared by all antibodies of the same class. The other part of this macromolecule is the variable (V) region, which is used to bind antigen. The composition of the V-region is unique for each antibody clone and differentiates it from other antibody clones.

The V-region is formed by combining a part from the heavy chain (V_H) and one part from the light chain (V_L). Each chain has three important complementary determining regions (CDRs), which together make up most of the antigen binding pockets. Of these six CDRs, the most important for antigen binding and incidentally also the most heterogeneous region is the heavy chain CDR3. The peptide sequence at this site so diverse that is often used to distinguish between different antibody clones much like fingerprints are utilized to identify people.

The human body can produce an almost infinite number of different antibody-specificities and this variability is attained by two different mechanisms:

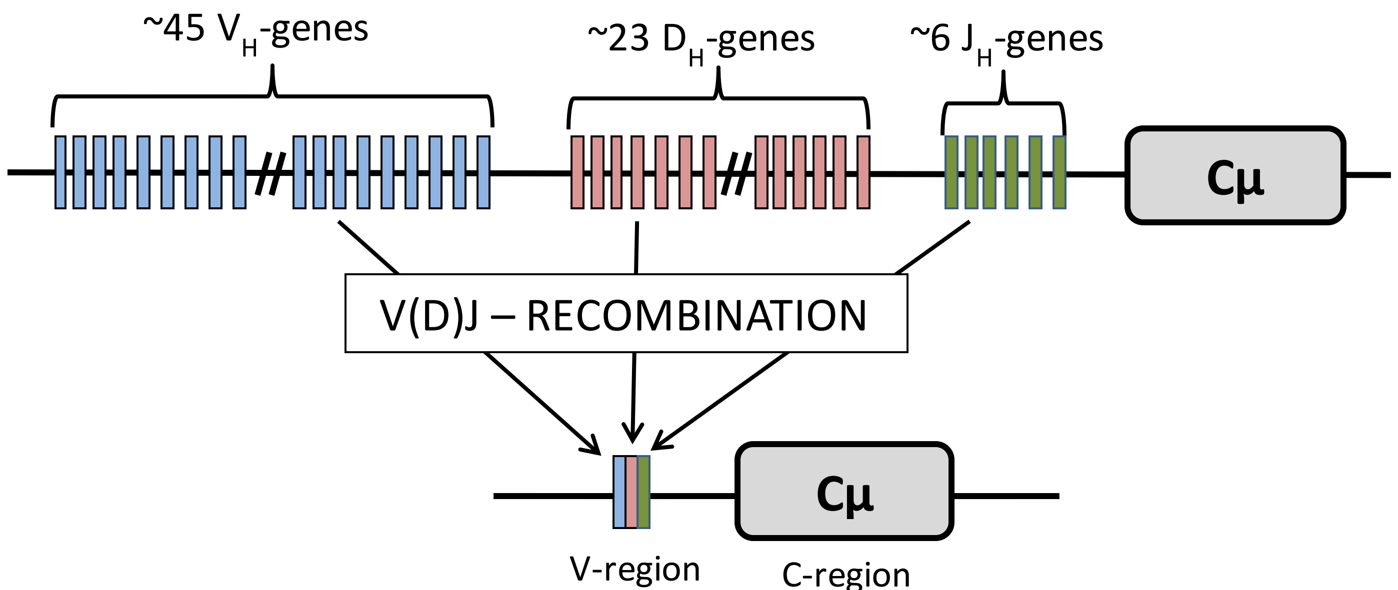
1. **Combinatorial diversity.** The human body has roughly 45 functional heavy V-genes, 23 D-genes and 6 heavy J-gene segments. One heavy chain is formed by combining one segment

from each of the three gene-families. Hence, it is mathematically possible to generate 45×23×6 = 6210 different heavy chains.

As above mentioned, there are two types of light chains, Kappa and Lambda. Kappa light chains are formed by combining one of 35 Vκ-genes with one of 5 Jκ-genes (35×5 = 175 possibilities). Lambda chains are formed by combining one of 33 Vλ-genes with one of 5 Jλ-genes (33×5 = 165 possibilities). Notice that there are no D-gene segments on light chains.

Finally, by joining 6210 possible heavy chains with either 175 possible Kappa chains or 165 possible Lambda chains, we arrive at 6210×175 + 6210×165 ≈ **2.1 million possible antibody Variable-regions**.

Figure 3. The generation of heavy chain combinatorial diversity.



2. **Junctional diversity.** The somatic recombination of V, D and J (heavy chains) or only V and J (light chains) is mediated by a collection of enzymes which together are referred to as the *V(D)J-recombinase*. When the chosen V, (D) and J gene segments are cut from the germline DNA and combined together, additional diversity is introduced by enzymes which may add or delete a random

number of nucleotides at the interface between two chosen gene-segments, often leading to frameshifts. This junction region encodes the previously-mentioned highly variable CDR3-region, which is so important for antigen recognition. Adding junctional diversity on top of combinatorial diversity creates an almost infinite number of possible antibody specificities (10^{11}).

The generation of diversity occurs in the bone marrow at the pre-B cell state. Progenitor cells committed to the B cell lineage first develop into pro-B cells before maturing into pre-B cells. Upon completion of recombination, the expression of functional IgM initiates a signaling cascade that silences the *V(D)J-recombinase* and heralds the advent of the immature B cell. After also acquiring IgD on its surface, the B cell is considered mature.

Table 1. Steps in the maturation of B lymphocytes

	Progenitor cell	**Pro-B cell**	**Pre-B cell**	**Immature B cell**	**Mature B cell**
Immunoglobulin DNA	Germline DNA	Germline DNA	Recombination in progress	Recombined DNA	Recombined DNA
Immunoglobulin expression	None	None	Cytoplasmic heavy chains	Surface IgM	Surface IgM and IgD

There are mainly two check-points in the development of the B cells that assures the generation of healthy, non-autoreactive B cells.

1. If recombination of the germline DNA at the pre-B cell stage fails to produce functional proteins, the nascent B cell enters apoptosis.

2. Immature B cells that through recombination have acquired functional antibodies that bind native molecules present in the bone marrow with high affinity either die by apoptosis or may activate the recombinase machinery again to try to obtain a different non-autoreactive specificity by fashioning a new light chain.

Antibodies are divided into five major classes based on the heavy chain constant region: IgM, IgD, IgA, IgE and IgG. The IgA and IgG classes are further subdivided into subclasses: IgA1, IgA2, IgG1, IgG2, IgG3 and IgG4. Each antibody type has its own special abilities, which are conferred/determined by the antibody Fc-region. This part is located at the tail of the Y-like antibody molecule. For example, only the Fc-region of IgE is able to dock to mast cells Fcε-receptor with high affinity.

Table 2. The immunoglobulin classes and their function

Function	IgM	IgD	IgG1	IgG2	IgG3	IgG4	IgA	IgE
Neutralization	+	-	++	++	++	++	++	-
Opsonisation	+	-	+++	-/+	++	+	+	-
Sensitization for NK cell killing	-	-	++	-	++	-	-	-
Sensitization of mast cells	-	-	+	-	+	-	-	+++
Complement activation	+++	-	++	+	+++	-	+	-
Mean serum concentration (mg/ml)	**1.5**	**0.04**	**9**	**3**	**1**	**0.5**	**2.1**	**$3x10^{-5}$**

All B cells initially express IgM. The rearranged V_H-region is literally adjacent to the Cμ and Cδ DNA segments and these are transcribed together into a single mRNA molecule. IgM and IgD are simply different splice variants of the same original mRNA. The Cγ (IgG), Cα (IgA) and Cε (IgE) gene segments are further away and are not transcribed in the naïve B cell. However, upon activation through stimulation by helper T cells and/or cytokines, the enzyme *activation-induced deaminase* (AID) is turned on and switch recombination may occur. Depending on the cytokine environment, the rearranged V_H-section is brought into proximity with Cγ, Cα or Cε and thus the B cell has undergone class-switch. It will still have the same specificity but no longer express IgM, instead it will produce IgA, IgG or IgE. Class-switch enables the B cells to produce antibodies with abilities tailored to the specific needs of the situation, e.g. IgE against parasite infection.

Resting B cells express their particular antibody on their surface as B cell receptors (BCRs). When a B cell encounters its target, it will bind the antigen with its BCRs and engulf the pathogen. After degrading the antigen, class II MHC-peptide complexes will be displayed on the cell surface for recognition by helper T cells. In this sense, B cells are also a kind of antigen presenting cell but unlike DCs, they may only present the antigen that they specifically recognize. When the B cell encounters a helper T cell that recognizes the peptide fragment displayed on the MHC class II, it activates the T cell which in turn activates the B cell through cytokines and the CD40 ligand. After receiving the “green light” from the helper T cell, the B cell undergoes proliferation, class-switch and differentiation. The progeny of this naïve B cell may differentiate into antibody factories (plasma cells) or memory cells.

Repeated or prolonged exposure to an antigen initiates a process dubbed affinity maturation. During the rapid proliferation in the germinal centers of lymphoid follicles, the rearranged V_H and V_L genes of B cells are subjected to a high frequency of random point mutations by a process involving *activation-induced deaminase* (AID). This somatic hypermutation among the proliferating B cells results in a multitude of variants of the initial antibody. All these variants compete for binding to antigen displayed by follicular dendritic cells. The B cells carrying BCRs with the highest affinity succeed in binding while other B cells die by apoptosis. The mutations are randomly introduced but because of the evolutionary pressure to produce higher affinity antibodies, the successful surviving B cells tend to have accumulated good mutations in the CDR-regions. The mutations also tend to be of the replacement type, i.e. mutations that cause an actual change in the polypeptide sequence. Silent mutations are defined as mutations that cause no change in the translated protein, e.g. nothing happens if the codon TCC is mutated to TCT since both code for the amino acid Serine. By convention, a ratio of Replacement to Silent mutations greater than 2.9 is indicative of affinity maturation *(Shlomchik, Marshak-Rothstein et al. 1987)*.

Sometimes, B cells may initiate immune responses without helper T cells. Polysaccharides and lipids can sometimes elicit antibody responses without helper T cells. These responses have thus been termed T-independent. In the case of bacterial polysaccharides, it is believed that their polymeric structure is able to crosslink a large number of BCRs and thus induce a strong activation signal in a B cell, which may then start to proliferate and produce IgM antibodies. Recent data has shown that class-switch and affinity maturation is also possible during this kind of immune responses *(Ueda, Liao et al. 2007, Herlands, Christensen et al. 2008, Yang, Ghosn et al. 2012)*. However, the subject of T-independent antigens in human biology is still poorly understood and the line between this kind of antibody responses and that of natural B1 cells is rather blurry. The third manuscript of this thesis does shed some light on this issue but much more research is needed.

1.1.2.2 Cellular immunity

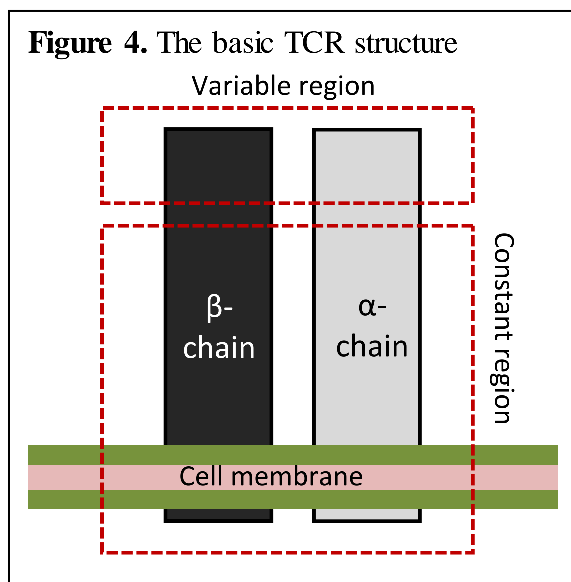


Figure 4. The basic TCR structure

The responsibility of the cellular immunity is to eradicate intracellular microbes, which may come from either direct invasion of host cells or ingestion by phagocytes. Cellular immunity is mediated by two types of T lymphocytes, helper T cells ($CD4^+$) and cytotoxic T cells ($CD8^+$), which both express T cell antigen receptors (TCRs). TCRs are essentially generated by the same recombination mechanisms as seen with the BCRs/antibodies and it has been estimated that each individual can produce 10^{16} unique TCRs, recognizing a wide array of peptides. TCRs consist of an α-chain and a β-chain, which both participate in antigen recognition. Just like heavy and light chains of BCRs/antibodies,

each of the two chains that make up the TCR contain a variable portion and a constant portion.

Differences between BCRs/antibodies and TCRs

- TCRs are membrane-bound proteins that are never secreted
- TCRs recognize processed peptide fragments of antigens presented on MHC class I (cytotoxic T cells) or class II (helper T cells). This is different from BCRs/antibodies, which recognize and bind to the three-dimensional surface of antigens.
- B cells mature in the bone marrow whereas T cells undergo their maturation process in the thymus.

T lymphocytes circulate through the peripheral lymphoid organs searching for foreign protein antigens. These are transported from the portals of microbial entry to the lymphoid organs by activated dendritic cells (DCs). At the lymph node, the DCs display processed peptides on MHC class II molecules for helper T cells. They may also "cross-present" viral antigens to cytotoxic T cells on MHC class I from infected host cells that have previously been ingested, thus priming cytotoxic T cells which are specific for that antigen. Activated cytotoxic T cells proliferate and differentiate into killer cells that limit the spread of intracellular pathogens by lysing infected host cells that display viral peptides on their MHC class I.

MHC class I and II

- MHC class II molecules are used by antigen presenting cells (APCs) to display peptide fragments from ingested and degraded antigens. These are recognized by helper T cells.
- MHC class I molecules are present on all cells and display peptide fragments from all proteins that are being translated inside the cell. Viruses utilize the host cell's ribosomes to produce viral proteins and infected cells will thus display viral peptides on their MHC class I, which may be recognized by cytotoxic T cells.

- T cells bind to the MHC molecules using their TCRs and an additional co-receptor. Helper T cells express CD4 while cytotoxic T cells express CD8. Binding to MHC class II requires CD4 and binding to MHC class I requires CD8.

A naïve helper T cells that recognizes its specific peptide fragment presented by an activated (i.e. also expressing co-stimulators) APC becomes itself activated. Depending on the type of stimulation, the naïve T cell may differentiate into one of several distinctive types of effector cells. The best characterized phenotypes are T_H1 and T_H2 but other subsets like T_H17 and T_{REG} are undoubtedly also immensely important. Each subtype produces a limited set of cytokines and performs different functions. For example, the T_H2 subtype which produces IL-4 and IL-5, directs B cells to produce anti-parasite IgE and activates eosinophils.

For the pathogenesis of atherosclerosis, the T_H1 subtype is generally regarded as most important. It secretes the pro-inflammatory IFN-γ which under normal circumstances tells phagocytes to eliminate ingested microbes and stimulates B cells to produce opsonizing/complement-recruiting IgG1. However, in the atherosclerotic plaque, IFN-γ exacerbates the destructive inflammation.

1.2 ATHEROSCLEROSIS – THE UNDERLYING CAUSE OF CVD

The acute ischemia associated with CVD, e.g. a stroke or myocardial infarction, occurs when the blood supply to an organ is blocked by the formation of a blood clot (thrombus). However, it is important to recognize that the intra-vascular formation of these clots is almost always preceded by the rupture of an atherosclerotic plaque.

Atherosclerosis is the progressive accumulation of cholesterol, triglycerides, fibrous tissue and inflammatory cells in sections of large- and medium-sized arteries throughout the body. It begins early in life as have been demonstrated in routine dissections of children where fatty streaks are typically observed in the arteries *(McGill, McMahan et al. 2000)*. These fatty streaks are believed be the precursors of the complicated atherosclerotic lesions, which manifest themselves later in life.

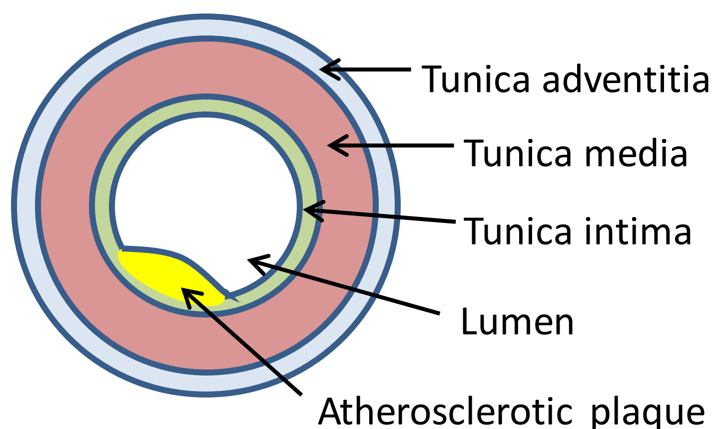
1.2.1 The pathogenesis of atherosclerosis

Atherosclerosis has traditionally been viewed as a gradual and passive deposition of cholesterol in the artery wall. This understanding was primarily derived from clinical observations of patients with familiar hypercholesterolemia who suffer from advanced atherosclerosis at an early age and then confirmed in the famous Framingham study, which identified high cholesterol as one of the risk factors for CVD. Statins were specifically developed by the drug industry to lower blood cholesterol and this class of drugs has been a huge success, decreasing the number of myocardial infarctions by 1/3 according to some estimates *(Mihaylova, Emberson et al. 2012)*. However, more recent studies have established that one of the main effects of statins is anti-inflammation *(Antonopoulos, Margaritis et al. 2012)* and some experts even argue that this might be the true protective effect of statins *(Ridker 2013)*.

The role of low grade inflammation at sites of plaque formation has steadily become more recognized over the past two decades and the paradigm on atherogenesis has slowly shifted from mainly emphasizing

lipids towards a holistic understanding that encompasses the complex interplay between lipids, oxidative stress and immune cells *(Libby, Lichtman et al. 2013)*. The importance of inflammation in atherosclerosis is an excellent example of rediscovered knowledge, for already in the 19th century Carl von Rokitansky and later Rudolf Virchow had discovered that there was inflammation in the atherosclerotic plaques *(Libby 2012)*.

Figure 5. Schematic anatomy of an arterial wall containing a plaque.



Atherosclerosis development is believed to be initiated when circulating low density lipoproteins (LDL) infiltrates the intima layer of the artery wall, either through passive diffusion or active transportation by endothelial cells *(von Eckardstein and Rohrer 2009)*. The LDL particles are trapped in this interstitial space and may be chemically modified by enzymes and/or reactive oxygen species *(Berliner and Heinecke 1996)*. Oxidized LDL (oxLDL) contains many pro-inflammatory substances, including lysophosphatidylcholine (LPC) and lipids that mimic platelet activating factor (PAF). These compounds activate the endothelial cells, which start to express adhesion molecules on their surfaces and secrete chemo-attractants that facilitate the infiltration of leukocytes (primarily monocytes and lymphocytes) into the intima part of the arterial wall *(Eriksson, Xie et al. 2001)*. Once in place, the monocytes differentiate into macrophages and start to engulf modified lipoproteins through their scavenger receptors. The result is the formation of lipid-laden foam cells which are characteristic for atherosclerosis *(Hansson and Jonasson 2009)*.

Macrophages are the most numerous inflammatory cells in human atherosclerosis but both T and B lymphocytes can also be identified, albeit at much lower numbers. Though few, the T cells are very important in the pathophysiology and Professor Libby at Harvard Medical School has likened them to generals leading masses of foot soldiers or conductors directing orchestras *(Libby 2012)*. The antigens to which the T cells react are believed to be modified low density lipoproteins and heat shock proteins *(Xu, Dietrich et al. 1992, Holvoet and Collen 1998)*. Besides the pro-inflammatory T_H1 cells that secrete IFN-γ, there are also regulatory T cells (T_{REG}) which ameliorate atherosclerosis by producing transforming growth factor-β and in some cases IL-10. The balance between T_H1 and T_{REG} cells is believed to be of vital importance to the stability of the atherosclerotic lesions *(Ait-Oufella, Salomon et al. 2006)*.The net effect of B cells in atherosclerosis development is currently unsettled. While some studies have shown that B cell depletion with anti-CD20 antibody decreases lesion size in mice *(Ait-Oufella, Herbin et al. 2010)*, it is also well known that natural antibodies (including anti-PC) are beneficial in experimental models *(Binder, Shaw et al. 2005)*.

While most scientists in the field believe the chronic inflammation associated with atherosclerosis is caused by modified lipoproteins, there are those who have proposed that the inflammation is a reaction to certain micro-organisms. The most commonly mentioned pathogens are *Chlamydia pneumonie, Porphyromonas gingivalis, Aggregatibacter actinomycetemcomitans* and cytomegalovirus which have all been detected in atherosclerotic plaques *(Rosenfeld and Campbell 2011)*. A problem for this infection hypothesis is that treatment studies with antibiotics have so far failed to deliver results. While some of these micro-organisms like the intracellular bacteria *Chlamydia pneumonie* might be difficult to treat, it is possible that their presence in plaque could be the result of a secondary infection. Several of these suggested bacteria, e.g. *Porphyromonas gingivalis,* are dental pathogens. While the link between poor dental status and cardiovascular disease has been known for a long time, it has been difficult to study the contribution of these

pathogens to CVD in isolation since poor dental is associated with a multitude of confounding factors. However, the American Heart Association has recently published a document stating that there is an independent correlation between dental infections and CVD *(Lockhart, Bolger et al. 2012)*. An interesting twist to this story is the finding that anti-PC antibodies, which we have proposed to be protective against CVD, are in fact induced by many bacteria, including dental pathogens. Many oral bacteria carry PC groups on the surface and induce robust anti-PC responses which are almost exclusively of the IgG2 isotype *(Schenkein, Gunsolley et al. 1999)*.

Atherosclerotic plaques are not randomly distributed throughout the arterial tree as they are often located at bifurcations and curvatures. The shear stress on endothelial cells which is exerted by blood flow has a profound effect on cytoskeletal arrangement, leukocyte adhesion, lipoprotein permeability and production of vasoactive compounds like nitric oxide *(Cunningham and Gotlieb 2005)*. The laminar shear stress at straight sections of the arterial tree is protective while the chaotic oscillatory flow pattern of swirls created at bifurcations predisposes plaque formation. Besides highlighting the importance of shear stress, this also underlines the importance of the endothelial cells in the atherosclerotic process.

1.2.2 Plaque rupture and Atherothrombosis

Besides being invaded by leukocytes from the circulation, the atherosclerotic plaque is also infiltrated by smooth muscle cells (SMC) from the neighboring tunica media layer of the arterial wall and/or circulating SMC precursors *(Caplice, Bunch et al. 2003)*. These SMC are the source of most of the interstitial collagen that lend strength to the fibrous cap that covers the necrotic lipid core of the plaque *(Schwartz, Virmani et al. 2000)*. This mass of connective tissue creates bulk, which might obstructs blood flow and cause insufficient oxygen supply to the myocardium during moments of increased cardiac demand, e.g. climbing stairs. However, clinicians have come to recognize that bulky plaques are

stable as long as they have a low degree of inflammation *(Glagov, Weisenberg et al. 1987)*. The plaques which are prone to rupture are characterized by heightened inflammation *(Finn, Nakano et al. 2010)*. The core of atherosclerotic plaques contain hefty amounts of pro-coagulants like Tissue Factor *(Mach, Schonbeck et al. 1997)* and even a slight rift of the fibrous cap will generate thrombi, which may occlude the artery and trigger acute tissue ischemia. The pro-inflammatory environment of vulnerable plaques is simmering with cytokines that promotes SMC apoptosis and stimulates macrophages to produce matrix metalloproteinases *(Galis, Sukhova et al. 1994)*. The decreased production of collagen in combination with induction of degrading enzymes jeopardizes the biomechanical stability of the fibrous cap that covers the plaque, which predisposes rupture *(Thim, Hagensen et al. 2008)*.

1.3 ANTIBODIES AGAINST THE PHOSPHORYLCHOLINE EPITOPE

During the early days of immunology research, the only source of monoclonal antibodies was murine myeloma cell lines. The clones were given names such as TEPC-15 and M167 and their antibodies were subject to intense study. Sometime in the 1960s, it was discovered that the antibodies of these cell lines were reactive against small molecules called haptens and in the case of M167 and TEPC-15 among others, this hapten was found to be phosphorylcholine (PC) *(Leon and Young 1971)*. PC was very a hot research topic in the 1970s and 80s as it was considered a model for humoral immunity *(Early, Huang et al. 1980)*. There are plenty of publications from this time period that report different aspects of PC-reactive antibody clones. Much of what is today considered basic immunological facts, such as somatic hypermutation and class-switching were validated in these early PC-studies which many times were published in top journals such as Nature and Cell *(Crews, Griffin et al. 1981, Gearhart, Johnson et al. 1981)*.

The main target of these PC-antibodies were hypothesized to be bacterial polysaccharides for PC was known to be an integral part of many bacteria, including some prominent human pathogens such as *Streptococcus pneumonie*, *Neisseria meningitis* and even the troublesome *Pseudomonas aeruginosa*. This theory was cemented by experiments showing that passive immunization with anti-PC could save fatally infected BALB/c mice from a certain death *(Yother, Forman et al. 1982)*. The importance of the PC-epitope for infectious defense can be further illustrated by C-Reactive Protein (CRP), which is rapidly produced by the liver in response to any infection. This evolutionary conserved acute phase protein is part of the innate immune system and aids phagocytosis/clearance by binding to PC-epitopes on microorganisms *(Mold, Nakayama et al. 1981)*. In the clinic, CRP is the single most important laboratory test for identifying patients with serious infections and almost every patient who seeks medical attention in an industrialized country has their CRP checked.

Just like CRP, anti-PC antibodies are also evolutionarily conserved and can be found across many species. Most basic research on the biology of anti-PC has been done in murine models where these antibodies are believed to be produced by natural $CD5^+$ B1 cells, independent of external antigen stimuli. This theory stems from a study that detected $TEPC\text{-}15^+$ B cells in the spleen of germ free mice *(Sigal, Gearhart et al. 1975)*. It has been shown that the mouse anti-PC repertoire is heavily dependent on one IGVH-gene (V1) and knocking out this one gene, greatly impairs the ability of the mice to produce high affinity anti-PC antibodies, even upon vaccination *(Mi, Zhou et al. 2000)*. The absolute majority of publications in the field of anti-PC have used mouse models and our present understanding of the human antibody response to PC is based on extrapolations on from mouse data. It has been a long-standing goal of the Frostegård laboratory to bridge this knowledge divide and elucidate the underlying biology of human anti-PC antibodies, particularly in relation to cardiovascular disease.

Interesting factoids on human anti-PC biology from the Frostegård lab.

- Women have significantly higher levels than men *(Su, Georgiades et al. 2006)*.
- The anti-PC titer decreases slowly with age *(Fiskesund, Su et al. 2012)*.
- Treating rheumatoid arthritis patients with anti-TNF therapy leads to decreased inflammation and increasing levels of anti-PC IgM *(Ajeganova, Fiskesund et al. 2011)*.
- However, treating rheumatoid arthritis with anti-CD20 (rituximab) lowers anti-PC IgM levels slightly but significantly over 12 months *(Ajeganova, Fiskesund et al. 2011)*.
- Following a gluten free vegan diet increases anti-PC IgM among rheumatoid arthritis patients *(Elkan, Sjoberg et al. 2008)*.
- People living a traditional life, unaffected by Western lifestyle, in the island of Kitava (New Guinea) have significantly higher titers of

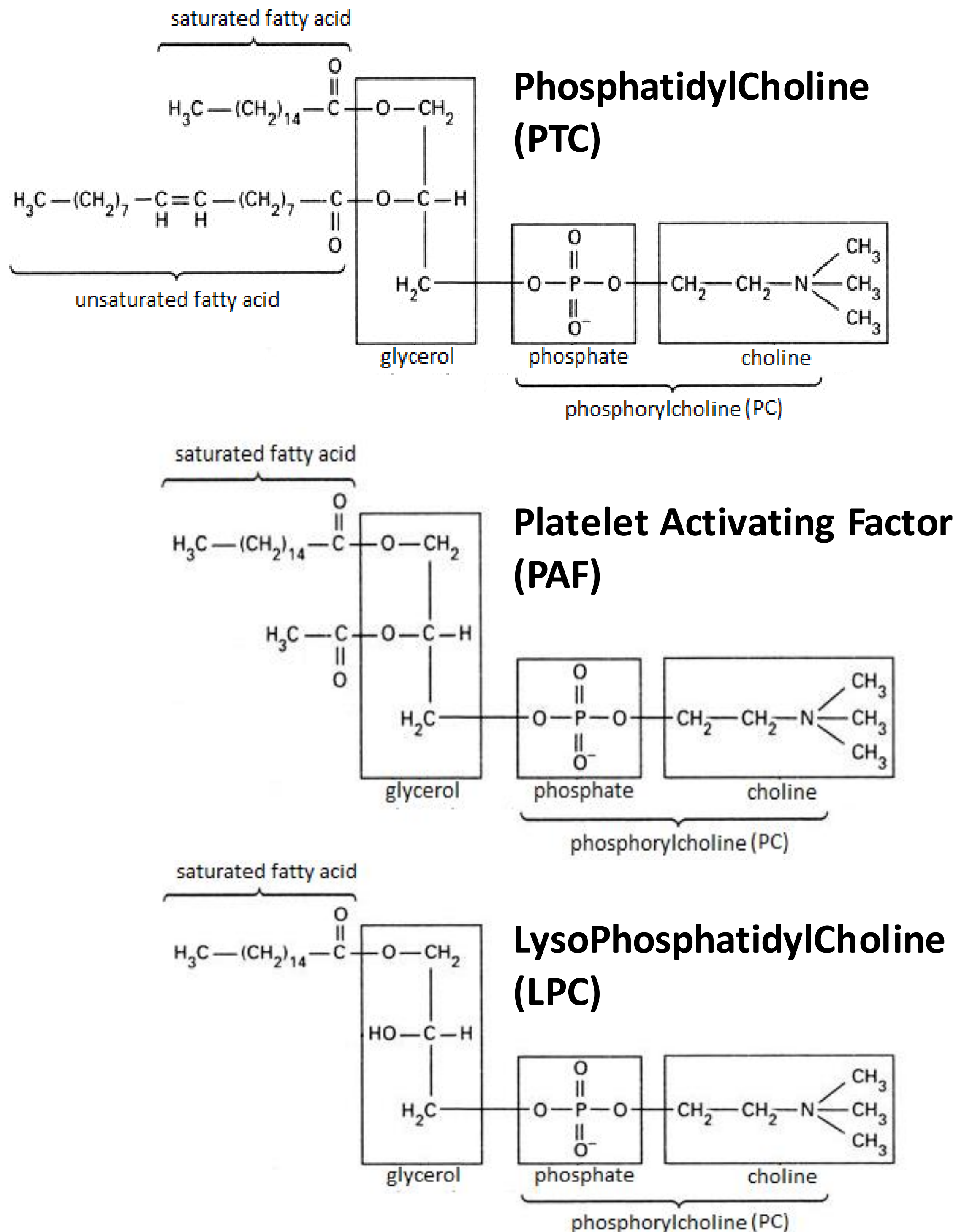
anti-PC IgM than a Swedish control population. This is particularly interesting because rheumatologic and cardiovascular diseases are unheard of on this island and it is not because people die young. If the Kitavans survive childhood, they live till old age. Professor Frostegård has hypothesized that diet is playing a role in maintaining high anti-PC levels among Kitavans but also exposure to microorganisms bearing PC-epitopes, including nematodes and parasites *(Frostegard, Tao et al. 2007)*.

- New born infants lack anti-PC antibodies (unpublished data). This has also been reported by the Witztum group in San Diego *(Chou, Fogelstrand et al. 2009)*.

As previously alluded to, anti-PC antibodies are ubiquitous in human serum and among the almost 10 000 people whose sera have been analyzed in the Frostegård laboratory, only one individual has been found to lack anti-PC IgM (unpublished data). Though everybody has them, the level of anti-PC in sera may differ 100-fold between individuals. On average, a human serum contains 320 µg/ml of anti-PC IgG and 110 µg/ml of anti-PC IgM *(Nishinarita, Sawada et al. 1990)*. For IgM, that corresponds to ~7.3% of the total circulating IgM pool which may seem unreasonably high. However, this is something that we have been able to confirm during anti-PC IgM extraction experiments. Starting from one bottle containing 25mg of purified human IgM (Sigma-Aldrich), we are typically able to isolate >2 mg of anti-PC IgM using affinity chromatography.

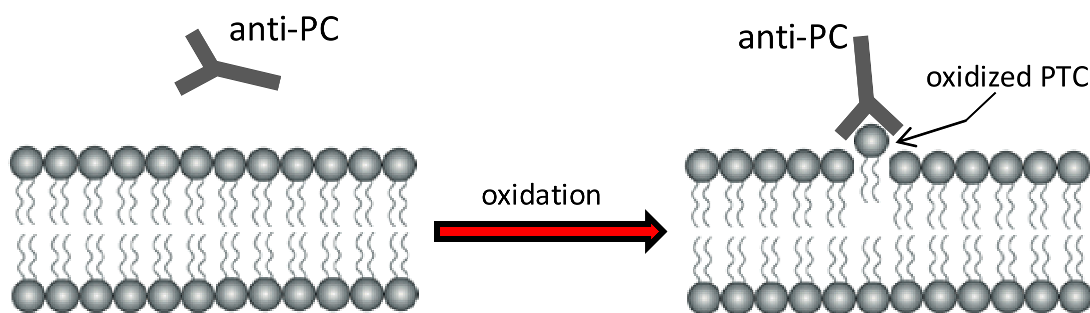
PC is not only found on invading pathogens, it also happens to be an integral component of the body's most abundant phospholipid, phosphatidylcholine (PTC). This phospholipid forms the outer layer of all cell membranes and coats the highly hydrophobic lipid core low density lipoprotein (LDL). PTC consists of one stable saturated fatty acid and one unsaturated fatty acid (Figure 6), which is prone to oxidation under the certain conditions. One such circumstance occurs during apoptosis, when the cellular anti-oxidation systems (e.g. glutathione) malfunctions and

Figure 6. Phosphatidylcholine (PTC) is the most common phospholipid in the body. It is structurally very similar to Lysophosphatidylcholine (LPC) and Platelet Activating Factor (PAF). All three molecules contain a PC-group.



exposes the membrane to reactive oxygen species (ROS). Introduction of oxygen atoms into certain PTC molecules of the membrane induces conformational changes in the membrane that expose PC-groups, which may be targeted by anti-PC antibodies *(Shaw, Horkko et al. 2000)*. It has been shown that mouse anti-PC binds to apoptotic cells and can be used in flow cytometry to visualize dead/dying cells much like a viability dye *(Shaw, Horkko et al. 2000)*. The group of professor Silverman has also demonstrated that mouse anti-PC IgM aids in the clearance of apoptotic cells *(Chen, Park et al. 2009)*. In normal tissue, the removal of dead/dying cells is so efficient that you can rarely see apoptotic cells in histological sections. However, sections from advanced atherosclerotic lesions are literally littered with dead cells *(Tabas 2005)* and anti-PC might play an important role in helping phagocytes clean up.

Figure 7. Oxidation induces conformational changes in the cell membrane that exposes the PC-epitope.



Besides being exposed on apoptotic cells, the PC-epitope is also found on oxidized low density lipoprotein *(Chou, Hartvigsen et al. 2008)*. This is also due to oxidation of PTC, which along with ApoB protein covers the hydrophobic core of triglycerides and cholesterol. Foam cells which are pathognomonic for atherosclerosis are generated when infiltrating macrophages ingest oxLDL using their scavenger receptors (ScRs). CD36 which is one of the main ScR that is responsible for oxLDL ingestion and foam cell generation recognizes the PC-epitope of oxLDL *(Boullier, Friedman et al. 2005)*. As one would expect, anti-PC IgM has been shown to interfere with this process *in vitro* by blocking the PC-groups on oxidized LDL *(de Faire, Su et al. 2010)*.

Figure 6 illustrates the structural relationship between the compounds PTC, platelet activating factor (PAF) and lysophosphatidylcholine (LPC), which all contain the PC-group. As previously discussed, PTC is inert and an important structural component of cell membranes, but it can be converted into highly pro-inflammatory agents by oxidation *(Hazen and Chisolm 2002)*. PAF is produced by many cell types but particularly by immune cells in response to danger stimuli. While the compound was named after its ability to activate platelets, it is probably more important in mediating inflammation. PAF has a relatively short half-life for it is quickly inactivated by *lipoprotein associated phospholipase A2* (Lp-PLA2), which converts it into LPC. While less potent than PAF, there are many publications on the detrimental effect of LPC in atherosclerosis (*Matsumoto, Kobayashi et al. 2007)*.

Oxidation of PTC typically generates PAF-like compounds like ox-PAPC, PGPC and notably POVPC, which has been shown to bind and activate the PAF-receptor *(Pegorier, Stengel et al. 2006)*. This mechanism has been hypothesized to be one of the main pathways for initiation of inflammation by oxLDL in the arterial wall *(Libby 2006)*. Interestingly, our laboratory has reported that anti-PC IgG can neutralize the pro-inflammatory effect of PAF on endothelial cells *(Su, Hua et al. 2008)*. PAF-like lipids are like PAF, hydrolyzed by Lp-PLA2 into LPC which is one of the main components of oxLDL *(Matsumoto, Kobayashi et al. 2007)*.

1.4 ANTI-PC AS RISK MARKER/FACTOR FOR CVD

Examining the risk factors behind a disease provides clues to its pathogenesis as well as important information that can be used to derive strategies for prediction, prevention and treatment. The distinction between risk markers versus risk factors is an important one. Risk factors are by definition causal while risk markers are indicators of disease processes and may or may not be causal *(Vasan 2006)*.

The town of Framingham in Massachusetts was selected in the 1940s by US researchers for a large epidemiological study on biological and environmental factors associated with CVD. The subsequent publications were among the most important scientific reports in the field of epidemiology and the results have had wide-reaching implications for the global population. Almost everybody in the industrialized world knows about the detrimental effects of *Smoking, Physical inactivity, Obesity, Family history of CVD, Hypercholesterolemia* and *Hypertension*. Together with *Diabetes*, *Old age* and *Male gender*, these attributes represent the traditional risk factors of CVD, which are routinely used in the clinic to stratify cardiovascular risk. This knowledge forms the backbone of modern preventive care.

The traditional risk factors are strongly associated with atherosclerotic disease and can explain 75% to 90% of all events *(Greenland, Knoll et al. 2003, McGill, McMahan et al. 2008)*. The chance of getting CVD is low if one does not have any of the aforementioned risk factors *(Lloyd-Jones, Leip et al. 2006)*. Although the traditional risk factors have proven to be very valuable, complacency is a mentality unknown to scientists. There is a need for further understanding of the disease in order to identify novel targets for treatment. Since the importance of the chronic inflammation component of CVD has become increasingly apparent over the last two decades, much attention has been given to biomarkers that are related to inflammation. High-sensitive CRP levels *(Devaraj, Singh et al. 2009)* and the activity of serum Lp-PLA2 enzyme *(Rosenson and Stafforini 2012)* have both been independently correlated with CVD. In the case of Lp-

PLA2, inhibitors have been developed that have shown promise in animal models *(Wilensky, Shi et al. 2008, Wang, Zhang et al. 2011)*.

Though the study of anti-PC antibodies went out of fashion during the late 1980s, in recent years, the field has undergone a renaissance. The discovery that the classic PC-reactive TEPC-15 clone recognizes oxidation specific epitopes *(Shaw, Horkko et al. 2000)* opened up a whole new avenue of investigation. Subsequent research has demonstrated that anti-PC aids in clearance of apoptotic cells *(Chen, Park et al. 2009)* and prevents the formation of foam cells in atherosclerosis by clearing oxidized low density lipoprotein *(de Faire, Su et al. 2010)*. Murine vaccination studies to induce robust humoral PC-responses have likewise shown strong beneficial effects on experimental atherosclerosis *in vivo* *(Binder, Horkko et al. 2003, Caligiuri, Khallou-Laschet et al. 2007)*. Given this body of evidence, professors Frostegård and de Faire at Karolinska Institutet began to advance anti-PC IgM deficiency as a novel risk marker for CVD *(de Faire and Frostegard 2009)*. This hypothesis has been tested and validated in several large CVD cohorts in Sweden. Low serum level of anti-PC IgM has consistently been linked to increased risk of CVD. This association is independent of the traditional risk and anti-PC IgM as a test might thus identify risk patients that the traditional risk factors do not pick up.

Table 3. Summary of the epidemiological studies on anti-PC IgM and CVD. Having a low serum level of anti-PC is associated with increased CVD-risk.

STUDY	Odds Ratio	95% Confidence Interval
VIP/MONICA ***(Gronlund, Hallmans et al. 2009)***	1.69	1.09 - 2.54
Malmö Diet Cancer ***(Sjoberg, Su et al. 2009)***	2.01	1.11 - 3.67
Castro II ***(Fiskesund, Stegmayr et al. 2010)***	1.62	1.11 - 2.35
Stockholm 60-year old men ***(de Faire, Su et al. 2010)***	1.96	1.09 - 3.55
Gothenburg ACS ***(Caidahl, Hartford et al. 2012)***	2.28	1.32 – 3.92

2 AIMS OF THE THESIS

- To study the relationship between anti-PC levels and the incidence of stroke in a population-based cohort from northern Sweden.
- To investigate the properties of anti-PC antibodies belonging to different classes/subclasses (IgM, IgA, IgG2 etc).
- To determine the stability/consistency of serum anti-PC IgM titers (within the same individuals) over time period of 4 years.
- To identify and characterize PC-reactive human B cells and produce human anti-PC monoclonal antibodies.

3 METHODOLOGICAL CONSIDERATIONS

All experiments in this thesis were conducted on human serum and cells originating from voluntarily donated blood. The studies were approved by ethical boards at Karolinska Institutet and Umeå University prior to initiation. This section contains detailed descriptions of the methods that were used in the experiments. The corresponding papers are referred to with roman numerals (I-III).

3.1 STUDY GROUPS

3.1.1 VIP/MONICA cohort (paper I)

The MONICA project was part of the World Health Organization (WHO) initiative to study trends in CVD. Every fourth year, 2000 to 2500 persons (25 to 74 years of age) living in two Swedish counties (Norrbotten and Västerbotten) were invited to a health examination. Doctors would record height, weight, blood pressure, smoking habits, serum cholesterol, and lifestyle habits *(Stegmayr, Lundberg et al. 2003)*. Participants were also encouraged to donate blood for future research, stored as heparin–plasma in the Northern Sweden Medical Research Bank *(Lenner, Hallmans et al. 1991)*. In paper I, we used data from the Northern Sweden MONICA project for the years 1985 through 1999.

VIP is an ongoing project in the Swedish county of Västerbotten. It was initiated in 1985 and was originally intended as a health promotion program for the population in this county (Västerbotten; ≈250 000 inhabitants). Every year, all persons reaching the ages of 30, 40, 50, and 60 are invited to their local health centers for a routine screening. The questionnaire for VIP is very similar to the one used in the MONICA project. All participants are asked to donate blood samples, which are then stored in the above-mentioned biobank. By December 2002, a total of 74 000 unique people had attended health examinations within the framework of the project.

The participation rate is 60% for VIP and 77.2% for MONICA. Previous publications on nonparticipants have shown that nonparticipants in VIP are quite similar to the participants from a socioeconomic standpoint, indicating a marginal social selection bias *(Weinehall, Hallgren et al. 1998)*.

3.1.2 Hypertension cohort (paper II)

The European Lacidipine Study on Atherosclerosis (ELSA) was a 4-year prospective study on hypertension treatment in relation to development of atherosclerosis in the carotid artery *(Zanchetti, Bond et al. 2002)*. The Swedish branch of this European collaboration consisted of 226 subjects who had established hypertension (diastolic pressure >95 mm Hg). During the admission process, information on age, gender, blood pressure, weight, height, smoking habits and previous medical history was recorded along with laboratory values of different parameters including creatinine, fasting glucose, total cholesterol, high-density lipoprotein (HDL) cholesterol and triglycerides. From this cohort, we have previously determined that high levels of anti-MDA-LDL, anti-oxLDL and anti-PC IgM are protection factors for atherosclerosis development *(Su, Georgiades et al. 2006)*. In paper II we explore the roles of anti-PC IgG1, IgG2 and IgA, different antibody idiotypes as well as the long term stability of anti-PC IgM levels. We have also used this cohort to study the predictive power of combining anti-PC with anti-MDA-LDL or anti-oxLDL.

3.2 CAROTID ULTRASOUND

The carotid Intima-Media Thickness (IMT) is often used as a surrogate marker for global atherosclerosis *(Mancini, Dahlof et al. 2004)*.

The mean of the maximum Intima-Media Thicknesses (IMT) in the far walls of common carotids and bifurcations (CBM_{max}) was determined by B-mode ultrasonography at the time of inclusion, and 4 years afterwards. All scans were performed with the Biosound 200 II device (All Imaging Systems inc., Irvine CA, USA) and read at the Ultrasound Coordinating Center with quality assurance accomplished as reported. The levels of the

different anti-PC antibody classes/subclasses at enrollment were evaluated with respect to increase or decrease in IMT at the 4-year follow up.

3.3 ENZYME LINKED IMMUNOSORBENT ASSAY (ELISA)

3.3.1 Determination of serum anti-PC levels

Detection of IgM anti-PC antibodies was performed with an ELISA kit from Athera Biotechnologies AB. The assay is based on PCs covalently linked to BSA and then coated onto 96-well Nunc Maxisorp microtiter plates. The assay was performed in accordance with manufacturer recommendations. All readings of results were performed on ELISA Multiscan Plus spectrophotometer (Molecular Devices, San Francisco). The coefficients of variation for the samples were <7%.

For determination of other immunoglobulin classes, this ELISA-kit was modified by switching the secondary antibody. The Athera-kit provides anti-human IgM coupled to *horse radish peroxidase* (HRP). When detecting anti-PC IgA, we simply switched to an anti-human IgA HRP from Sigma-Aldrich (goat anti-human IgA HRP). The secondary antibodies against the IgG subclasses were purchased from Invitrogen. The Athera-kit comes with an anti-PC IgM standard curve. For the other immunoglobulin classes/subclasses, we devised our own standard curve using serial dilutions of donated serum and defined arbitrary units (Units/ml).

3.3.2 Determination antibody levels against modified LDL

LDL was isolated from plasma of healthy donors by sequential preparative ultra-centrifugation and oxidized using copper ions (oxLDL) or modified with MDA (MDA-LDL). These were then coated on microplates, which were later blocked with 20% adult bovine serum in PBS (20% ABS-PBS). Diluted serum samples were incubated overnight at 4 °C. The presence of specific antibodies in the serum was detected using goat anti-human IgG ALP/anti-human IgM ALP in combination with substraste (pNPP) and read at 405 nm.

3.3.3 Characterization of the fine-specificities

The binding specificity of human anti-PC IgG1, IgG2, IgM and IgA were determined in a competitive ELISA with p-nitrophenylphosphorylcholine (NPPC) hapten or phosphorylcholine (PC) hapten in accordance with previously published work *(Brown, Schiffman et al. 1984)*. Briefly, hapten was mixed with pooled IgA or affinity purified anti-PC from pooled IgG/IgM and incubated in the wells of the ELISA microplate. Antibodies of each isotype were then detected with the above-mentioned class/subclass specific secondary antibodies.

3.3.4 Polyreactivity ELISA

Besides PC-reactivity, the human anti-PC mAbs that we produced in paper III were also screened for reactivity against unspecific antigens as previously described *(Wardemann, Yurasov et al. 2003)*. Antibodies were classified as polyreactive if they recognized at least two out of three analyzed antigens (double-stranded DNA, insulin and LPS). All ELISAs were developed with HRP–conjugated goat anti-human IgG (Jackson ImmunoResearch) and revealed using the chromogenic substrate 3,3’,5,5’-tetramethylbenzidine (Bio-Rad). Plates were read at 450 nm with a reference of 650 nm. To be considered reactive the results for any given antibody had to be confirmed in at least two independent experiments.

3.4 PURIFICATION OF SERUM ANTI-PC IgG AND IgM

Pooled human IgG (Baxter, Deerfield IL, USA) and IgM (Sigma Aldrich, St. Louis MO, USA) was passed over a column containing PC-Sepharose (Biosearch technologies, Novato CA, USA). The column was then washed with Phosphate Buffered Saline (PBS) pH 7.4 with Tween20 to remove non-bound immunoglobulins. These non-anti-PC antibodies were collected to be used as control antibodies (flowthrough immunoglobulins) in later experiments. The bound PC-specific antibodies were then eluted with 0.01 M acetic acid and concentrated/buffer exchanged to PBS pH 7.4 using Centricon Plus-70 centrifugation filter units (Millipore, Billerica MA, USA).

3.5 CELL VIABILITY ASSAY

Peripheral blood mononuclear cells (PBMC) were isolated from buffy coats using the standard protocol of Ficoll density gradient centrifugation. The freshly produced PBMC were counted and resuspended in RPMI 1640 before being seeded into 24-well plates at a concentration of 3×10^6 cells per ml.

L-α-Lysophosphatidylcholine (LPC) from egg yolk (Sigma) was first dissolved in ethanol and then further diluted in RPMI 1640 to a working stock solution. LPC was added to the cells of each well, either by itself, together with purified anti-PC IgM, total IgM or flowthrough IgM. After a 18 hour incubation period, cell viability was evaluated with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay. Live cells with functioning mitochondria metabolize MTT to formazin, which absorbs light at 570 nm. The viability of the cells in each well was thus quantified by collecting the insoluble formazin formed in each well, dissolving it in DMSO and reading the optical density (OD) at 570 nm.

3.6 BIOCONJUGATION OF PC TO PHYCOERYTHRIN

Phosphorylcholine hydroxyphenylacetic acid (Biosearch Technologies) was mixed with EDC along with sulfo-NHS and the resultant amine-reactive PC-compound was added to pure phycoerythrin (PE) protein. After one hour, the protein-hapten product (PC-PE) was isolated using a PD-10 Desalting Column (GE Healthcare).

PC-reactive TEPC-15 IgA from mouse myeloma (Sigma Aldrich) was passively adsorbed onto 5µm latex beads (Invitrogen) and subsequently blocked with BlockAce (AbD Serotec). The beads were stained with equal amounts of either PC-PE or native PE and analyzed on a LSR Fortessa flow cytometer (Becton Dickinson) for the purpose of confirming successful conjugation of PC to PE.

Further validation of the PC-dye was performed by Ig-ELISpot using commercial ELISpot-kits (Mabtech) and isolated B cells from buffy coats. Individual wells on ELISpot-plates were coated overnight at 4°C with either 10µg/ml of PC-BSA (Biosearch Technologies) or anti-IgM (provided in the kit) and then blocked with culture medium (RPMI 1640 + 10% FCS). B cells from four healthy individuals were stained with antibodies before being sorted into ELISpot-plates (100-200 cells/well). Sorted B cells were allowed to secrete immunoglobulins for 16 hours at 37°C with 5% CO_2. The frequency of spot-generation by PC^+ cells sorted on PC-BSA surface (nr of true PC-reactive B-cells) was compared to that of PC^+ cells sorted on anti-Ig surface (total nr of sorted viable B cells) and found to be comparable.

3.7 PHENOTYPIC CHARACTERIZATION OF PC-REACTIVE B CELLS

B cells from 19 healthy individuals were stained with antibodies and reagents and analyzed on a LSRFortessa flow cytometer with an acquisition target of $3x10^6$ cells. The data was then dissected in FlowJo v7.6.5 (TreeStar). Delineation of $CD5^+$ in B cells was done by FMO control. Total B cell count was estimated using CD19 BV421 in conjunction with CountBright Beads (Invitrogen) according to Becton Dickinson's lyse-no-wash protocol.

3.8 SINGLE B CELL SORTING

Freshly isolated B cells from ten healthy individuals were kept overnight in RPMI 1640 + 10% FCS and sorted the following morning. Total B cells were divided into IgG^+, naïve, IgM^+ memory and $CD27^+CD43^+$ subsets based on surface markers and subjected to first round of sorting using a FACSAria III (Becton Dickinson). PC^+ B cells from each subset were collected during the 4-way sort was then re-sorted using the same gating strategy and deposited as single cells into 96-well Twintec PCR-plates (Eppendorf) containing lysis buffer as previously described *(Tiller, Meffre et al. 2008)*. The purity of PC-reactive B cells was typically 67% and 100% after the first and second rounds of sorting, respectively.

3.9 cDNA SYNTHESIS, PCR AMPLIFICATION AND SEQUENCING

Single cell cDNA was synthesized in the original 96-well PCR plate by addition of nucleotides and SuperScript III RT (Invitrogen). Individual IgH (γ or μ) and IgL chain (κ or λ) genes rearrangements were amplified independently, using the cDNA as template, by two successive rounds of PCR (50 cycles each) as previously described *(Tiller, Meffre et al. 2008)*. The PCR-products were then sequenced using BigDye 3.1 terminator chemistry (Applied Biosystems) and read in an ABI 3730 instrument (Applied Biosystems). Light chains were sequenced once and heavy chains were sequenced twice (once from 3'-end and once from 5'-end) for highest fidelity.

3.10 IMMUNOGLOBULIN GENE SEQUENCE ANALYSIS

Ig gene usage and somatic hypermutations were analyzed by IgBLAST (http://www.ncbi.nlm.nih.gov/igblast/). Replacement (R) and silent (S) mutations frequencies in framework regions (FWRs) and CDRs were calculated for each region based on the absolute number of nucleotides in all analyzed sequences as defined by IgBLAST. IgG isotype subclasses were determined using the international IMmunoGeneTics information system (http://imgt.cines.fr).

The heavy-chain sequences were translated and the HCDR3 regions were aligned by means of the ClustalW peptide alignment algorithm. Clustered antibodies were considered to be of the related if their associated light chains utilized the same $V_{\lambda/\kappa}$ and $J_{\lambda/\kappa}$ genes. Phylogenetic mapping of clonally related antibodies from the same donor was performed by counting shared and unique mutations in relation to the corresponding germline IGHV-gene.

3.11 CLONING AND ANTIBODY PRODUCTION

Restriction sites for expression vector cloning were introduced by using gene-specific primers and first PCR products as template as previously described *(Wardemann, Yurasov et al. 2003, Tiller, Meffre et al. 2008)*. The digested PCR products from each single cell were cloned into

expression vectors containing human Igγ1, Igκ, or Igλ constant regions *(Wardemann, Yurasov et al. 2003, Tiller, Meffre et al. 2008)*. Ligation reactions were performed using the quick ligase kit (NEB) according to the manufacturer's instructions. Expression vectors containing IgH and the corresponding IgL genes were isolated from transformed-DH5alpha bacteria (Invitrogen) using plasmid DNA purification kits (NucleoSpin®Plasmid, Macherey-Nagel) and then sequenced to confirm consistency with the original PCR products.

Antibodies were produced by transient co-transfection of exponentially growing 293 human embryonic kidney fibroblasts using PEI-Max (Polysciences). Supernatants were collected after seven days of culture, and antibodies were purified by binding to protein G–Sepharose 4FF (GE Healtcare). Expression of antibodies consisting of both heavy and light chains, as well as the protein purity, was verified by SDS-polyacrylamide gel electrophoresis.

3.12 SURFACE PLASMON RESONANCE

In order to determine the affinity of ten human mAbs to PC, we performed surface plasmon resonance (SPR) analyses on a Biacore X100 instrument (GE Healthcare). The selection process of mAbs for the SPR analysis was conducted to generate a representative group of mAbs from different B cell subsets, using different Ig-genes and include both κ and λ clones. Monoclonal human antibodies were covalently bound at 10µg/ml to CM7 sensor chips through amine coupling in accordance with the manufacturer´s instructions. Once the antibodies had been immobilized, 13 different concentrations (0-200 µM) of free PC-hapten in solution were sequentially injected at a flow rate of 30 µl/min. Data was collected using multi-cycle kinetics and the bindings were analyzed using the Biacore X100 Evaluation software, version 2.0 (GE Healthcare) and fitted with a mathematical steady state affinity model.

3.13 STATISTICS

Serum antibody levels were dichotomized or determined as continuous variables as indicated. We calculated percentiles based on data from the whole study group. Wilcoxon rank sum tests or *t* tests were applied depending on the distribution of data. The association between antibodies and incident stroke were determined by conditional logistic regression models with calculation of odds ratios and 95% CIs. Age, gender, and geography were matched for by the design of the study (paper I). The association between antibodies and the progression of atherosclerosis over a 4-year period were determined by estimating increases in IMT (yes or no) using conditional logistic regression analysis (paper II). Adjustments were made for possible confounders including smoking habits, body mass index, diabetes, hypercholesterolemia (serum cholesterol ≥5.0 mmol/L), and hypertension (≥140/90 mm Hg systolic/diastolic) (paper I and II). A two-tailed *P* value <0.05 was considered significant. SAS was used for statistical analyses of epidemiological data (release 9.1; SAS Institute Inc.).

Student's t-test was used to test statistical differences in the *in vitro* experiments. The r^2-value and statistical significance of the correlation between serum anti-PC IgM and number of $PC^+CD27^+CD43^+$ B cells in blood was calculated according Pearson. The significance of IgG-subclass distributions was determined through Fischer's exact test. These analyses were performed in GraphPad Prism v6.02 and R v2.13.2.

4 RESULTS AND DISCUSSION

4.1 ANTI-PC IGM AND RISK OF STROKE

Paper I is a classic nested case-control study, which is based on a cohort that was created by our co-authors in Umeå. They had access to blood samples and life-style data from the huge MONICA and VIP initiatives (see methodology section). Using the Swedish stroke registry, they identified 227 people who had participated in the health checks and later developed a first-time stroke. They also selected 455 age- and sex-matched controls from the same MONICA/VIP material.

We measured the anti-PC IgM levels in this cohort and reported that the median levels of IgM anti-PC levels did not differ significantly between stroke cases and controls. However, when analyzing relative risks for stroke at various cut-off levels for anti-PC levels, significance was reached at the 30th percentile (multivariately adjusted odds ratio (OR) 1.62; CI, 1.11 to 2.35). This association was independent of and unaffected by other risk factors, including smoking habits, body mass index, diabetes, hypercholesterolemia, and hypertension. This finding confirms and extends our previous reports regarding an inverse relationship between anti-PC and atherosclerosis or CVD.

When gender-specific analyses were performed, significant relative risks were seen for women at levels of IgM anti-PC <52 U/mL, corresponding to the median, for both crude data and after multivariate adjustments (OR 1.92; CI, 1.13 to 3.28). Although already significant below the median, the relationship gets even more pronounced in the lower percentiles (OR 2.65; CI 1.41 to 4.95). To our disappointment, no association was seen among the men in this study.

In a subgroup analysis of our previously published Malmö Diet Cancer study, low anti-PC levels came out as a significant predictor for ischemic stroke, with a relative risk as high as 4 among men *(Sjoberg, Su et al.*

2009). However, there were too few females in that study to provide reliable data on women. The results of the subgroup analysis in the Malmö Diet Cancer study differ from the present study because here we only noted low anti-PC to be related to an increased stroke risk among women, whereas in the previous report, this association was only significant among men. Our finding here was therefore a bit unexpected.

There could be several explanations for this discrepancy (power issues, differences in population structure, age distributions, etc). First, in the present study, stroke was the primary and only endpoint. Second, in the Malmö study, the number of women was relatively low, providing analyses with low power. Further on, in addition to the geographical, genetic, socioeconomic, and dietary differences between the cohorts, the most striking difference is the age structures of the two studies. The subjects in the current study are on average of 5 years younger than those included in the Malmö Diet Cancer study, and we have a greater spread of age in the current study, with some stroke cases occurring at 30 years of age. The average age at stroke incidence in this cohort was just above 60 years, which is relatively young in the context of stroke.

Both the current study and the Malmö Diet Cancer study are based on post hoc analyses. This is a limitation to both studies and the possibility of chance findings can never be completely ruled out. There might also, as always, be unknown confounders. However, of note is that adjustment for available confounders does not weaken our results. Future studies on various populations are warranted to reliably and consistently estimate risk associations.

Persons who developed stroke and incidentally had atrial fibrillation were included among stroke cases because the study design does not make distinctions between subtypes of stroke. This is partly because it is sometimes hard to correctly identify cardioembolic stroke cases and partly because the majority (85%) of ischemic strokes are attributable to atherosclerosis anyway *(Warlow 1998)*. The number of possible atrial

fibrillation–related strokes was most likely not very common because our stroke cases were comparably young and atrial fibrillation is rather uncommon in this age group.

The mechanisms by which anti-PC could protect against stroke might be related to the anti-inflammatory properties of anti-PC, inhibiting the inflammatory effects of PC-containing inflammatory phospholipids including platelet-activating factor.

In conclusion, we reported in this study that anti-PC IgM levels below the 30th percentile could be used to predict increased risk for stroke in a population-based study from Northern Sweden. In subgroup analyses, this association was only present among women. The findings presented here may potentially open up for novel immunotherapy regimens against atherosclerosis and stroke or other forms of CVD either through passive transfer of anti-PC antibodies or active immunization using PC as an antigen. However, additional large-scale studies are needed to determine the exact role of anti-PC in women and men.

4.2 ANTI-PC SUBCLASSES AND IMT-PROGRESSION

The European Lacidipine Study on Atherosclerosis (ELSA) was a 4-year prospective study on hypertension treatment in relation to development of atherosclerosis in the carotid artery *(Zanchetti, Bond et al. 2002)*. From this cohort, we had previously determined that high levels of anti-oxLDL IgM, anti-PC IgM (but not IgG) and antibodies against malonyldialdehyde modified LDL (anti-MDA-LDL) are protection markers for atherosclerosis development *(Su, Georgiades et al. 2006)*. In the present study we wanted to go further and explore the roles of anti-PC IgG1, IgG2 and IgA as well as the long term stability of anti-PC IgM levels.

Measurable levels of anti-PC IgG1 and IgG2 were found in most subjects whilst levels of IgG3 and IgG4 were generally undetectable. Thus only anti-PC IgG1 and IgG2 were included in the subsequent investigation. We found no correlation between anti-PC IgG2 and IMT-changes (OR 0.96, CI 0.38 to 2.43, p=0.94 above the 90th percentile). However, a high level of the IgG1 subclass at baseline was strongly predictive of no increase in IMT after four years (OR 0.22, CI 0.08 to 0.60, p<0.01 above the 90th percentile). Testing serum for anti-PC IgA showed that subjects with low levels of anti-PC IgA had increased risk for IMT-progression (OR 2.46, CI 1.22 to 4.99, p=0.01 below the 25th percentile). A protective effect at high levels was not seen for anti-PC IgA.

Low levels of anti-PC IgM has consistently been correlated with CVD in previous studies *(de Faire, Su et al. 2009, Gronlund, Hallmans et al. 2009, Sjoberg, Su et al. 2009, Fiskesund, Stegmayr et al. 2010)*. This study introduces two new biomarkers, anti-PC IgG1 and IgA. Spearman rank correlation coefficients show that levels of anti-PC IgM, IgA and IgG1 are all closely associated. This implies that it is only necessary to measure one antibody class since measuring more classes would be redundant. Given that all previous publications have used anti-PC IgM and the availability of a ready-to-use ELISA kit, it is wise to continue the

use anti-PC IgM for risk assessments. However, the role of anti-PC IgA deserves further study due to its intricate connection with gut immunity.

4.3 FINE SPECIFICITY DIFFERENCES AMONG THE ANTI-PC ISOTYPES

Anti-PC antibodies are subdivided into two populations based on their affinity for phosphorylcholine (PC) and *p*-nitrophenylphosphorylcholine (NPPC) *(Brown, Schiffman et al. 1984)*. The fine specificity profiles of anti-PC IgM, IgA, IgG (total), IgG1 and IgG2 were determined in pooled fractions of immunoglobulins to minimize the impact of individual variation. Human anti-PC IgM and IgA were found to be exclusively Group I (similar to the murine T-15 clone). The IgG fraction, however, was determined to contain both Group I and Group II antibodies. Detailed examination with subclass specific antibodies revealed a significant discrepancy between anti-PC IgG1 and IgG2 with regard to specificity. Whereas the IgG1 pool was found to be mostly Group I, the IgG2 pool was clearly made up of Group II anti-PC antibodies.

Patients suffering from periodontal diseases have an elevated risk for CVD *(Blaizot, Vergnes et al. 2009)* even though they develop high titers of anti-PC IgG2 *(Schenkein, Gunsolley et al. 1999)*. This finding might seem to contradict the “house-keeping” hypothesis, in which high levels of anti-PC antibodies are supposed to prevent atherosclerosis and CVD. However, our new data provides an explanation. We have demonstrated that only antibodies of the Group I idiotype are associated with decreased atherosclerosis progression. The level of anti-PC IgG2 (Group II) was not associated with IMT-changes at all (p=0.94). Given that IgG2 antibodies are used to counter carbohydrate antigens *(Scott, Shackelford et al. 1988),* it is likely that anti-PC IgG2 is produced in response to PC-bearing bacteria. Human anti-PC IgG2 has, in fact, been implicated as a bactericidal agent against *Streptococcus pneumonie* and *Haemophilus influenza (Goldenberg, McCool et al. 2004)*.

4.4 COMPOSITE MARKERS IMPROVES THE RISK ASSESSMENT

Combining data on anti-PC IgM with anti-MDA-LDL strengthened the negative association with IMT-progression at levels above the 90th percentile for both antibodies (OR 0.10, CI 0.01 to 0.85, $p < 0.05$). This improved predictive value was also noted between anti-PC IgM and anti-oxLDL IgM at levels above the 90th percentile for both antibodies (OR 0.12, CI 0.03 to 0.42, $p < 0.05$). These combinations are stronger than each of the individual markers by themselves. Anti-MDA-LDL like anti-PC is a natural antibody that has been widely studied in the context of CVD *(Chou, Fogelstrand et al. 2009)*. The finding that the two antibodies can act in synergy opens up exciting avenues related to *in vitro* experiments as well as immunization strategies involving induction of both anti-PC and anti-MDA-LDL. Although combining two different antibodies produces impressive odds ratios (OR), the best OR is obtained by only looking at the very highest levels (top 5th percentile) of anti-PC IgM alone (OR is 0.05, CI 0.006 to 0.40, $p < 0.001$). Among the twelve study participants in the top 5th percentile, only one had IMT-progression after four years (8.3%). For those below the 95th percentile, the incidence of IMT-progression was 137 cases in 214 subjects (64%). While the limited number of individuals in this group urges caution, it is still a striking finding.

4.5 ANTI-PC INHIBITS LPC INDUCED CYTOTOXICITY ON CELLS

Both purified anti-PC IgM and total IgM was able to inhibit LPC-induced cytotoxicity ($p<0.05$) in human immune cells. The effect seen with the flowthrough IgM fraction (largely depleted of anti-PC IgM) was not significant.

We have previously demonstrated that anti-PC can inhibit the formation of foam cells *(de Faire, Su et al. 2010)* and neutralize the pro-inflammatory effect of PAF *(Su, Hua et al. 2008)*. In this study, we have identified an additional mechanism through which anti-PC could confer protection against CVD and atherosclerosis, where dead cells are

abundant. Apoptosis is known to weaken advanced atherosclerotic plaques *(Seimon and Tabas 2009)* and inhibiting LPC-induced cell death could be very important in stabilizing plaques that might otherwise rupture, especially considering the richness of LPC in plaques *(Portman and Aexander 1969)*.

4.6 SERUM LEVEL OF ANTI-PC IgM IS DOES NOT CHANGE

The level of anti-PC IgM was measured in serum samples taken at two time points, four years apart (admission and follow-up). During this time period, levels of anti-PC IgM changed remarkably little in most patients. The Spearman rank correlation coefficient between the samples taken 4 years apart was determined to be 0.92 with a *p*-value of less than 0.0001.

Long term stability is important quality for a biomarker that makes claim to predict atherosclerosis progression and cardiovascular events many years in the future. A previous study indicated that anti-PC IgM was constant over a period of many weeks *(Padilla, Ciurana et al. 2004)*. Here we have, for the first time, shown that the levels are steady over a four year period.

4.7 PC-REACTIVE B CELLS ARE ABUNDANT IN BLOOD

In order to isolate PC-reactive B cells, we conjugated PC-hapten onto phycoerythrin (PE) for flow-cytometric identification of B cells carrying PC-reactive surface immunoglobulin. The brightly fluorescent end-product, PC-PE was validated for specificity and then used in combination with a panel of monoclonal antibodies to divide $CD19^+$ B cells into four subsets, [1] IgM^+ memory, [2] IgG-switched, [3] naïve B cells and [4] $CD27^+CD43^+$ B cells. Each B cell subset was then further subdivided into PC-reactive and PC-negative B cells. Single PC-reactive B cells from each subset were isolated following two successive rounds of sorting.

PC-reactive B cells were readily identifiable in all examined B cell subsets and found to be particularly enriched in the IgM^+ memory population, which is consistent with the view that this subset is important for combating carbohydrate and lipid antigens *(Weller, Braun et al. 2004)*. While PC-reactive B cells in the $CD27^+CD43^+$ population were the hardest to come by (approximately 10 cells per million B cells), a large proportion of these cells were spontaneously secreting IgM. The relative number of these $CD27^+CD43^+$ B cells as identified by flow-cytometry was significantly correlated to the serum level of anti-PC IgM (r^2=0.42; p=0.0025).

Regarding the nature of the $CD27^+CD43^+$ population, which has recently been described as human equivalents of mouse B1 cells *(Griffin, Holodick et al. 2011)*, we could confirm that they do spontaneously secrete IgM. However, many of our subsequent findings contradict the innate nature of these cells. Antibodies cloned from $CD27^+CD43^+$ B cells were as mutated as those from class-switched B cells. Furthermore, a large number of PC-reactive cells in the $CD27^+CD43^+$ population were found to be clonally related to cells from other memory populations as visualized by phylogenetic trees. Although $CD27^+CD43^+$ B cells may not be innate lymphocytes, they definitely exist and probably execute important tasks. Here, we have shown that the number of PC-reactive $CD27^+CD43^+$ is

significantly correlated with serum anti-PC IgM, suggesting that these are key antibody-secreting cells.

In mice, CD5 is an established marker for natural B1 cells, including murine PC^+ B cells *(Griffin, Holodick et al. 2011)*. In order to assess whether CD5 is also preferentially expressed on human PC^+ B cells, we analyzed CD5-expression among human B cells in ten individuals. We here report that approximately 8% of $CD27^+$ memory B cells and 30% of naïve B cells were $CD5^+$, however, CD5 was equally expressed on PC^+ and PC^- B cells. These data show that CD5 is not a useful marker for PC^+ B cells in man.

4.8 THE HUMAN ANTI-PC REPERTOIRE IS DIVERSE AND MUTATED

We sorted single PC-reactive B cells from ten healthy individuals in order to characterize the anti-PC repertoire in a healthy population. Complete sequencing data was obtained from 1388 PC-reactive cells and after analysis, we found that the human anti-PC specificity can be conferred by a wide array of immunoglobulin gene combinations. Although the anti-PC repertoire is arguably skewed towards certain families of Ig-genes (IGHV3, IGKV3 and IGKV4), it still does not change the narrative of a broad immune-response towards this small epitope.

Although PC-reactive B cells likely operate with minimal T-cell help, the antibodies showed significant levels of mutations. Granted that this seems counterintuitive, recent publications have unequivocally demonstrated that affinity maturation and class-switching can occur without T-cells, albeit the process is more cumbersome *(Ueda, Liao et al. 2007, Herlands, Christensen et al. 2008, Aranburu, Ceccarelli et al. 2010, Yang, Ghosn et al. 2012)*.

With the exception of immunoglobulins obtained from the naïve B cells; all PC-reactive antibodies in this study were extensively mutated. The mutations were primarily located in the complementarity determining regions (CDRs) of the antibodies and a majority of them were replacement

mutations. Antigen driven selection processes are characterized by the accumulation of replacement mutations. By consensus, a skewed replacement to silent mutation ratio (R/S) of >2.9 in the CDR regions is considered indicative of antigen driven affinity maturation *(Shlomchik, Marshak-Rothstein et al. 1987).* In our study, the antibodies generated from memory PC$^+$ B cells typically had CDR regions with R/S-ratios of >5. These data suggest that human PC-reactive antibodies mainly arise from classical antigen-driven immune responses involving somatic hypermutation. This discripancy between our data and the prevailing paradigm, which is based on mouse data, was unexpected as we had hypothesized that the human anti-PC repertoire would be narrow and unmutated. However, humans do not live under specific pathogen free (SPF) conditions like inbred laboratory mouse strains and the broad anti-PC repertoire of adult humans that we report in this article, is likely reflective of multiple immunological challenges by PC-antigens. While our data does not preclude the possibility of a germline encoded innate anti-PC in humans, it seems clear that in adults, if present, this putative innate response is overshadowed by an adaptive T-independent process mediated by conventional B cells. Innate or not, this is still a clinically relevant immune response, and multiple studies have consistently confirmed that having a low titer of anti-PC is an independent risk factor for cardiovascular disease *(de Faire, Su et al. 2009, Gronlund, Hallmans et al. 2009, Fiskesund, Stegmayr et al. 2010, Caidahl, Hartford et al. 2012).*

Sequencing data from IgG-switched cells, on which we had performed single-cell PCR (*n*=247), identified IgG2 as the predominant subclass (64%) among the switched PC-reactive B cells. Sorted and cloned PC-negative IgG-switched control cells, were as expected mostly IgG1 (59%). This skewed subclass distribution towards IgG2 that we report, is consistent with previous studies that have characterized the quantity of each anti-PC IgG-subclass in human blood *(Scott, Briles et al. 1987).*

4.9 COMPARATIVE ANALYSES OF THE HCDR3 REGION

The HCDR3 region of an antibody is unique and is often considered to be a fingerprint region that can be used to distinguish between different clones. Among the hundreds of naïve PC^{+} cells that we examined, we only detected two cells with similar HCDR3. Conversely, within the memory compartments of each and every donor, we observed an abundance of clonally related B cells with identical VH/D/JH-gene usage and matching HCDR3 sequences. This phenomenon was particularly pronounced in the IgG-switched and $CD27^{+}CD43^{+}$ subsets, where a majority (60%) of PC-reactive B cells had clonal relatives. The lower degree of clonal expansion (32%) and hence broader anti-PC repertoire found among IgM^{+} memory B cells might support the hypothesis that this subset constitutes a reservoir memory compartment *(Defrance, Taillardet et al. 2011)*. Many of the clonally related clusters of B cells that were identified within individual donors were dispersed across multiple B cell subsets and this seems to suggest a common origin for all three memory subsets.

Whilst comparing antibody HCDR3s from different donors by means of alignment with the ClustalW algorithm, we identified 18 idiotypes that were present in two or more donors. These highly similar clones found in multiple individuals, utilize the same heavy and light chain gene configuration and possess strikingly similar HCDR3 and LCDR3 sequences.

4.10 EXPRESSED HUMAN ANTI-PC MONOCLONALS BIND TO PC

In order to functionally characterize the properties of human anti-PC antibodies, we endeavored to express our most interesting clones as recombinant mAbs using a previously published human IgG1-construct *(Tiller, Meffre et al. 2008)*. The selection process was primarily focused on covering the common idiotype families. Of the 35 expressed antibodies, 34 bound to PC-conjugated bovine serum albumin (PC-BSA) in ELISA. Strong binders constituted 40% of the mAbs and these were primarily found among clones from the IgG-switched and $CD27^{+}CD43^{+}$ subsets. We also assayed the polyreactivity profiles of the mAbs using an established

ELISA method *(Wardemann, Yurasov et al. 2003)*. Polyreactivity was observed in approximately 26% of our human anti-PC mAbs which is in the same range of what has been reported for other specificities *(Amara, Steen et al. 2013)*.

Using surface plasmon resonance, we determined the affinity of ten mAbs to free monomeric PC-hapten and the results were compared with the previously reported affinity of mouse TEPC-15 *(Gearhart, Johnson et al. 1981)*. Several of the tested human mAbs had affinities similar to TEPC-15. However, a clone from the most prevalent idiotype family (found in 6 out of 10 indivuals) exhibited an affinity of $2.25x10^{-7}$ M, almost 200 times greater than the germline mouse TEPC-15 antibody.

5 CONCLUDING REMARKS

The importance of immune cells and inflammation in the development of atherosclerosis is now widely accepted. This concept was first conceived in the 1980s when inflammatory cells like activated macrophages and T cells were detected in atherosclerotic plaques. Subsequent studies have proven this theory and thus catalyzed a paradigm shift in the cardiovascular field. Along with the new scientific stance on its pathogenesis came new ideas about immunomodulatory treatments and immunological biomarkers for atherosclerosis.

PC is an epitope that is exposed on oxLDL, apoptotic cells and certain human pathogens such as *Streptococcus pneumonie*. Anti-PC antibodies are ubiquitous in mammalian sera and it has been hypothesized that these antibodies carry out important functions in homeostasis. This belief stems from multiple studies, which have demonstrated the importance of anti-PC antibodies in infectious defense and clearance of oxLDL/apoptotic cells. Previous studies originating from the Frostegård group have correlated low levels of anti-PC IgM with increased incidence of cardiovascular events and high levels with slower atherosclerosis progression. This thesis builds upon the previous knowledge but has taken the anti-PC concept further by consolidating the theory and exploring the molecular ontogeny of human anti-PC antibodies.

In paper I, we showed that low level of anti-PC IgM (below the 30th percentile) is associated with increased risk of stroke in a large population based cohort from northern Sweden. Like in our previous epidemiological studies on anti-PC, this association is independent of traditional risk factors.

In paper II, we demonstrated that high serum level of anti-PC IgM, IgA and IgG1 are associated with decreased atherosclerosis progression. Anti-PC IgG2 was not correlated to atherosclerosis progression in any way. We speculate that this discrepancy could be due to the fact that anti-PC IgG2

has a different fine-specificity and that the serum level of anti-PC IgG2 is mainly influenced by previous infections by PC-bearing bacteria, which we assume is a random event. In paper II, we also showed that the serum level of anti-PC IgM does not change much over a period of four years and that serum anti-PC IgM could be used in conjunction with serum anti-MDA or anti-oxLDL IgM to further enhance our ability to predict atherosclerosis progression.

The main finding of paper III is that the human anti-PC antibody response is heterogeneous though some very similar antibody clones were found in multiple study subjects. Furthermore, human anti-PC antibodies were discovered to be heavily mutated, including those of the IgM-class, and the mutation pattern was indicative of affinity maturation.

Immunomodulation as a treatment option against atherosclerosis and CVD has been gaining appreciation among scientist over the last two decades. Though the inflammatory nature of atherosclerosis has been broadly understood for quite some time now, novel drugs have yet to appear. The scientific work in this thesis represents one avenue of research in the quest for better diagnostic tools and treatment options for patients with CVD. Based on the many promising studies on anti-PC in CVD, natural or induced antibodies against PC could potentially be used to treat patients with low levels of anti-PC and I believe that the work in this thesis, which has focused on understanding the human anti-PC antibody response, is one step in that direction.

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late 1980s and though he is not in biomedical research, he was a constant source of inspiration during my childhood. I fondly remember performing simple chemical experiments with him as a little child, like dissolving metal in acid and turning a lemon into a battery. Thinking back, I believe that he is the reason for my enduring infatuation with science and thus, I have chosen to dedicate this thesis ***to my father*.**

7 REFERENCES

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